INSIGHTS IN EXERCISE PHYSIOLOGY: 2021

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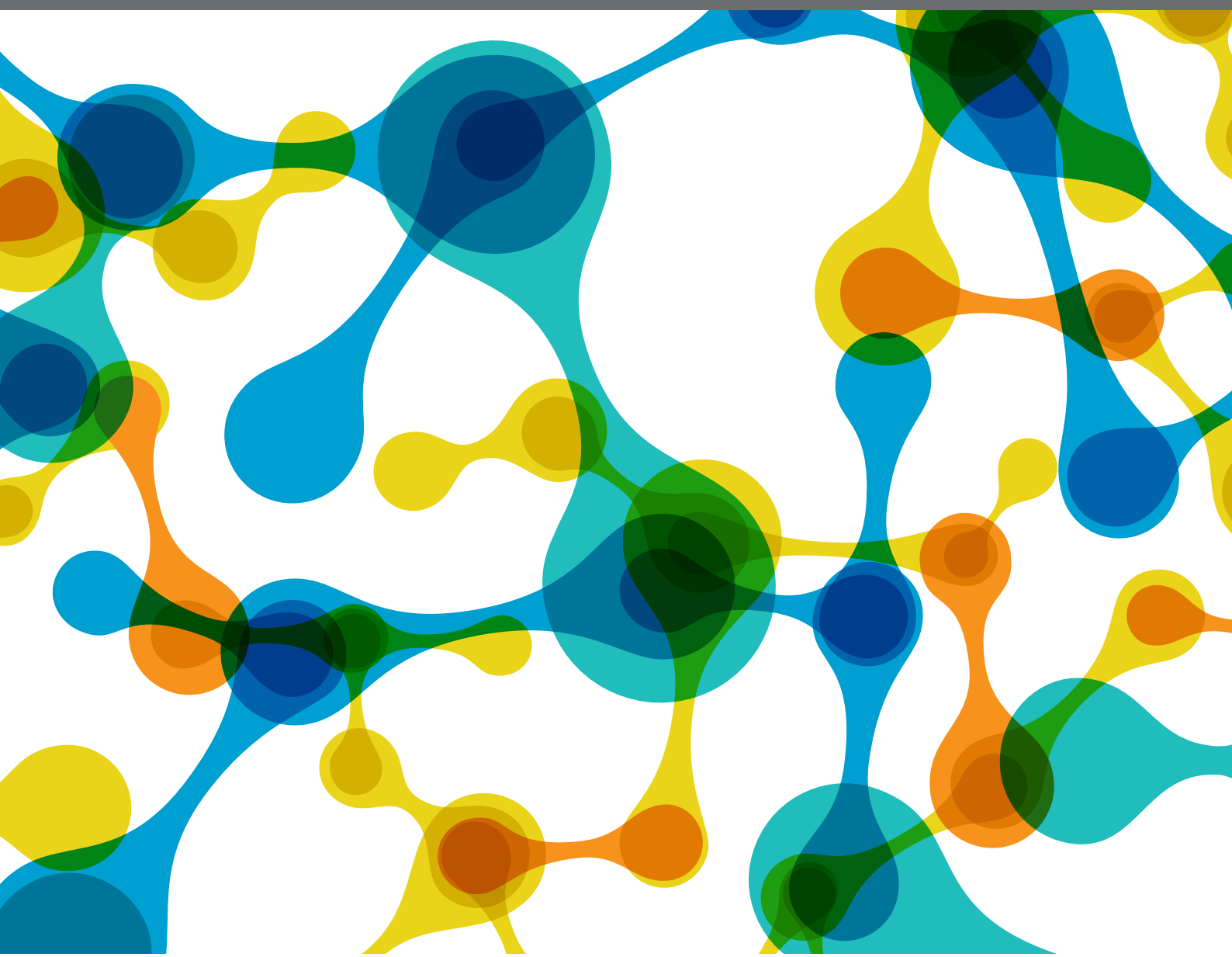

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INSIGHTS IN EXERCISE PHYSIOLOGY: 2021

Topic Editors:
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Editorial: Insights in exercise physiology: 2021

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Editorial on the Research Topic
Insights in Exercise Physiology: 2021

The roots of exercise physiology date back into antiquity, when Susruta (about 600 B.C.) in India was likely the first physician to prescribe moderate daily exercise, and Hippocrates (460–370 B.C.) in Greece was the first to provide a written exercise prescription, and Galen (129–210 A.D.) recommended to include regular physical activity in the management of avoiding illness (Tipton, 2014). While physiological concepts at that time were far from current understanding, it were findings from seminal research work published in the first three decades of the 20th century that laid the foundations of modern exercise physiology (Lindinger, 2022). For instance, August Krogh (regulation of oxygen supply to working muscles) and Archibald Vivian Hill (production of heat in the muscle and concept of maximal oxygen uptake) pioneered contemporary exercise physiology, both being awarded the Nobel Prize in Physiology or Medicine in 1920 and 1922, respectively. Subsequently hundreds of exercise laboratories have been set up around the world and thousands of publications contributed and still contribute to a more complete understanding of exercise physiology. In particular, during the past few decades, significant advances have been made in analytical laboratory techniques, where the fields of biochemistry, genetics and molecular biology pushed forward exercise science into a new era (Gomes et al., 2020).

Part of the current scope of research in exercise physiology is represented by 11 papers contributing to the Research Topic "Insights in Exercise Physiology: 2021," from different areas.

Cardiovascular exercise physiology

It is widely accepted that opportunely dosed exercise has the potential to treat chronic cardiovascular diseases, including coronary artery disease (CAD), heart failure, and hypertension but several open issues remain on the mechanisms underlying the cardiac and vascular benefits, the optimization and individualization of exercise type, intensity, and duration, and the identification of the limiting factors of the exercise

tolerance in patients. Overall, studies published in the present Research Topic shed new light onto some important aspects of the field including the vascular response to exercise in the presence of heart failure/CAD and the role of innovative rehabilitation approaches in cardiopathic patients.

Mannozzi and colleagues examined whether baroreflex dysfunction in heart failure exacerbated ventricular-vascular uncoupling at rest, and during exercise in response to baroreceptor unloading by performing bilateral carotid occlusions in chronically instrumented conscious canines (Mannozzi et al.). The findings indicate that the already challenged orthostatic and exercise tolerance is impaired by the enhanced ventricular-vascular uncoupling during baroreceptor unloading, negatively affecting exercise tolerance and quality of life in heart failure patients (Mannozzi et al.).

Montalvo and colleagues evaluated exercise-induced blood flow patterns in the carotid artery during various types of exercise (maximal tests on a treadmill, cycle-ergometer, and arm-ergometer, and 1-repetition maximum tests of the squat, bench press, and biceps curl) at 3 different intensities (low, moderate, high) (Montalvo et al.). Simultaneous real-time ultrasound image and blood flow of the carotid artery showed that all exercise intensities across all modalities resulted in turbulent blood flow. However, endothelial shear stress (ESS) was greatest during treadmill at a high intensity, while bench press and biceps curls yielded the least ESS (Montalvo et al.).

Fanget and colleagues proved tele-rehabilitation as effective and safe alternative for cardiac rehabilitation during the COVID-19 period, suggesting this approach also to be used to facilitate the continuity of care for patients unable to participate in center-based cardiac rehabilitation (Fanget et al.).

Labeix and colleagues demonstrated benefits of a specific inspiratory muscle training (IMT) during cardiac rehabilitation of patients suffering from coronary artery disease with moderate obstructive sleep apnea (Labeix et al.). Additional IMT resulted in a decrease of the apnea-hypopnea index in those patients.

Molecular adaptations to exercise

Despite the tremendous advancements in the knowledge of the molecular modifications underlying the musculoskeletal plastic response to exercises, many aspects deserve further study. In particular, doubts still exist about the fine-tune mechanisms triggering the underlie response based on variations in exercise quality, intensity, duration and volume and their countless potential combinations, both in health and disease. In the present RT three works investigated fundamental molecular aspects of the individual response to training including the impact of volume and intensity onto the biochemical muscular adaptations, the role of miRNAs in the individual response to exercise, and the significance of mitochondrial regulator protein cyclophilin-D, involved in cell death due to ischemia/reperfusion injury, in the adaptative response to exercise.

Vann and colleagues studied the effects of higher-load (HL) versus (lower-load) higher-volume (HV) resistance training on skeletal muscle hypertrophy, strength, and muscle-level molecular adaptations in young men (Vann et al.). Single leg training was performed over 6 weeks. Cross-sectional area of the vastus lateralis muscle was only increased after HV, but HL improved leg extensor strength more than HV (Vann et al.). Biochemical assays revealed that integrated non-myofibrillar protein synthesis rates were higher in HV compared to HL.

Witvrouwen and colleagues evaluated changes in plasma-derived miRNAs by acute and chronic exercise in heart failure with reduced ejection fraction (HFrEF) in order to assess whether these can mechanistically be involved in the variability of exercise-induced adaptations (Witvrouwen et al.). The authors demonstrated some interesting relationships between miRNAs and exercise responses, e.g., baseline miR-23a predicted VO_2peak response to training.

Radhakrishnan and colleagues investigated whether acute cyclophilin-D (Cyp-D) ablation, using tamoxifen-induced ROSA26-Cre-mediated, would downregulate oxygen consumption and trigger an adaptive response that manifests in higher exercise efficiency in conditional knockout mice (Radhakrishnan et al.). The authors demonstrated (as previously shown for constitutive Cyp-D ablation) that acute Cyp-D ablation also induces a state of increased O_2 utilization efficiency, associated with a metabolic switch toward preferential utilization of glucose via AMPK-TBC1D1 signaling nexus (Radhakrishnan et al.).

Exercise injury and recovery

In the field of exercise physiology researchers often focus on the study of the mechanisms that underlie the appearance of exercise-induced injuries. The latter must be considered in the frame of the complexity and peculiarity of the exercise gesture and often include problems that go far beyond the simple musculoskeletal and tendon involvements. In fact, new research horizons appear in relation to the identification of the mechanisms of exercise-induced damage, both at the gastrointestinal and renal levels. All these aspects have an obvious great practical impact in the context of the physical preparation and rehabilitation.

In the present RT, Kirsten Legerlotz and Tina Nobis comprehensively dealt with the complexity regarding effects of fluctuating female hormones on the Injury risk in women (Legerlotz and Nobis). The authors discuss existing knowledge on the relationships between female hormones, musculoskeletal properties, neurophysiological changes and the risk to suffer from an injury. They also emphasize that a multitude of questions need to be answered, several contradictions to be

solved, and several correlations to be explained (Legerlotz and Nobis).

Sichting and colleagues performed an explorative cross-sectional study in 40 identical twin pairs, investigating the effects of regular recreational exercise activities on Achilles tendon mechanical properties (Sichting et al.). The authors found a 28% greater Achilles tendon stiffness in physically active twins compared to their inactive twin. Furthermore, they found sport-specific adaptations, i.e., by demonstrating that the stiffness of tendons responded more to exercise activities with an "aerial phase" such as running and jumping (Sichting et al.).

Hernando et al. monitored renal function in 76 marathon finishers (14 females) starting from the day before the marathon until 192 h after completing the race. Recovery effects were investigated under 3 different conditions: total rest (REST), continuous running at their ventilatory threshold 1 (VT1) intensity (RUN), and elliptical workout at their VT1 intensity (ELLIPTICAL). Unexpectedly, the ELLIPTICAL group showed a significantly lower glomerular filtration rate compared to the RUN and the REST group.

Air pollution and exercise

The known changes in climatic and environmental conditions, also related to the extreme diffusion of pollutants and increased average temperature, on the one hand, and the increasing outdoor sports practice on a large scale, on the other, require great attention to the physiological aspects of adaptations to exercise carried out in critical conditions, both in acute and chronic settings. The study of these interactions represents a major research challenge for the future, with fundamental predictable social implications.

In the present RT, You et al. explored potential effects on health when physical exercise (PE) is combined with exposure to air pollution (AP), by applying cluster, co-citation, and co-occurrence analysis using CiteSpace and VOSviewer software (You et al.). The research distribution, major topics, and relevant hotspots of the PE-AP field have been described by the knowledge-map strategy. At least with regard to the respiratory system, the first barrier of the body to defense against air pollutants, the benefits of exercise might be offset under AP conditions. Finally, the authors highlight a couple of research questions that should be considered by future studies (You et al.).

Concluding remarks

Exercise physiology amazes for the enormous variety of research fields, each of which has a direct potential impact on the human's quality of life.

Despite this heterogeneity a *trait d'union* is the intrinsic value of exercise in determining a plethora of physiological adaptations whose impact depend on the actual conditions and objectives.

From the role of polypill to fundamental determinant of the athletic performance, exercise represents a field of extreme interest, with transversal entanglements in the various scientific disciplines, from engineering to medical fields, and in its multidisciplinary nature is its beauty.

This will ensure that research in this area will not find moments of stalemate or reduction of interest on the part of those who dedicate their lives to research.

Author contributions

GA and MB wrote the manuscript and both approved the final version of this editorial.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Plasma-Derived microRNAs Are Influenced by Acute and Chronic Exercise in Patients With Heart Failure With Reduced Ejection Fraction

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Background: Exercise training improves VO_2peak in heart failure with reduced ejection fraction (HFrEF), but the effect is highly variable as it is dependent on peripheral adaptations. We evaluated changes in plasma-derived miRNAs by acute and chronic exercise to investigate whether these can mechanistically be involved in the variability of exercise-induced adaptations.

Methods: Twenty-five male HFrEF patients (left ventricular ejection fraction $<$ 40%, New York Heart Association class $\geq$ II) participated in a 15-week combined strength and aerobic training program. The effect of training on plasma miRNA levels was compared to 21 male age-matched sedentary HFrEF controls. Additionally, the effect of a single acute exercise bout on plasma miRNA levels was assessed. Levels of 5 miRNAs involved in pathways relevant for exercise adaptation (miR-23a, miR-140, miR-146a, miR-191, and miR-210) were quantified using RT-qPCR and correlated with cardiopulmonary exercise test (CPET), echocardiographic, vascular function, and muscle strength variables.

Results: Expression levels of miR-146a decreased with training compared to controls. Acute exercise resulted in a decrease in miR-191 before, but not after training. Baseline miR-23a predicted change in VO_2peak independent of age and left ventricular ejection fraction (LVEF). Baseline miR-140 was independently correlated with change in load at the respiratory compensation point and change in body mass index, and baseline miR-146a with change in left ventricular mass index.

Conclusion: Plasma-derived miRNAs may reflect the underlying mechanisms of exercise-induced adaptation. In HFrEF patients, baseline miR-23a predicted VO_2peak response to training. Several miRNAs were influenced by acute or repeated exercise. These findings warrant exploration in larger patient populations and further mechanistic *in vitro* studies on their molecular involvement.

Keywords: microRNA, HFrEF—heart failure with reduced ejection fraction, VO_2peak, peak oxygen uptake, response, exercise training, adaptation

INTRODUCTION

Heart failure (HF) is an increasingly prevalent syndrome with substantial mortality and morbidity due to exercise intolerance and dyspnea at exertion (Ponikowski et al., 2016). Apart from pharmacological treatment, exercise training is a successful multisystem approach in patients with heart failure with reduced ejection fraction (HFrEF) as it significantly improves morbidity and quality of life (Ponikowski et al., 2016). However, the individual response to exercise training in terms of peak oxygen consumption (VO_2peak) is highly variable, with 55% of HF patients showing insufficient increase (Bakker et al., 2018). Importantly, these VO_2peak non-responders carry an adverse prognosis, independent of other risk factors, and early identification is mandatory (Tabet et al., 2008). The mechanisms driving the variability in response remain incompletely understood, but evidence is pointing toward both genetic and epigenetic regulation (Gevaert et al., 2019; Witvrouwen et al., 2019).

MicroRNAs (miRNAs) are epigenetic modulators of protein coding genes that act at the post-transcriptional level (Peschansky and Wahlestedt, 2014). They are involved in pathways that are relevant for adaptation to exercise, such as changes in skeletal muscle function and angiogenesis, reduction of inflammation and response to hypoxia (Wada et al., 2011; Hecksteden et al., 2016; Welten et al., 2016; Seo et al., 2017; An et al., 2018; Zheng et al., 2018). We recently identified 5 circulating miRNA (miR-23a, miR-140, miR-146a, miR-191, and miR-210), that predicted the training-induced change in VO_2peak in HFrEF patients. In a bio-informatics analysis of their gene targets, this miRNA panel showed intriguing relations with biological pathways that could be involved in cardiovascular adaptation to exercise, such as vascular endothelial growth factor (VEGF) and mitogen-associated protein kinase (Witvrouwen et al., 2021). Furthermore, these miRNAs have been related to endothelial function and angiogenesis, skeletal muscle mass and function, and inflammatory processes, all relevant to exercise adaptation (Wada et al., 2011; Zhou et al., 2011; Zhu et al., 2016; Seo et al., 2017; Sun et al., 2017; Mitchell et al., 2018; Zheng et al., 2018; Du et al., 2019; Liu et al., 2019; Qiao et al., 2020).

Abbreviations: Aix, Augmentation index; AIx75, Heart rate corrected AIx; CBC, Complete blood count; CRT, Cardiac resynchronization therapy; ET, Exercise training; FMD, Flow mediated dilation; ICD, Implantable cardioverter defibrillator; IVS, Interventricular septum; IVSd, Interventricular septal end diastole; LAVi, Left atrial volume index; LVEDV, Left ventricular end diastolic volume; LVEF, Left ventricular ejection fraction; LVMi, Left ventricular mass index; PWV, Carotid-femoral pulse wave velocity; RCP, Respiratory compensation point; RER, Respiratory exchange ratio; UC, Usual care.

Previously, it has been shown that miR-146a levels at peak exercise are positively related with VO_2max, and miR-210 was negatively related to VO_2max in healthy subjects (Baggish et al., 2011; Bye et al., 2013). Both miR-146a and miR-210 have also been associated with the diagnosis of HF (Vegter et al., 2016). Furthermore, circulating miRNA levels are dynamically regulated by acute and chronic exercise. In healthy subjects, some miRNAs are down- or upregulated immediately after an acute exercise bout, and return to resting levels 24 h after an extended-duration acute exercise bout, depending on the tissues of origin or targets affected by exercise (Baggish et al., 2011, 2014; Nielsen et al., 2014). However, whether circulating miRNAs in HFrEF patients are dynamically regulated after a period of exercise training or by an acute exercise bout is currently unknown.

In this prospective cohort study, we aimed to evaluate whether plasma levels of miR-23a, miR-140, miR-146a, miR-191, and miR-210 are influenced by a 15-week exercise training program. In addition, we assessed the effect of an acute exercise bout on plasma miRNA levels, both in the untrained and trained status.

MATERIALS AND METHODS

Patients and Study Design

In this prospective cohort study, consecutive HFrEF patients that were referred for a 15-week supervised combined strength and moderate-intensity aerobic training program to the Cardiac Rehabilitation Centre of the Antwerp University Hospital (ET group) were compared to age-matched HFrEF patients receiving usual care without exercise training (UC group). Randomization into a training and non-training group was considered as non-ethical in view of the strong indication for exercise training in HFrEF (Class IA indication) (Ponikowski et al., 2016). Patients were included when they completed at least 30 of the 45 sessions. The study complied with the Declaration of Helsinki and was approved by the ethics committee of the Antwerp University Hospital. Written informed consent was obtained from all participants.

The change in miRNA levels after a 15-week exercise training program was investigated in the ET group and compared the UC group, and baseline miRNA levels were related to the change in VO_2peak. In the ET group only, the relation between baseline miRNA levels and change in cardiopulmonary exercise test (CPET), cardiac and vascular adaptation, and muscle strength was studied, and the effect of an acute exercise bout on the miRNA panel was assessed (**Figure 1**).

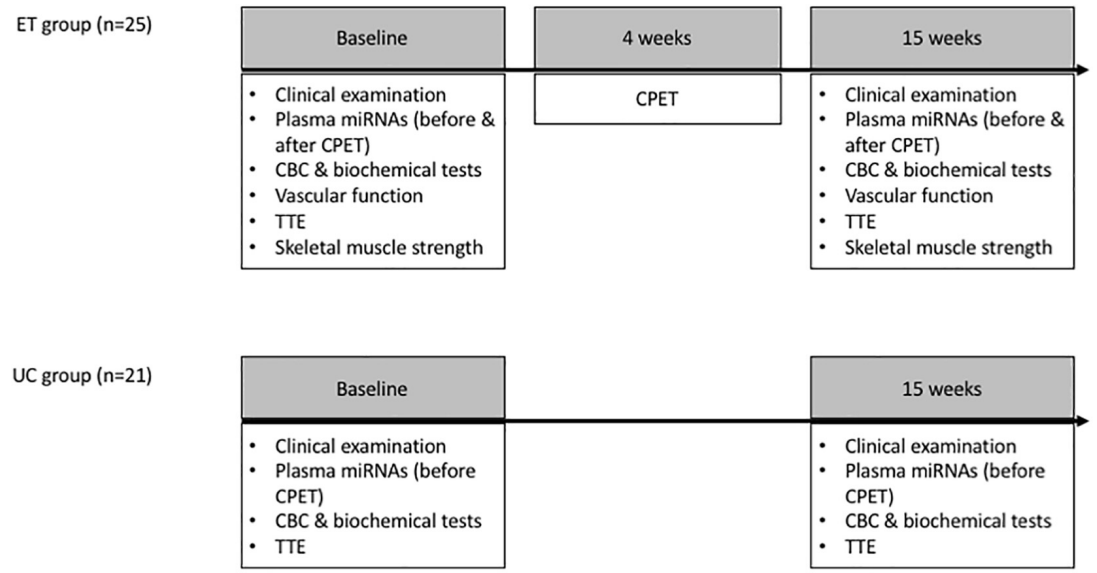

FIGURE 1 | Study design: Plasma miRNA levels were assessed at baseline and after 15 weeks in the ET and UC group. Vascular function, strength characteristics and the effect of an acute exercise bout (CPET) on the miRNA levels were evaluated in the ET group only. Since the relation between miRNAs and the central/peripheral determinants of VO_2peak (e.g., endothelial function and skeletal muscle strength) was not the primary objective of this study, these determinants were not assessed in the UC group. Vascular function measurements included flow mediated dilation of the brachial artery, pulse wave velocity and heart rate corrected augmentation index. Maximal strength of quadriceps, pectoral, latissimus dorsi, triceps, and deltoid muscles was assessed. CBC, complete blood count; CPET, cardiopulmonary exercise test; ET, exercise training; TTE, transthoracic echocardiography; UC, usual care.

Power Calculation

The sample size was calculated at 20 individuals per group. This offers 80% power to detect a difference in change in VO_2peak between the 2 groups of 0.9 standard deviations (SD) at a significance level of 5%. Previous studies indicate that the standard deviation of the change in VO_2peak is typically around 1.4 ml/kg/min (Belardinelli et al., 1995). Hence, a difference of 1.26 ml/kg/min in change in VO_2peak between the two groups is detectable.

In- and Exclusion Criteria

Patients with a left ventricular ejection fraction (LVEF) $< 40\%$, symptoms and signs of HF [New York Heart Association class (NYHA) $\geq$ II], clinically stable and optimally medically treated for $\geq$ 6 weeks, aged $\geq$ 18 and $\leq$ 80 years were eligible. To avoid the effect of sex-differences in epigenetic regulation, only male patients were included. Exclusion criteria were severe valvular pathology, severe renal failure (eGFR CKD-EPI < 30 ml/min/1.73m^2), acute coronary syndrome < 4 weeks ago, uncontrolled hypertension or arrhythmias, cognitive impairment, severe pulmonary disease (FEV1 $< 60\%$ predicted, severe decrease in diffusion capacity, chronic obstructive pulmonary disease GOLD III-IV), auto-immune disorders, oncologic disease, or inability to exercise.

Exercise Training

Supervised in-hospital exercise training consisted of combined aerobic and resistance training, 3 sessions/week (58 min/session) for 15 weeks. Aerobic training intensity was set at 90% of heart rate (HR) at the respiratory compensation point (RCP). When RCP was not reached, exercise intensity was calculated using the Karvonen formula [exercise heart rate = rest heart rate + (0.70* heart rate reserve)] (Karvonen et al., 1957). Strength exercise was an important component of the training program, with the focus primarily on gaining strength during the first 8 weeks. Afterward, aerobic training became more prominent (**Supplementary Figure 1**).

Clinical Assessments

CPET was performed on a treadmill (Medical Jaeger, Würzburg, Germany) with a graded protocol (equivalents of 40 W + 20 W/min or 20 W + 10 W/min) (Beckers et al., 2011), with an identical protocol for the follow-up test (Cardiovit CS-200 Ergo-Spiro, Schiller AG, Baar, Switzerland). Gas exchange measurements and 12-lead electrocardiogram were recorded continuously. Blood pressure was measured every minute. VO_2peak was determined as the mean VO_2peak during the final 30 s of exercise. Percent predicted VO_2peak was calculated using the Jones equation (Jones and Campbell, 1982). The RCP was estimated from the systematic increased ventilatory equivalent for VCO_2 (VE/VCO_2) and the systematic decrease in end tidal partial pressure of CO_2 ($PETCO_2$) (Whipp et al., 1989; Algul et al., 2017).

Echocardiography was performed on a Vivid E95 cardiac ultrasound using the 4V transducer for 3-D imaging and analyzed on Tomtec Arena). Left ventricular ejection fraction (LVEF), left ventricular mass index (LVMi), left ventricular end diastolic volume (LVEDV), left atrial volume index (LAVi),

interventricular septum (IVS) thickness and diastolic parameters (E/A, E/e') were recorded. In the UC group, LVEF was calculated using Simpson's monoplane (4 chamber view) method on M5S transducer, AGFA IMPAX Agility 8.1.2, Vivid E95.

Endothelial-dependent vasodilation of the brachial artery was evaluated by flow mediated dilation (FMD) as previously described (ProSound alfa6, Hitachi-Aloka Medical Ltd.) (Van Craenenbroeck et al., 2015c; Mannaerts et al., 2019). FMD was expressed as the percent change in peak vessel diameter from the baseline value [(peak diameter − baseline diameter)/baseline diameter]. Endothelial-independent dilation was calculated accordingly after sublingual administration of nitroglycerine. Arterial stiffness was assessed with carotid-femoral pulse wave velocity (PWV) and pulse wave analysis (PWA) that calculates augmentation index (AIx) and heart rate corrected AIx (AIx75) using SphygmoCor (Atcor Medical), as previously described (Van Craenenbroeck et al., 2015b). All measurements were done in triplicate.

Bioelectrical impedance analysis was performed on an Omron BF306 Body Fat Monitor (Omron Healthcare Co., Ltd., Kyoto) using 2 electrodes (1 handle in each hand) to provide estimates of total lean mass and fat mass.

Plasma MicroRNA Levels

Whole blood was collected after an overnight fast prior to the CPET in ethylenediaminetetraacetic acid tubes (EDTA). The first 3 ml of blood was discarded to prevent contamination with skin epithelial cells and endothelial cells. Samples were centrifuged within 30 min after collection (1,500 g, 15 min) at room temperature, and plasma was stored at −80°C.

MiR-23a, miR-140, miR-146a, miR-191, and miR-210 were quantified in plasma samples using miRNA RT-qPCR. In brief, plasma samples were thawed on ice and centrifuged for 10 min (4°C, 16,000 g). RNA enriched for small RNAs (including miRNAs) was isolated using the mirVana Paris Kit (Thermo Fisher Scientific). Four hundred microliter 2X Denaturing Solution was added to 400 µl of plasma. RNA was extracted using acid-phenol:chloroform and ethanol. The aliquoted eluate was immediately stored at −20°C. Reverse transcription and preamplification were performed using TaqMan miRNA primers (Thermo Fisher Scientific) and multiplex qPCR was done in a CFX96 thermal cycler (BioRad) as previously described (Van Craenenbroeck et al., 2015a). Raw Cq values were calculated in BioRad CFX manager software v.3.1 using automatic baseline and threshold settings. Cq values that were undetermined or > 35 were removed from the analysis, to minimize statistical confounding by high quantification cycle values. Data were normalized using geNorm and relative miRNA levels were expressed as $\log(2^{-\Delta Cq*}10^4)$ (Gevaert et al., 2018).

Statistical Data Analysis

Data were analyzed using SPSS 26.0 and R version 3.6.0.

Normality of continuous variables was evaluated using Shapiro-Wilk test. Normally distributed data are expressed as mean ± standard deviation (SD), skewed variables as median and range (1st–3rd quartile). Fisher-exact test was used for comparison of categorical variables, independent samples *T*-test or Mann-Whitney *U*-test for comparison of continuous variables. To assess changes with 15 weeks of ET or with acute exercise, linear mixed models were fitted using time and group or visit as fixed effects and patient ID as random effect, or paired samples *T*-test was used as appropriate.

Correlations were assessed using Pearson correlation analysis. Multiple linear regression analyses adjusting for age and baseline LVEF were performed to assess independent determinants of VO_2peak. A two-sided p-value < 0.05 was considered significant.

RESULTS

Baseline Patient Characteristics and MicroRNA Expression

Twenty-five patients were included in the ET group and 21 patients in the UC group. Baseline patient demographics, clinical, pharmacological, CPET characteristics, and circulating miRNA levels are shown in **Table 1**.

At baseline, ET and UC were similar with regard to demographics and clinical characteristics, except for BMI, which was higher in UC ($p = 0.042$). Ischemic cardiomyopathy was more common in ET compared to UC ($p = 0.033$), and implantable cardioverter defibrillator (ICD) was less common in ET compared to UC ($p = 0.022$). Pharmacological therapy was comparable between ET and UC. CPET characteristics were similar between groups, except for work economy, which was lower in ET compared to UC group (6.4 vs. 7.3, $p = 0.015$).

Baseline miRNA expression was similar between groups, except for miR-23a which was higher in patients referred for ET compared to CG ($p = 0.043$).

At baseline, better heart (LVEF) and kidney (creatinine) function were associated with higher VO_2peak (respectively, $r = 0.303$, $p = 0.043$, and $r = -0.514$, $p < 0.001$, **Supplementary Figure 2**). Patients with lower LVEF had higher miR-210 levels ($r = -0.321$, $p = 0.032$, **Supplementary Figure 2**) independent from age (β = −9.455, $p = 0.035$, 95%C.I. −18.192, −0.717). None of the other baseline miRNA levels were related with LVEF. No significant correlation was found between baseline miRNA levels and baseline VO_2peak.

Exercise Training-Induced Changes in MicroRNA Expression

Changes in aerobic capacity and clinical characteristics after 15 weeks of follow-up are shown in **Table 2** and **Supplementary Figure 3**. Change in VO_2peak was significantly different between the ET and UC group (+ 0.95 vs. − 0.64 ml/kg/min (difference 1.59, 95% CI 0.06, 3.12, $p = 0.041$). NYHA class, peak load and load at RCP significantly improved in ET. Both ET and UC patients performed a maximal exercise test, evidenced by a high respiratory exchange ratio (RER).

After 15 weeks of follow-up, plasma levels of miR-146a significantly decreased in the ET group, whereas in the UC group plasma levels remained unaltered (p interaction < 0.05, **Figure 2** thick black lines). A significant different evolution in expression levels of miR-191 was observed in ET compared to UC (decrease

TABLE 1 | Baseline patient characteristics and training adherence.

	ET (n = 25)	UC (n = 21)	p-value
Clinical characteristics			
Age (years)	55.6 ± 13.4	60.0 ± 9.4	0.199
Male sex	100%	100%	1.0
BMI (kg/m^2)	26.3 ± 4.7	29.2 ± 4.7	0.042
Diabetes (n, %)	7 (28%)	1 (5%)	0.055
Arterial hypertension	13 (52%)	8 (38%)	0.346
History of smoking	21 (84%)	13 (62%)	0.089
NYHA class	II = 15 (60%) III = 10 (40%)	II = 17 (81%) III = 4 (19%)	0.124
Ischemic origin of HF	15 (60%)	6 (29%)	0.033
CRT or ICD	ICD = 5 (20%); CRT = 4 (16%)	ICD = 11 (52%); CRT = 4 (19%)	0.022 1.0
Creatinine (mg/dl)	1.25 (0.98–1.54)	1.22 (0.96–1.59)	0.947
eGFR (ml/min/1.73 m^2)	69.2 ± 27.6	66.6 ± 21.8	0.726
Echo characteristics			
LVEF (%)	32.5 (25.0–37.0)	30.0 (22.5–37.0)	0.576
Pharmacological therapy			
RAAS blocker	25 (100%)	21 (100%)	1.0
Beta blocker	22 (88%)	19 (90%)	1.0
Aldosteron antagonist	18 (72%)	13 (62%)	0.467
Diuretic	16 (64%)	10 (48%)	0.264
CPET characteristics			
Resting heart rate (bpm)	66.0 (60.0–71.5)	63.0 (55.0–71.5)	0.440
Baseline VO_2peak (ml/kg/min)	21.0 ± 6.3	19.2 ± 5.8	0.321
% Predicted VO_2peak (%—ml/kg/min)	73.0 ± 20.6	71.4 ± 16.8	0.780
RER	1.19 ± 0.1	1.18 ± 0.1	0.736
Work economy (watt/ml/kg/min)	6.4 ± 1.0	7.3 ± 1.3	0.015
Peak systolic blood pressure (mmHg)	140 ± 31.5	129 ± 37.3	0.289
Peak load (Watt)	133.6 ± 39.9	140.5 ± 46.5	0.592
VE/VCO_2 slope	35.7 ± 6.8	33.5 ± 7.7	0.296
miRNA expression [log($2^{-\Delta Cq}$*10^4)]			
miR-23a	1.49 ± 0.4	1.23 ± 0.5	0.043
miR-140	2.50 ± 0.2	2.46 ± 0.2	0.432
miR-146a	3.66 ± 0.2	3.62 ± 0.3	0.557
miR-191	3.83 ± 0.2	3.87 ± 0.2	0.519
miR-210	1.48 ± 0.3	1.41 ± 0.3	0.401
Training adherence			
Sessions completed (max. 45)	41 (39-43)	NA	NA

Data are expressed as mean ± SD, as median (1st–3rd quartile) or as number of subjects (%).
BMI, body mass index; ET, exercise training; CPET, cardiopulmonary exercise test; CRT, cardiac resynchronization therapy; eGFR, estimated glomerular filtration rate; ICD, implantable cardioverter defibrillator; HF, heart failure; LVEF, left ventricular ejection fraction; RAAS, renin-angiotensin-aldosterone system blockers; n, number of subjects; NA, not applicable; NYHA class, New York Heart Association functional class; RER, respiratory exchange ratio; UC, usual care.

vs. increase, p interaction < 0.05), but within group differences did not reach significance (dotted-dashed lines, **Figure 2**). None of the other miRNAs had a significant different evolution between the groups (p-interaction > 0.05).

Acute Exercise-Induced Changes in MicroRNA Expression

A single exercise bout (CPET) resulted in a rapid and significant decrease in miR-191 levels in untrained HFrEF patients ($p = 0.043$). Intriguingly, exercise training resulted in a blunted and even reversed response to acute exercise (**Figure 3**); a non-significant increase ($p = 0.120$) after training was observed (p-interaction = 0.003). No significant effect on the other plasma-derived miRNAs was observed, but the same trend of reversal of the miRNA response was observed (except for miR-210).

MicroRNAs as Predictors for Response to Exercise Training

After 15 weeks of follow-up, VO_2peak significantly changed in ET compared to UC.

In the ET group only, changes in CPET, echocardiographic, muscle strength and vascular function parameters were assessed as secondary characteristics of adaptation to training. Following training, peak load, load at RCP, VO_2 at 50% of peak load during

CPET1, BMI, LVEF, LVMi, lean mass and strength characteristics significantly improved (see **Table 2**).

Baseline MicroRNAs and Change in VO_2peak

Baseline miR-23a was significantly associated with percent change in VO_2peak (r = 0.387, p = 0.009, **Figure 4**), and this was confirmed by multiple linear regression adjusted for age and baseline LVEF (β = 11.307, p = 0.017, 95% CI 2.113, 20.500). Other miRNAs were not significantly related to VO_2peak changes.

Baseline MicroRNAs and Training-Induced Changes in Clinical Variables

Baseline miR-140 was related with the percent change in load at RCP ($r = -0.505$, $p = 0.033$) as well as the percent change in BMI ($r = -0.454$, $p = 0.023$). Baseline miR-146a correlated with the percent change in LVMi ($r = -0.446$, $p = 0.026$, **Figure 5**). None of the other baseline miRNAs were related with training-induced changes in clinical variables.

The percent change in BMI, percent change in peak load and percent change in lean mass were not related with the percent change in strength characteristics.

DISCUSSION

In this prospective cohort study, we investigated the effect of 15 weeks of exercise training as well as an acute exercise bout on plasma miRNA levels in HFrEF patients. Moreover, we studied the relation of miRNA levels with VO_2peak training response to unravel the underlying mechanisms of adaptation to chronic exercise. The principal findings include:

TABLE 2 | Change in clinical characteristics, CPET variables, echocardiographic findings, skeletal muscle strength, and vascular function after 15 weeks of either exercise training (ET) or usual care (UC).

	ET (n = 25)		UC (n = 21)		p-value for interaction
	Baseline	**15 weeks**	**Baseline**	**15 weeks**	
BMI	26.3 ± 4.7	27.0 ± 4.7*	29.2 ± 4.7	29.1 ± 4.6	0.006
NYHA class (n, %)	II = 15 (60%), III = 10 (40%)	I = 9 (36%), II = 13 (52%) III = 2 (8%), IV = 1 (4%)*	II = 17 (81%), III = 4 (19%)	I = 1 (5%), II = 14 (67%), III = 6 (28%)	0.002
VO_2peak (ml/kg/min)	21.0 ± 6.3	21.95 ± 7.5	19.2 ± 5.8	18.56 ± 6.2	0.041
Peak load (Watt)	133.6 ± 39.9	156.4 ± 47.9*	140.5 ± 46.5	143.3 ± 45.3	<0.001
RER	1.19 ± 0.1	1.21 ± 0.1	1.18 ± 0.1	1.18 ± 0.1	0.675
VE/VCO_2 slope	35.7 ± 6.8	37.2 ± 9.4	33.5 ± 7.7	35.5 ± 9.5	0.751
Load at RCP (Watt)	110.5 ± 38.5	127.6 ± 40.7**	128.8 ± 46.2	113.8 ± 52.6	0.031
VO_2 at RCP (ml/kg/min)	19.2 ± 6.1	20.1 ± 6.1	18.5 ± 6.7	17.7 ± 7.3	0.370
VO_2 at 50% of peak load during CPET1 (ml/kg/min)	14.1 ± 4.1	12.9 ± 3.8**	11.6 ± 3.9	12.0 ± 4.7	0.022
LVEF (%)	31.17 ± 7.4	37.15 ± 9.9*			
LVMi (g/m2)	161.32 ± 72.0	135.45 ± 63.0**			
RWT	0.33 ± 0.09	0.32 ± 0.08			
LAVi (ml/m2)	45.36 ± 19.3	42.47 ± 16.7			
IVSd (mm)	10.66 ± 2.3	10.55 ± 2.0			
LVEDV (ml)	194.17 ± 55.9	193.58 ± 54.8			
E/A	1.29 ± 0.8	1.22 ± 0.7			
E/e' (med)	17.4 ± 8.2	19.1 ± 14.6			
E/e' (lat)	13.8 ± 8.2	12.7 ± 9.1			
Lean mass (kg)	59.6 ± 8.5	61.5 ± 8.1**			
Bio-impedance (%)	26.0 ± 7.2	26.1 ± 6.9			
Quadriceps (kg)	37.07 ± 18.0	51.20 ± 19.2*			
Latissimus dorsi (kg)	46.25 ± 12.3	54.20 ± 12.3*			
Triceps, pectoral and deltoid muscles (kg)	55.80 ± 14.3	63.95 ± 11.6**			
Pectoral muscles (kg)	28.88 ± 10.9	41.33 ± 10.2*			
PWV (m/s)	7.96 ± 2.0	7.63 ± 1.9			
FMD (%)	4.89 ± 3.2	5.18 ± 2.3			
AIx75 (%)	17.06 ± 13.4	17.5 ± 13.0			

*Data are expressed as mean ± SD or as number of subjects (%). *p < 0.001, **p < 0.05.*

AIx75, heart rate corrected augmentation index; BMI, body mass index; CPET, cardiopulmonary exercise test; ET: exercise training; FMD, flow-mediated dilation; IVSd, interventricular septal end diastole; LAVi, left atrial volume index; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVMi, left ventricular mass index; n, number of subjects; NYHA class, New York Heart Association class; PWV, pulse wave velocity; RCP, respiratory compensation point; RER, respiratory exchange ratio; UC, usual care.

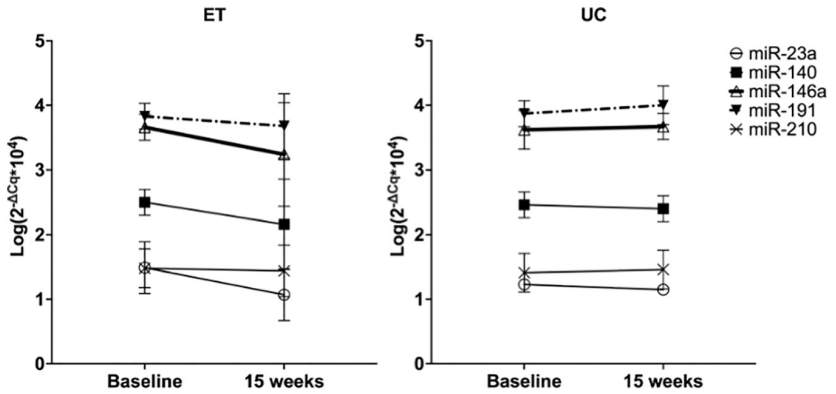

FIGURE 2 | Effect of 15 weeks of training on plasma levels of miRNAs in ET compared to 15 weeks of follow-up in UC. Data are expressed as the mean logarithm of the relative expression of the respective miRNA ± SD at baseline and after 15 weeks. Each line represents the change in plasma miRNA levels with 15 weeks of training in ET (n = 25) and 15 weeks of follow-up in UC (n = 21).

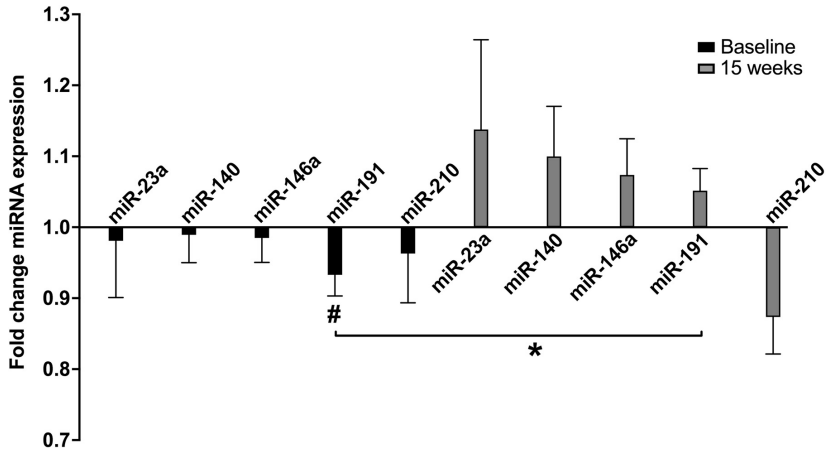

FIGURE 3 | Fold change miRNA expression with acute exercise at baseline and after 15 weeks of exercise training in ET. ET, exercise training group (n = 25); Fold change, post CPET/pre CPET miRNA expression. Data are expressed as mean and error. #within group $p < 0.05$, *p-value for interaction < 0.05.

- miR-146a levels decrease following 15 weeks of training compared to controls
- A single bout of acute exercise results in a decrease in miR-191 in untrained patients
- Baseline miR-23a predicts the percent change in VO_2peak following 15 weeks of training
- miRNA change in response to exercise may provide insights in the mechanisms driving VO2peak variability.

Dynamic Regulation of MicroRNA Expression Following Chronic Exercise

As previously reported, expression levels of circulating miRNAs change with acute or chronic exercise training (Baggish et al., 2011; Denham and Prestes, 2016). In the present study, we observed a significant decrease in relative expression of miR-23a, miR-140, and miR-146a in the ET group with 15 weeks of training. However, the evolution was only significantly different for miR-146a when compared to the UC group. Our findings are in contrast with Baggish et al. (2011) who observed no change in miR-146a levels with 90 days of rowing training. This difference might be related to the population studied i.c. athletes. To date, evidence on the physiological role of circulating miRNA in the adaptation to exercise is scarce, and to the best of our knowledge, virtually non-existent in the response to training in HFrEF patients. Hence, we can only speculate that the differences in circulating miRNA levels after training that we observed, may result from an underlying active and selective miRNA process that is involved in pathways relevant to exercise adaptation in HFrEF patients, rather than reduced passive release of these miRNAs.

In HFrEF patients, capillary density in skeletal muscle is reduced (Duscha et al., 1999). Both miR-23a and miR-146a were previously shown to stimulate angiogenesis (Zhou et al., 2011; Zhu et al., 2016). Therefore, reduced miR-23a and miR-146a levels after 15 weeks of training may reflect a diminished need for angiogenesis since capillary density increases with endurance and resistance training (Ingjer, 1979; Hudlicka et al.,

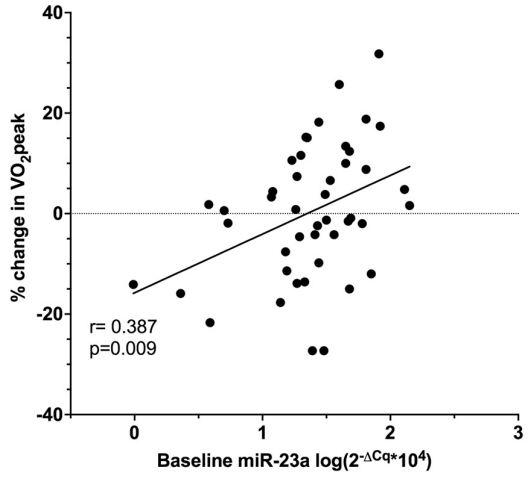

FIGURE 4 | Pearson correlation of baseline relative miR-23a expression and the percent change in VO_2peak in ET (n = 25) and UC (n = 20).

1992; Holloway et al., 2018). Also, a transient increase in miR-23a and miR-146a may be expected during the training program, reflecting the exercise-induced angiogenesis, but this needs to be explored in future experiments.

Furthermore, HFrEF patients often have skeletal muscle wasting, especially with more advanced disease status, and this contributes to typical HF symptoms and signs such as dyspnea and exercise intolerance, which results in lower VO_2peak and load during CPET (Ponikowski et al., 2016). Exercise training improves skeletal muscle mass and function and has beneficial effects on LVEF and LV remodeling in HFrEF patients (Adams et al., 2017; Tucker et al., 2019). An important driver of skeletal muscle wasting is the ubiquitin-proteasome system (Adams et al., 2021). Both miR-23a and miR-140 were shown to protect against skeletal muscle atrophy through inhibiting the ubiquitin-proteasome pathway and Wnt family member 11 expression, respectively (Wada et al., 2011; Liu et al., 2019). Hence, after training, sufficient skeletal muscle hypertrophy may result in lower miR-23a and miR-140 levels. However, this contrasts the finding that baseline miR-140 was inversely correlated with the change in load at RCP and BMI.

In the present study, we observed a differential expression between ET and UC in miR-23a. This could be attributed to the non-randomized study design, where ET patients might have had more skeletal muscle wasting compared to stable sedentary HFrEF controls, as BMI was significantly lower in ET compared to UC. Unfortunately, we do not have strength characteristics of the UC group. After 15 weeks of combined resistance and aerobic training, BMI significantly increased in the ET group, which could be attributed to increases in skeletal muscle mass, as indicated by higher strength characteristics in ER and coinciding increase in lean mass. However, no correlations with strength characteristics, or between (fold change) miR-23a and percent change in strength or lean mass were observed. Regarding the effect on cardiac hypertrophy, both miR-23a, miR-140, and miR-146a mediate cardiac hypertrophy through targeting the ubiquitin-proteasome pathway, GATA binding protein 4 and dihydrolipoyl succinyltransferase, respectively (Wang et al., 2012; Heggermont et al., 2017; Li et al., 2019). In contrast, we observed an inverse correlation between baseline miR-146a and percent change in LVMi in the ET group.

Baseline miR-210 was inversely related to LVEF. Since miR-210 has been related to hypoxia and upregulates VEGF in endothelial cells (Zheng et al., 2018), the inverse relation with LVEF could reflect the reduced oxygen delivery to the periphery that coincides with worsening LVEF and cardiac output in HFrEF (Piepoli et al., 2010).

Dynamic Regulation of MicroRNA Expression Following Acute Exercise

In addition, miRNA levels can be altered by acute exercise bouts. Previous research in patients with chronic kidney disease showed a rapid downregulation of circulating miR-146a following an acute exercise bout (Van Craenenbroeck et al., 2015a). In patients with heart failure (average LVEF 47.7%), Xu et al. (2016) observed an increase in circulating miR-21, miR-378, and miR-940 with acute exercise. However, in this study no distinction between heart failure with reduced, preserved or mid-range ejection fraction was made. In healthy athletes, miR-146a and miR-222 were shown to be upregulated by acute exercise both before

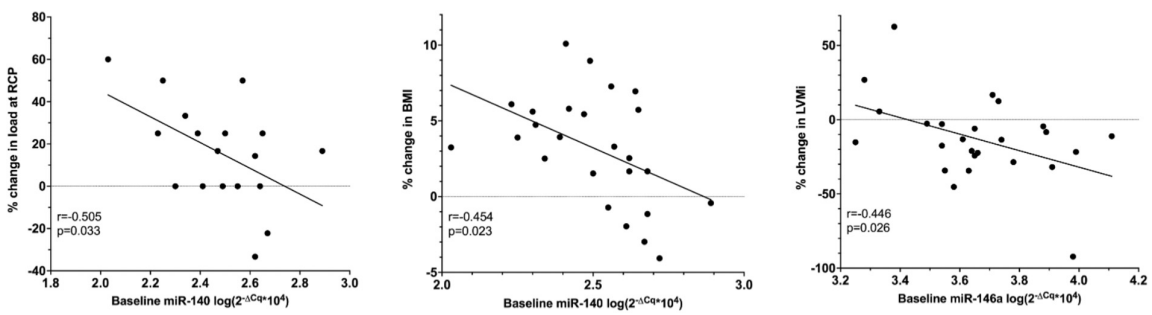

FIGURE 5 | Pearson correlation of baseline relative miRNA expression, the percent change in LVMi, percent change in load at RCP and the percent change in BMI in ET only. BMI, body mass index (n = 25); LVMi, left ventricular mass index (n = 25); RCP, respiratory compensation point (n = 18).

and after a 90-day rowing training, whereas miR-21 and miR-221 were only upregulated by acute exercise before the training period (Baggish et al., 2011). In the present study, at baseline all miRNA tended to decrease following an acute exercise bout, but this was only significant for miR-191. Intriguingly, this response reversed after 15 weeks of ET, which also suggests a selective training-induced effect on the miRNA expression.

MiR-191 has inhibitory effects on angiogenesis in endothelial cells (Gu et al., 2017; Du et al., 2019) and it stimulates myogenesis (Mitchell et al., 2018). As an acute exercise bout in sedentary patients elicits a hypoxic state, this triggers pro-angiogenic mechanisms. The fact that miR-191 has been shown to inhibit angiogenesis therefore could explain the decreased miR-191 levels observed at baseline. However, this needs to be confirmed in *in vitro* experiments. Regarding the effect on myogenesis, a single exercise bout provokes acute muscle damage after which myogenesis is established, and therefore lower levels of miR-191. After this initial decrease in myogenesis, we speculate to observe a rise in miR-191 and stimulation of myogenesis to repair the damaged skeletal muscle cells and to increase skeletal muscle hypertrophy. However, we only collected blood samples immediately after CPET so this hypothesis needs to be confirmed. In addition, increased angiogenesis and reduced myogenesis due to lower circulating miR-191 levels following an acute exercise bout may be conflicting. This could be explained by the fact that miRNA are tissue and disease specific, and circulating miRNA levels not always reflect intracellular levels (Pigati et al., 2010).

Finally, we hypothesize that with repeated acute exercise bouts (i.e., the effect of a 15-week training program) in HFrEF patients, the triggers for angiogenesis and myogenesis might have faded out due to increased capillarity and skeletal muscle mass, resulting in the opposite change of miRNA expression levels.

Predicting Change in Aerobic Capacity Based on Baseline Plasma MicroRNA Levels

More than half of the HFrEF patients who participate in an ET program may not increase their VO_2peak (Bakker et al., 2018) and despite many efforts, a predictive biomarker for VO_2peak response to training is still lacking. In our previous study, we identified several miRNA that were upregulated in patients with an unfavorable VO_2peak response (Witvrouwen et al., 2021). Among these miRNAs, miR-23a, miR-140, miR-146a, miR-191, and miR-210 were involved in pathways relevant for exercise adaptation processes. In the current study, we observed a significant change in VO_2peak in ET compared to UC; however, the increase within ET was not significant, which could be explained by the fact that BMI significantly increased in ET. Consequently, the observed change in VO_2peak in ml/kg/min is underestimated. Furthermore, we confirmed that baseline miR-23a predicts the change in VO_2peak with training, which may reflect the underlying mechanisms of exercise adaptation since miR-23a was shown to stimulate angiogenesis and to protect against skeletal muscle atrophy (Wada et al., 2011; Zhou et al., 2011). However, we observed clear improvements in muscle strength, but no correlations with miR-23a. This could be attributed to the low sample size. Nevertheless, miRNAs could emerge as promising epigenetic biomarkers of training response.

Limitations and Future Perspectives

Whereas aerobic training is known to improve endothelial function in stable coronary artery disease and HFrEF patients (Van Craenenbroeck et al., 2010, 2015c), and both aerobic, resistance and combined aerobic/resistance training showed similar improvements in FMD in patients with hypertension or prehypertension (Pedralli et al., 2020), we did not observe significant improvements in vascular function with 15 weeks of ET. This could be attributed to this subgroup analysis lacking statistical power to draw definitive conclusions.

Furthermore, the study can be biased due to the non-randomized design. However, as stated in the methods, randomizing patients to a training and control group would have been unethical in view of the class IA recommendation of ET in HFrEF patients with favorable effects on morbidity, mortality and quality of life (Ponikowski et al., 2016). As findings of this study are hypothesis generating, they should be validated in larger prospective trials and in *in vitro* experiments. Future pre-clinical studies could investigate and compare the expression levels in tissue (skeletal muscle, endothelial cells) to the observed changes in plasma levels. Hence, the contribution of miRNA to exercise adaptation processes can be examined, as either miRNA post-transcriptionally influence gene expression or they can be an exercise-induced epiphenomenon in these tissues (f.ex. exercise-induced skeletal muscle hypertrophy results in an increased release of miRNAs in the circulation). This will aid in further unraveling of the underlying mechanisms of response to acute and chronic exercise.

CONCLUSION

The effect of acute and chronic exercise on the expression levels of 5 circulating miRNAs involved in pathways relevant for exercise adaptation (miR-23a, miR-140, miR-146a, miR-191, and miR-210) was investigated in HFrEF patients admitted to a 15-week combined strength and aerobic training program and compared to the sedentary usual care group.

MiR-146a levels decreased following 15 weeks of training compared to the UC group. A single bout of acute exercise resulted in a decrease in miR-191 levels before, but not after training. Baseline miRNA-23a levels were related with the change in VO_2peak. Furthermore, baseline miR-140 was inversely related to the percent change in load at RCP and BMI, and baseline miR-146a was inversely related to the percent change in LVMi following 15 weeks of training.

Therefore, miR-23a, miR-140, miR-146a, and miR-191 may provide insights in skeletal muscle, cardiac hypertrophy and angiogenic response to exercise in HFrEF patients. These findings warrant further exploration in larger patient populations and in molecular biology set-ups.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article are available from the corresponding author upon request, for non-commercial purposes, without breaching participant confidentiality.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Antwerp University Hospital Drie Eikenstraat 655, 2650 Edegem, Belgium. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IW, AG, AVC, and EVC: conceptualization and writing—original draft. IW, NP, BE, TS, IG, WH, and PB: data collection. IW and EB: formal analysis. AG, WH, PB, AV, HH, AVC, and EVC: supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.736494/full#supplementary-material

Supplementary Figure 1 | Training protocol including aerobic exercises (blue) and strength training (yellow). Latissimus dorsi, pectoral, triceps, deltoid, and quadriceps muscles were resistance trained. c-down, cool-down; ex, exercises; w-up, warming-up; reps, repetitions; R, respiratory compensation point at start; RCPi, respiratory compensation point at 4w CPET; RM, repetition maximum.

Supplementary Figure 2 | Pearson correlation of baseline LVEF and creatinine with VO_2peak, and baseline LVEF and relative miR-210 expression in ET (n = 25) and UC group (n = 21). ET, exercise training; LVEF, left ventricular ejection fraction; UC, usual care.

Supplementary Figure 3 | Distribution of percent change in VO_2peak in all participants (n = 46). Gray, non-responders (<6% increase in VO_2peak); black, responders (≥6% increase in VO_2peak).

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Enhanced Oxygen Utilization Efficiency With Concomitant Activation of AMPK-TBC1D1 Signaling Nexus in Cyclophilin-D Conditional Knockout Mice

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We have previously reported in HEK 293T cells and in constitutive cyclophilin-D (Cyp-D) knockout (KO) mice that Cyp-D ablation downregulates oxygen consumption (VO_2) and triggers an adaptive response that manifest in higher exercise endurance with less VO_2. This adaptive response involves a metabolic switch toward preferential utilization of glucose *via* AMPK-TBC1D1 signaling nexus. We now investigated whether a similar response could be triggered in mice after acute ablation of Cyp-D using tamoxifen-induced ROSA26-Cre-mediated (i.e., conditional KO, CKO) by subjecting them to treadmill exercise involving five running sessions. At their first treadmill running session, CKO mice and controls had comparable VO_2 (208.4 ± 17.9 vs. 209.1 ± 16.8 ml/kg min^{-1}), VCO_2 (183.6 ± 17.2 vs. 184.8 ± 16.9 ml/kg min^{-1}), and RER (0.88 ± 0.043 vs. 0.88 ± 0.042). With subsequent sessions, CKO mice displayed more prominent reduction in VO_2 (genotype & session interaction $p = 0.000$) with less prominent reduction in VCO_2 resulting in significantly increased RER (genotype and session interaction $p = 0.013$). The increase in RER was consistent with preferential utilization of glucose as respiratory substrate (4.6 ± 0.8 vs. 4.0 ± 0.9 mg/min, $p = 0.003$). CKO mice also performed a significantly higher treadmill work for given VO_2 expressed as a power/VO_2 ratio ($7.4 \pm 0.2 \times 10^{-3}$ vs. 6.7 ± 0.2 10^{-3} ratio, $p = 0.025$). Analysis of CKO skeletal muscle tissue after completion of five treadmill running sessions showed enhanced AMPK activation (0.669 ± 0.06 vs. 0.409 ± 0.11 pAMPK/β-tubulin ratio, $p = 0.005$) and TBC1D1 inactivation (0.877 ± 0.16 vs. 0.565 ± 0.09 pTBC1D1/β-tubulin ratio, $p < 0.05$) accompanied by increased glucose transporter-4 levels consistent with activation of the AMPK-TBC1D1 signaling nexus enabling increased glucose utilization. Taken together, our study demonstrates that like constitutive Cyp-D ablation, acute Cyp-D ablation also induces a state of increased O_2 utilization efficiency, paving the way for exploring the use of pharmacological approach to elicit the same response, which could be beneficial under O_2 limiting conditions.

Keywords: oxygen consumption, conditional knockout, cyclophilin-D, exercise capacity, treadmill exercise

INTRODUCTION

Cyclophilin-D (Cyp-D) is a mitochondrial matrix-resident peptidyl-prolyl isomerase involved in various pleotropic functions of the cell including energy metabolism, metabolic adaptation, and nuclear/mitochondria signaling, and recently in nuclear translocation of apoptosis inducing factor (Elrod et al., 2010; Tavecchio et al., 2013; Tubbs et al., 2014; Radhakrishnan et al., 2015, 2019; Qamar et al., 2021). We first reported in HEK 293 T cells that Cyp-D interacts with mitochondrial transcription factors and that Cyp-D silencing downregulates the expression of mitochondrial genes initiated from the heavy strand promoter 2 (HSP2) encoding respiratory complex proteins leading to a reduction in oxygen consumption (VO_2; Radhakrishnan et al., 2015). Further work in our laboratory showed that Cyp-D ablation using constitutive Cyp-D knockout (KO) mice also downregulated respiratory complex I and IV activities and thereby VO_2 (Radhakrishnan et al., 2015). The VO_2 downregulation was accompanied by a reduction in VCO_2 but to a lesser extent resulting in an increased respiratory exchange ratio (RER) consistent with a metabolic shift favoring glucose utilization over fat. This effect was accompanied by enhanced exercise capacity demonstrating increased oxygen utilization efficiency (Radhakrishnan et al., 2019). The response was mediated in part *via* adenosine monophosphate-activated protein kinase (AMPK) and its downstream partner tre-2/USP6, BUB2, cdc16 domain family member 1 (TBC1D1), the so-called "AMPK-TBC1D1 signaling nexus."

We now investigated whether a similar adaptive response could be triggered in animals developed with normal Cyp-D expression after acutely inducing the Cyp-D ablation. To address this question, we used a conditional KO (CKO) mouse – tamoxifen-induced ROSA26-Cre-mediated – and asked whether the acute Cyp-D ablation in CKO mice also: (i) reduces VO_2 and improves exercise capacity, (ii) elicits a metabolic shift favoring glucose utilization, and (iii) whether AMPK-TBC1D1 signaling is involved. If the metabolic shift could be induced in adult mice born without the defect, the possibility of pharmacological manipulation became attractive, specially using small molecules to selectively destabilize the interaction between Cyp-D and mitochondrial transcription factors without necessarily affecting other Cyp-D functions and be beneficial for oxygen limiting clinical conditions.

MATERIALS AND METHODS

The studies were approved by the Institutional Animal Care and Use Committee at Rosalind Franklin University of Medicine and Science (Protocol B17-20) and by the Edward Hines VA Hospital Institutional Animal Care and Use Committee (Protocol H17-014).

Animals

Mice were purchased from the Jackson Laboratory (Bar Harbor, ME, United States) and conditional Cyp-D KO mice were generated as described below in the Methods section. Mice were bred and group-housed in the Biologic Resource Facility (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care) at the Rosalind Franklin University of Medicine and Science. Lights were set at the recommended illumination levels with a 12/12-h cycle controlled *via* automatic timers and the temperature maintained between 68 and 74°F. Mice were fed high-quality commercial laboratory diets *ad libitum*.

Materials

NativePAGE Novex Bis-Tris gels (3–12%), NativePAGE running buffer, NuPAGE Bis-Tris gels (4–12%), NuPAGE MOPS SDS running buffer, NuPAGE transfer buffer, PVDF membrane, and SuperSignal West Femto maximum sensitivity substrate were obtained from Thermo Fisher Scientific Inc. (Brookfield, WI, United States). The antibodies phospho-AMPK (Thr 172), phospho-TBC1D1 (Ser 660), and β-tubulin were from Cell Signaling Technology (Danvers, MA, USA). Antibody for peroxisome proliferator activator receptor-γ coactivator 1α (PGC-1α) was from Santa Cruz Biotechnology (Santa Cruz, CA, United States). All other fine chemicals were obtained from Sigma-Aldrich (St. Louis, MO, United States).

Study Design

Experiments were conducted in intact animals to assess the effects of conditional Cyp-D ablation *in vivo* on oxygen metabolism by measuring VO_2 and derived parameters at rest and during exercise. Two groups of mice, age and gender matched, representing control ($n = 10$) and conditional Cyp-D KO ($n = 10$), were subjected to a treadmill running protocol until exhaustion, during which VO_2, carbon dioxide production (VCO_2), and RER, total running time before exhaustion, total running distance, and work performed were measured. Fuel utilization was calculated using VO_2 and VCO_2 data. Five running sessions were performed. After the running sessions, mice skeletal muscle tissues were harvested, quickly frozen in liquid N_2, and stored at −80°C for Western blotting.

METHODS AND MEASUREMENTS

Generation of Conditional Ppif Knockout Mice

The Jackson Lab has cryopreserved sperm (stock 005737, Ppiftm1Mmos/J) that was heterozygous for the Cyp-D floxed allele ($Ppif^{Fl/+}$). This stock was originally generated by Dr. Stanley J. Korsmeyer's lab (Schinzel et al., 2005). Jackson Lab performed a cryorecovery from the frozen sperm using wild-type female (stock 000664, C57BL/6J) as oocyte donors. A heterogeneous Ppif colony ($Ppif^{Fl/+}$) was generated by the Jackson Lab.

$Ppif^{Fl/+}$ mice from two different donating mothers were inter-crossed to generate homozygous Cyp-D floxed mice ($Ppif^{Fl/Fl}$). Then, $Ppif^{Fl/Fl}$ mice were crossed with mice homozygous for the ROSA26-Cre^{ERT2} (Cre/Cre) transgene (stock 008463;

Gt(ROSA)26Sortm1(cre/ERT2)Tyj) to generate mice heterozygous for the Cyp-D floxed allele and hemizygous for ROSA26-Cre^{ERT2} transgene ($Ppif^{Fl/+}$-$Cre/^{+}$). Next, the $Ppif^{Fl/+}$-$Cre/^{+}$ mice were crossed with $Ppif^{Fl/Fl}$ mice to generate conditional Cyp-D knockout mice ($Ppif^{Fl/Fl}$-$Cre/^{+}$) and controls ($Ppif^{Fl/Fl}$).

Genotyping

Genotyping was performed according to the recommendations by the Jackson Lab. Briefly, tail biopsy (~2 mm) from 21-day-old pups were digested overnight at 55°C using 0.5 ml/sample of DNA isolation buffer (100 mM Tris.Cl, pH 8, 5 mM EDTA, 0.2% SDS, and 200 mM NaCl) containing 5 µl of 20 mg/ml proteinase K. The samples were then heated at 95°C for 10 min to inactivate Proteinase K. Equal volume of phenol: $CHCl_3$: IAA mixture was then added, mixed gently for 5 min to allow precipitation of proteins, and centrifuged at 12, 000 *g* for 5 min at 4°C for phase separation. The supernatant was carefully removed, 2 volumes of 100% ethanol were added, incubated at room temperature for 5 min, and then centrifuged at 12,000 *g* for 5 min at 4°C for DNA precipitation. The supernatant was discarded, and the pellet (DNA) was washed with 1 ml of 70% ethanol. The pellet was then dried at 37°C for 5 min and dissolved in TE buffer. The DNA concentration was determined by absorbance at 260 nm and 150 ng of DNA was used for the PCR. Two PCR reactions per sample were performed: one to detect Cre gene product and another one to detect Flox gene product. For Cre PCR, the primers used were as: oIMR 3621 (F: 5' to 3' CGT GAT CTG CAA CTC CAG TC) and oIMR 9074 (R: 5' to 3' AGG CAA ATT TTG GTG TAC GG). For Flox PCR, the primers used were as: oIMR 5116 (F: 5' to 3' GCT TTG TTA TCC CAG CTG GCG C) and oIMR 5115 (R: 5' to 3' TTC TCA CCA GTG CAT AGG GCT CTG). PCR was performed in 12 µl of reaction volume using the kit (KAPA2G Robust Hotstart Readymix PCR kit) using an applied biosystems thermal cycler (GeneAmp PCR system 2700). The thermal cycler conditions were set according to the genotyping protocol for mice stock # 005740 by the Jackson Lab and the PCR products separated using 0.7% agarose gel electrophoresis.

Tamoxifen Injection

Tamoxifen base (T5648, Sigma) was dissolved in peanut oil (10 mg/ml) and was given as 40 mg/kg as described by Andersson et al. (2010). Eight-week-old $Ppif^{Fl/Fl}$-$Cre^{/+}$ mice (Flox/flox/cre, Cyp-D CKO) and $Ppif^{Fl/Fl}$-$Cre^{/-}$ mice (Flox/flox, control mice) received intraperitoneally 1 mg of tamoxifen per day for five consecutive days. Administration of tamoxifen induces activation of the ubiquitously expressed Cre^{ERT2} recombinase, which stimulates recombination and excision of floxed Cyp-D in all tissues. Since $Ppif^{Fl/Fl}$ mice do not contain Cre gene, they will not undergo Cyp-D recombination and serve as control.

Treadmill Running

After 2 weeks of completion of tamoxifen injections (i.e., on day 19 as shown in **Figure 1**), treadmill running was started to assess effects on oxygen metabolism (i.e., VO_2, VCO_2, and RER) in CKO and control mice at rest and during treadmill exercise using a calorimeter (Oxymax System, Columbus Instruments).

Treadmill running had two components: (1) Training protocol and (2) Treadmill exercise protocol.

Treadmill Training Protocol

It was performed as previously described (Radhakrishnan et al., 2019). Each mouse was first subjected to a 3-day habituation period. On day 1, the mouse ran 10 min at 10 m/min without inclination; on day 2, the mouse ran 10 min at 10 m/min with 20 degree inclination; and on day 3, the mouse ran 20 min at the same speed and inclination. An electrical grid placed at the start of the treadmill ramp delivered an aversive shock when the mouse stopped running and slid into the grid to elicit avoidance of subsequent shocks by running (i.e., avoidance conditioning). The mouse was discarded from the study if more than three shocks in one session were required to promote running.

Treadmill Exercise Protocol

It was performed as previously described (Pederson et al., 2005; Radhakrishnan et al., 2019). Each mouse ran for a total of five sessions with the treadmill at a constant 20° inclination. At the start of each session, the mouse was allowed to acclimate in the chamber for 30 min before running. Baseline measurements were obtained between min 25 and 30. The treadmill was then started at 10 m/min and its speed increased by 2 m/min every 4 min to a maximum of 26 m/min for session one and to 30 m/min in sessions two through five. The mouse was allowed to run at the maximum speed for up to 90 min or until exhaustion. The strength of the shock was kept at 2 mA for the first 30 min running at maximum speed, after which the current was reduced to 0.5 mA. Exhaustion was defined as sliding into the shock grid and sustaining a shock (0.5 or 2 mA) for a minimum of 2 s for the third time or sustaining a single shock of ≥5 s (instead of running). The investigators were blind to the mice genotype. The treadmill was cleaned between animals with 70% ethanol, wiped with napkins, and air dried. Each running session was completed by the mice cohorts (20 mice total) in 4 days. There was a 3-day resting period afterward resulting in a week for completion of one running session by the mice cohorts. It took a total of 32 days to complete the five sessions.

VO_2, VCO_2, and Derived Measurements

The measurements were performed as previously described (Radhakrishnan et al., 2019). Power (Joules/min) was calculated by dividing work performed (Joules) by time spent on treadmill (min). Fuel utilization (carbohydrate, g/min and fat, g/min) was calculated using VO_2 and VCO_2 data as previously described (Radhakrishnan et al., 2019) according to Carbohydrate (mg/min) = [−3.226 g carbohydrate/$LO_2 \times VO_2$ (L/min) + 4.585 g carbohydrate/$LCO_2 \times VCO_2$ (L/min)] × 1,000; Fat (mg/min) = [1.695 g fat/$LO_2 \times VO_2$ (L/min) − 1.701 g fat/$LCO_2 \times VCO_2$ (L/min)] × 1,000 (Petrosino et al., 2016).

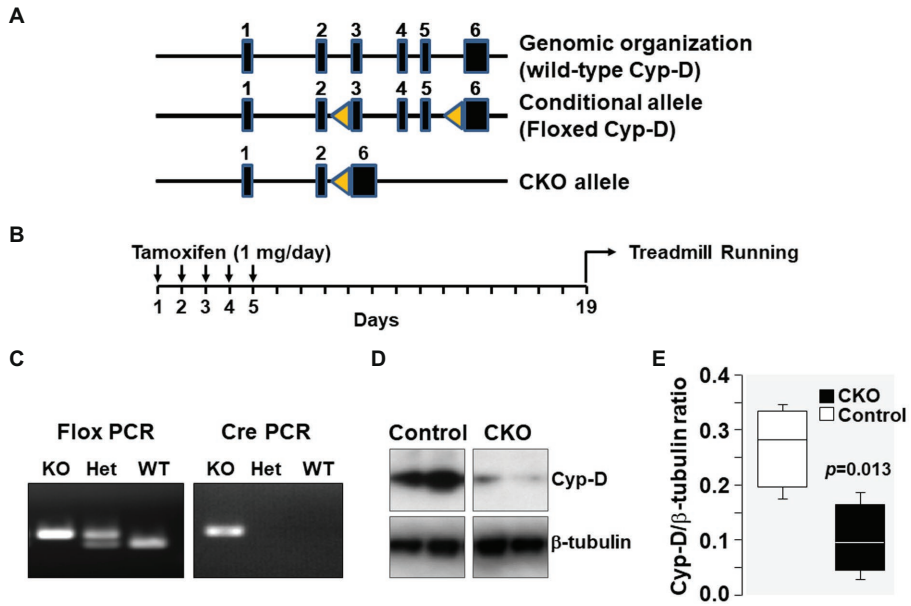

FIGURE 1 | (A) Genomic organization of Cyp-D encoding ppif gene in mouse chromosome 14 is shown. The ppif gene has six exons which are represented by closed boxes. Genomic organization of floxed Cyp-D in which exons 3–5 has flanking lox-P sites (yellow triangles) is shown as conditional allele. The floxed Cyp-D mouse was generated by Stanley J. Korsmeyer's lab. Genomic organization of conditional knockout (CKO) allele showing deletion of exons 3–5. **(B)** Schematic demonstrating time of tamoxifen treatment. **(C)** $Ppif^{Fl/+}$-$Cre^{/+}$ mice pups were genotyped to confirm the presence of $Ppif^{Fl/+}$ and Cre genes. **(D)** Western blot showing Cyp-D protein in skeletal muscle tissue homogenates of control and cyclophilin-D conditional knockout (CKO) mice. **(E)** Box plots denote densitometry of Cyp-D normalized to β-tubulin.

Western Blotting

The Western blotting was performed as previously described (Radhakrishnan et al., 2019).

Statistical Analysis

Linear mixed effect model was used to assess the effect of genotype and running sessions and their interaction on metabolic variables treating running sessions as discrete variable (SPSS 24.0, IBM Corp.; Armonk, NY, United States). Student's *t*-test was used to assess (i) the effect of genotype on metabolic variables collected at baseline and at exercise and (ii) the effect of genotype on molecular signaling implementing t-test (when normality test passed) and Mann-Whitney Rank Sum test (when normality test failed; SigmaPlot v.12.5). Strength of relationships between variables was assessed by linear regression and Pearson's product moment correlation analysis (SigmaPlot v.12.5). Data are presented as means ± SD in the text and means ± SEM in the figures. A two-tail value of $p \leq 0.05$ was considered significant.

RESULTS

Confirmation of Cyp-D Knockout

Genomic organization of the mouse genome at chromosome 14 and the strategy for conditional KO generation is shown in **Figure 1**. As mentioned, $Ppif^{Fl/+}$-$Cre^{/+}$ mice have Ppif allele flanked between two lox-P sites which upon tamoxifen injection will undergo Cre-mediated recombination resulting in deletion of the Ppif allele. $Ppif^{Fl/+}$-$Cre^{/+}$ mice pups were genotyped to confirm the presence of $Ppif^{Fl/+}$ and Cre genes. Results showed that the products of expected size were generated by flox PCR (400 bp) and by Cre PCR (200 bp). Tamoxifen injection resulted in Cre-mediated deletion of lox-P flanked Ppif allele and was confirmed by Western blotting detection of Cyp-D levels in mice skeletal muscle tissue homogenates (**Figure 1**).

Effect of Conditional Cyp-D Knockout on VO_2, VCO_2, and RER

At their first treadmill running session, as shown in **Figure 2A**, CKO mice and controls had comparable VO_2, VCO_2, and RER. However, with subsequent sessions, the VO_2 decreased in CKO mice without a significant change in VCO_2. The lower VO_2 for a given VCO_2 manifested in a significantly higher RER that remained statistically significant for most of the sessions (**Figure 2A**). This behavior is consistent with a metabolic adaptation induced by exercise training in CKO mice.

All individual VO_2, VCO_2, and RER measurements obtained at baseline – before starting treadmill exercise – combining the five sessions are shown in **Figure 2B**. CKO mice had a lower VO_2 ($p = 0.003$) and a lower VCO_2 ($p = 0.014$) but of lesser magnitude yielding a slightly higher RER which did not attain statistical significance ($p = 0.201$).

All individual VO_2, VCO_2, and RER measurements obtained during treadmill running combining the five sessions are shown

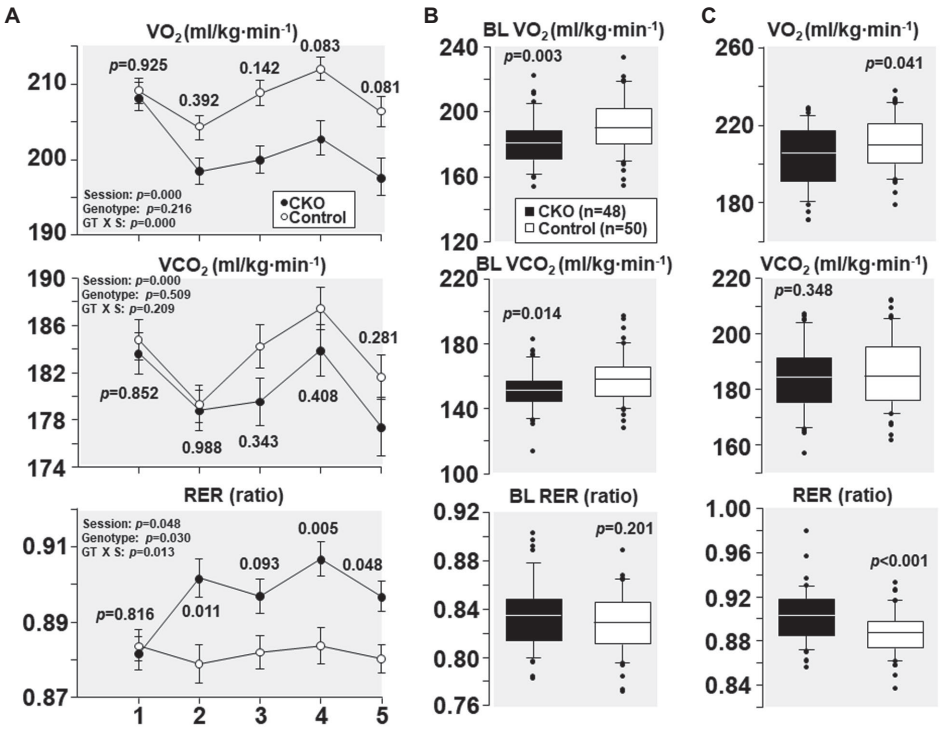

FIGURE 2 | (A) Oxygen consumption (VO_2), carbon dioxide production (VCO_2), and respiratory exchange ratio (RER) of cyclophilin-D conditional knockout (CKO; $n = 10$) and control mice ($n = 10$) at five running sessions. Values are shown as mean ± SEM. Differences were analyzed by a linear mixed-effects model. Data from one CKO mouse were lost at running sessions four and five due to refusal of running. **(B)** All individual oxygen consumption (VO_2), carbon dioxide production (VCO_2), and respiratory exchange ratio (RER) measurements obtained under baseline (BL) conditions in cyclophilin-D conditional knockout (CKO) and control mice are shown as box plots, comparing the groups using t-test. **(C)** Box plots depicting all individual data points of oxygen consumption (VO_2), carbon dioxide production (VCO_2), and respiratory exchange ratio (RER) during exercise at five running sessions by conditional cyclophilin-D knockout (CKO) and control mice. Differences were analyzed by t-test.

in **Figure 2C**. CKO mice also exhibited a lower VO_2 ($p = 0.04$) but without a significant difference in VCO_2 ($p = 0.348$), yet with a highly significant increase in RER ($p < 0.001$).

Effect of Conditional Cyp-D Knockout on Exercise Capacity

From the first and the subsequent sessions, CKO mice had a higher power and power/VO_2 but did not attain statistical significance (**Figure 3**). Yet, when all individual power and power/VO_2 ratio throughout the five running sessions were combined, CKO mice had a statistically significant higher power and power/VO_2 (**Figure 3**), consistent with increased oxygen utilization efficiency.

Effect of Conditional Cyp-D Knockout on Fuel Utilization

As shown in **Figure 4A**, at their first running session, the fuel utilization was comparable between CKO mice and controls. Yet, with subsequent running sessions, carbohydrate utilization was higher in CKO mice but the difference was not statistically significant. Likewise, fat utilization was also comparable between the genotypes at the first running session. However, with subsequent running sessions, fat utilization was lower in CKO mice attaining statistical significance in most of the running sessions (**Figure 4A**).

All individual carbohydrate and fat utilization at baseline – before starting treadmill exercise – combining the five sessions demonstrated comparable carbohydrate and fat utilization in CKO mice and controls (**Figure 4B**). All individual carbohydrate and fat utilization during exercise throughout the five running sessions showed increased carbohydrate and decreased fat utilization in CKO mice (**Figure 4C**).

Effect of Conditional Cyp-D Knockout on AMPK-TBC1D1 Nexus and Its Downstream Signaling

AMPK phosphorylation at Thr 172 – indicative of its activation – was increased by 64% and TBC1D1 phosphorylation Ser 660 – indicative of its inactivation – was increased by 55% in CKO mice skeletal muscles compared to control (**Figure 5**). Analysis of relationship between pAMPK and pTBC1D1 (**Figure 5**) showed a positive correlation which did not attain statistical significance ($R = 0.639$; $p = 0.08$).

PGC-1α levels increased by 162% and GLUT4 levels increased by 103% in CKO mice skeletal muscles compared to control (**Figure 6**). Analysis of relationship between GLUT4 and AMPK

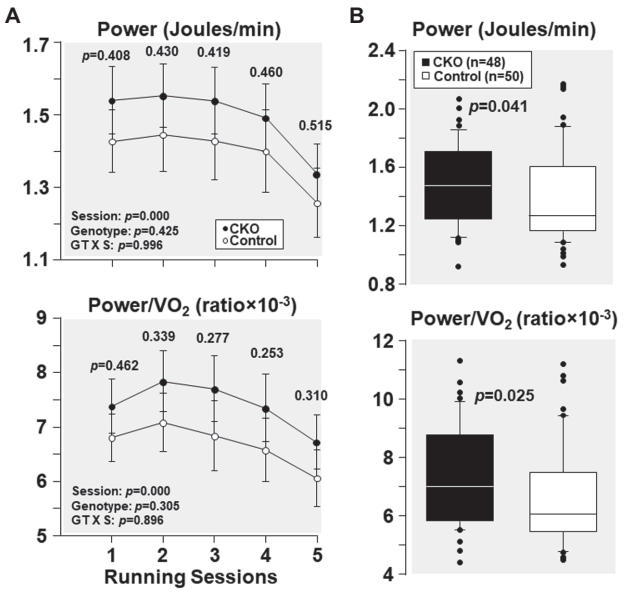

FIGURE 3 | (A) Power and power/VO_2, at five running sessions by conditional cyclophilin-D knockout (CKO; $n = 10$) and control mice ($n = 10$). Differences were analyzed by a linear mixed-effects model. Data from one CKO mouse were lost at running session four and five due to refusal of running. **(B)** Box plots depicting power (Joules/min) and power/VO_2 ratio during exercise in conditional cyclophilin-D knockout (CKO) and control mice. Differences were analyzed by Mann-Whitney rank sum test.

demonstrated a positive correlation without statistical significance ($R = 0.686$; $p = 0.061$).

The power/VO_2 exhibited a positive correlation with the pAMPK/β-tubulin ratio ($R = 0.753$; $p = 0.03$) and the PGC-1α/β-tubulin ratio ($R = 0.708$; $p = 0.04$; **Figure 7**).

DISCUSSION

Consistent with our previous studies showing that Cyp-D ablation resulted in downregulation of VO_2 in HEK 293 T cells and in constitutive KO mice, with increased oxygen utilization efficiency in the latter, the present studies demonstrate for the first time that conditional Cyp-D ablation in adult mice also reduces systemic VO_2 and increases oxygen utilization efficiency. Reduction in VO_2 was accompanied by higher power during treadmill exercise for a given VO_2 and was accompanied by increased RER consistent with preferential utilization of glucose over fat. We also found direct evidence of AMPK activation with concomitant TBC1D1 inactivation and increased GLUT4 expression in skeletal muscle tissues of Cyp-D CKO mice after exercise. Taken together, these studies demonstrate that conditional Cyp-D ablation in adults leads to an adaptive response that improves oxygen utilization efficiency and exercise capacity associated with activation of AMPK signaling, an effect that invites work on options for pharmacologically recapitulating the effect, which would be beneficial under oxygen limiting conditions.

Conditional Cyp-D Ablation Reduces VO_2

We have previously reported in HEK 293 T cells that Cyp-D silencing results in downregulation of mitochondrial transcripts and concomitant reduction in cellular oxygen consumption (Radhakrishnan et al., 2015). Further studies in constitutive Cyp-D KO mice demonstrated downregulation of VO_2 at rest and during endurance exercise (Radhakrishnan et al., 2019). In the present studies, we demonstrated that VO_2 in conditional Cyp-D KO adult mice at rest and during exercise was comparable to control mice in the first running session and decreased only with subsequent sessions. Decrease in VO_2 was accompanied by increased RER indicative of an adaptational response and training effect in CKO mice.

Conditional Cyp-D Ablation Improves Exercise Capacity and Induces Metabolic Switch With Concomitant Activation of AMPK-TBC1D1 Signaling Nexus

During exercise, in contracting muscles, activation of AMPK occurs consequent to AMP accumulation as ATP consumption increases (Winder and Hardie, 1996). AMPK activation occurs mainly through activation of the upstream liver kinase B1(kinase LKB1) through Thr172 phosphorylation of its catalytic domain in response to increased cellular AMP:ATP ratio (Hawley et al., 2003; Woods et al., 2003). Activated AMPK inactivates its downstream target TBC1D1 (Kramer et al., 2006; Treebak et al., 2006; Taylor et al., 2008; Ferrari et al., 2019; de Wendt et al., 2021) by phosphorylation (an interaction also known as "AMPK-TBC1D1 signaling nexus"; Chavez et al., 2008; Taylor et al., 2008; Vichaiwong et al., 2010; Treebak et al., 2014; Kjøbsted et al., 2016; Ferrari et al., 2019). TBC1D1 inactivation during exercise has been reported in mouse skeletal muscles (Taylor et al., 2008; Vichaiwong et al., 2010; de Wendt et al., 2021) and in human skeletal muscles (Jessen et al., 2011; Tobias et al., 2020). Activation of AMPK-TBC1D1 signaling nexus results in increased GLUT4 expression and translocation from the cytosol to the plasma membrane enabling increased glucose uptake. Glucose enters the glycolytic pathway and is metabolized in the cytosol to pyruvate and then to lactate by lactate dehydrogenase (LDH). The conversion of pyruvate to lactate is favored over alternative pyruvate fates given the near-equilibrium nature of the LDH reaction (Lambeth and Kushmerick, 2002) and a much higher activity than regulatory enzymes of the glycolytic and oxidative pathways (Rogatzki et al., 2015). Lactate then enters the mitochondria where it is converted to pyruvate by the LDH present on the mitochondrial inner membrane (Hashimoto et al., 2006; Gladden, 2008). Pyruvate is then converted to acetyl coA and metabolized in the Krebs cycle. Thus, the AMPK-TBC1D1 signaling nexus increases glucose uptake and enhances glucose utilization through glycolysis and the Krebs cycle. Consistent with this effect, we observed in the present study activation of AMPK-TBC1D1 signaling nexus (**Figure 5**) and increased GLUT4 expression (**Figure 6**) in CKO mice after exercise. Consistently, several other studies have also showed increased expression of skeletal muscle GLUT4 in rats and human after exercise (Ivy et al., 1999;

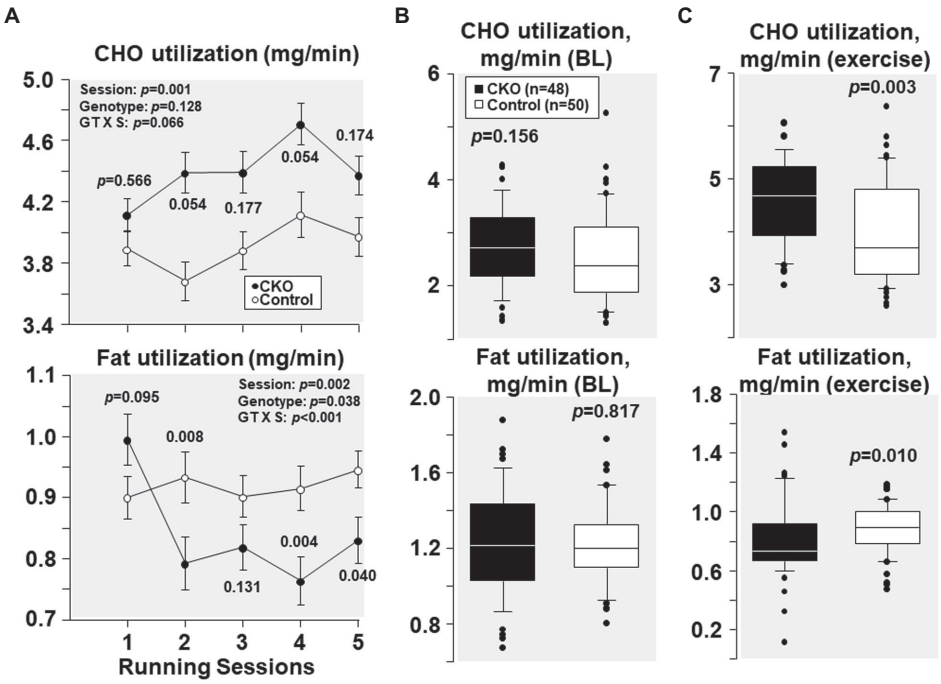

FIGURE 4 | (A) Carbohydrate (CHO) and fat utilization at five running sessions by conditional cyclophilin-D knockout (CKO; $n = 10$) and control mice ($n = 10$). Differences were analyzed by a linear mixed-effects model. Data from one CKO mouse were lost at running session four and five due to refusal of running. **(B)** Box plots depicting estimation of carbohydrate and fat utilization by conditional cyclophilin-D knockout (CKO) and control mice at baseline (BL) and **(C)** during exercise (all individual data points of all five running sessions). Differences were analyzed by t-test when normality test passed (CHO utilization) and by Mann-Whitney Rank Sum test when normality test failed (Fat utilization).

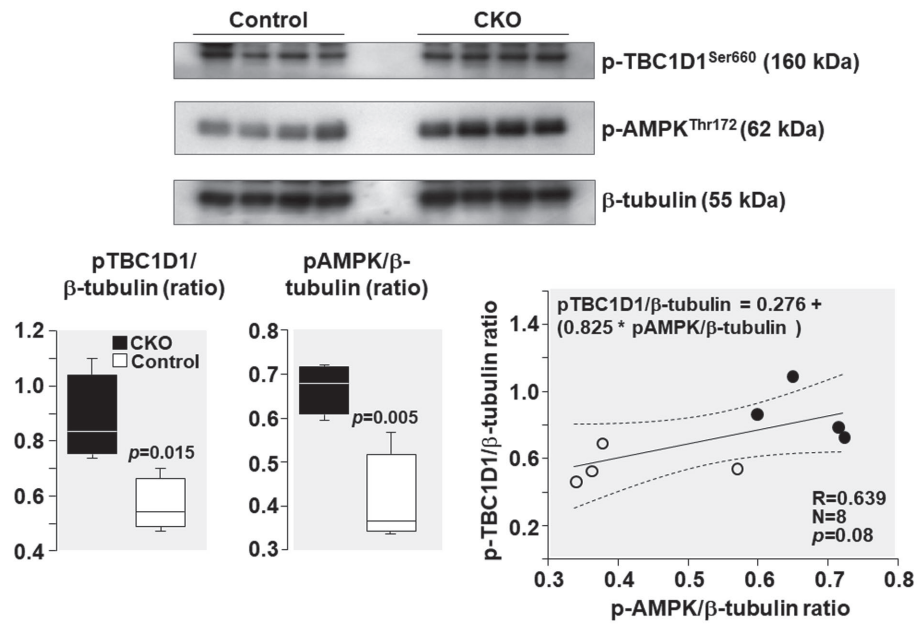

FIGURE 5 | Western blots demonstrating levels of AMPK phosphorylation at threonine 172 (Thr172), TBC1D1 phosphorylation at serine 660 (Ser 660), and β-tubulin (loading control) in mice skeletal muscles after exercise. Box plots denote densitometry of pAMPK and pTBC1D1 normalized to β-tubulin. $N = 4$ from each group. Relationship between pAMPK/β-tubulin ratio and pTBC1D1/β-tubulin ratio is shown. The regression line is shown with the corresponding 95% confidence interval.

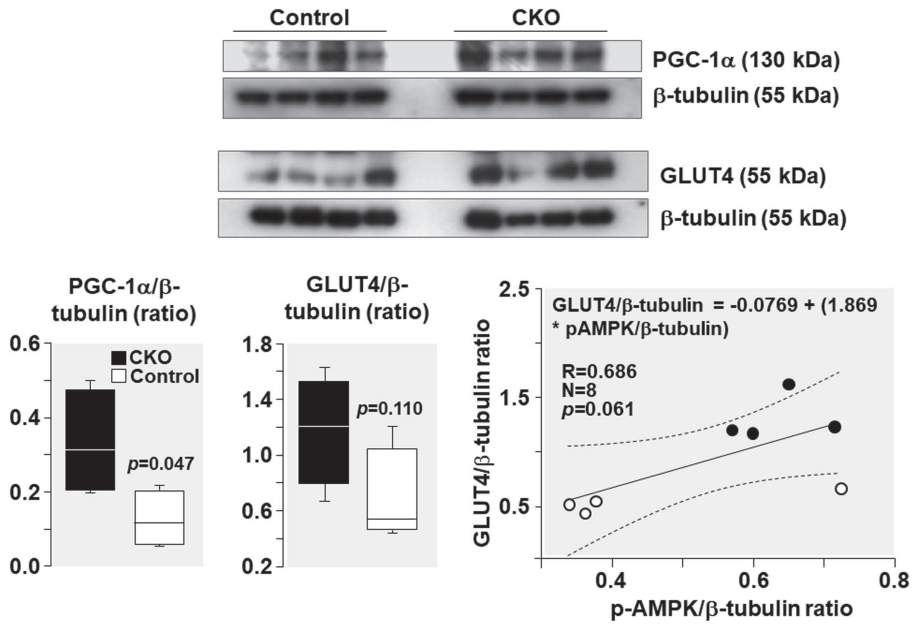

FIGURE 6 | Western blots demonstrating levels of PGC-1α, GLUT4, and β-tubulin (loading control) in mice skeletal muscle after exercise. Box plots denote densitometry of PGC-1α and GLUT4 normalized to β-tubulin. *N* = 4 from each group. Relationship between pAMPK/β-tubulin ratio and GLUT4/β-tubulin ratio is shown. The regression line is shown with the corresponding 95% confidence interval.

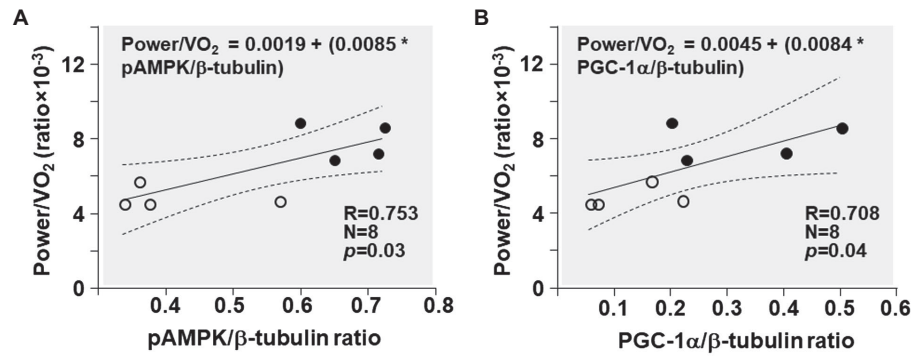

FIGURE 7 | Shown are the relationships between **(A)** pAMPK/β-tubulin ratio and power/VO_2 ratio and **(B)** PGC-1α/β-tubulin ratio and power/VO_2 ratio in CKO mice (black circles) and controls (white circles). The regression line is shown with the corresponding 95% confidence interval.

Kuo et al., 1999; MacLean et al., 2000; Flores-Opazo et al., 2020). Increased GLUT4 expression has been correlated with increased glucose transport and utilization (Kern et al., 1990; Ivy et al., 1999; MacLean et al., 2000).

In addition, AMPK activation also increases the expression of PGC-1α (as also shown in **Figure 6**). The effect occurs by phosphorylation at its threonine-177 and serine-538 residues prompting self-dependent activation of its promoter and gene expression (Jäger et al., 2007). PGC-1α further increases GLUT4 expression and thereby glucose uptake (Jäger et al., 2007). In addition, PGC-1α activates mitochondrial biogenesis and other pathways, which could improve the exercise capacity after training. Several lines of evidence suggest that PGC-1α is a key factor in mediating exercise training induced adaptations in mitochondria (Olesen et al., 2010) in response to AMPK activation. For example, AICAR – the AMPK activator – improves exercise capacity in mice with concomitant increase in PGC-1α gene expression (Narkar et al., 2008). In addition, AICAR injections fail to increase levels of mitochondrial and metabolic proteins in skeletal muscles of PGC-1α KO mice (Leick et al., 2010). Thus, AMPK in CKO mice could induce additional adaptive responses, including mitochondrial biogenesis *via* PGC-1α, leading to enhanced exercise capacity.

In our previous studies in constitutive Cyp-D KO mice, the aforementioned metabolic effects were associated with downregulation of electron transport complexes I and IV activities consequent to reduced expression of subunits encoded by the mitochondrial heavy strand promoter 2 (Radhakrishnan et al., 2015, 2019). The expression of these subunits

requires the interaction of Cyp-D with the mitochondrial transcription factors B1 and B2 (Radhakrishnan et al., 2015). The reduced activity of electron transport complexes I and IV would create an "energy-stress" (Gowans et al., 2013) during exercise prompting an increase in AMP:ATP ratio and the consequent activation of the AMPK-TBC1D1 signaling nexus. Other studies have also reported complex I inhibition as the mechanism of AMPK activation. In fact, the mechanism of pharmacological activation of AMPK by metformin involves electron complex I inhibition (Owen et al., 2000; Zhou et al., 2001; Mohsin et al., 2019). However, metformin abrogates exercise-mediated increase in skeletal muscle mitochondrial respiration (Konopka et al., 2019).

In our previous study in constitutive Cyp-D KO mice, we have documented a metabolic shift – i.e., increased glucose utilization over fat. Tavecchio et al. (2015) have documented in their study that Cyp-D constitutive KO mice had increased transcription of genes involved in glucose metabolism resulting in increased glucose utilization and impaired fatty acid utilization. In the present study in conditional Cyp-D KO mice, we have documented a similar adaptive response. In the present study, AMPK activation was accompanied by increased glucose utilization over fatty acids (as shown in **Figure 4**) that is energetically advantageous. Glucose oxidation per mole yields 6.3 moles of ATP, whereas fatty acid yields only 5.6 moles of ATP (Opie, 1991; Stanley and Chandler, 2002) resulting in a 12.5% increase in energy production with glucose oxidation. Thus, this metabolic switch enhances oxygen utilization efficiency during exercise and improves exercise capacity as evidenced by the significant increase in power during treadmill exercise for given oxygen consumed (power/VO_2 ratio; as shown in **Figure 3**).

Comparison Between Constitutive and Conditional Cyp-D KO

Both constitutive and conditional Cyp-D KO had their Cyp-D knocked-out in all tissues. Cyp-D ablation was carried out at an embryonic stage in constitutive KO, whereas Cyp-D ablation was carried out at the adult stage in CKO mice. Yet, the CKO mice displayed similar effects – VO_2 downregulation and metabolic switch – as that of constitutive KO but of lesser magnitude. The metabolic switch in CKO mice was prominent at the second running session and thereafter, as documented by increased glucose utilization over fatty acids (**Figure 4**). The first and second running sessions were performed at the third and the fourth week, respectively, after tamoxifen treatment for 5 days. This hints to the possibility that the metabolic switch can be triggered probably only 4 weeks after completion of pharmacological interventions. The time delay for this metabolic switch – we rationalize – includes the time required for cre/lox-P recombination-dependent Cyp-D ablation and subsequent respiratory chain downregulation. In addition, exercise training induced mitochondrial adaptations involving PGC-1α could also have played role in improving the exercise capacity. These results point to the fact that selectively destabilizing interaction between Cyp-D and mitochondrial transcription factors to reduce oxygen consumption without affecting other functions of Cyp-D in adults could have potential positive impact on oxygen utilization efficiency and it could be highly beneficial under oxygen limiting conditions.

Conclusion

Conditional Cyp-D ablation reduced VO_2 and increased power/VO_2 ratio, demonstrating increased oxygen utilization efficiency consistent with our previous work in constitutive KO mice. Conditional Cyp-D ablation also triggered the adaptive response which involved a metabolic switch toward increased glucose utilization with concomitant activation of AMPK-TBC1D1 signaling nexus and increased GLUT4 expression in skeletal muscles after exercise. Our study raises the possibility that the metabolic switch can probably be triggered in 4 weeks after completion of pharmacological intervention. Developing tools for modulation of this adaptive response could be beneficial in a myriad of physiological and clinical conditions where oxygen availability is limited.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at Rosalind Franklin University of Medicine and Science (Protocol B17-20) and by the Edward Hines VA Hospital Institutional Animal Care and Use Committee (Protocol H17-014).

AUTHOR CONTRIBUTIONS

JR and RJG conceived and designed the research and wrote the manuscript and agreed on the final version. JR performed the experiments with the assistance of AB. AB developed the software necessary for the experiments and data analysis. JR and AB analyzed the data. All authors contributed to the article and approved the submitted version.

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An Identical Twin Study on Human Achilles Tendon Adaptation: Regular Recreational Exercise at Comparatively Low Intensities Can Increase Tendon Stiffness

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Achilles tendon adaptation is a key aspect of exercise performance and injury risk prevention. However, much debate exists about the adaptation of the Achilles tendon in response to exercise activities. Most published research is currently limited to elite athletes and selected exercise activities. Also, existing studies on tendon adaptation do not control for genetic variation. Our explorative cross-sectional study investigated the effects of regular recreational exercise activities on Achilles tendon mechanical properties in 40 identical twin pairs. Using a handheld oscillation device to determine Achilles tendon mechanical properties, we found that the Achilles tendon appears to adapt to regular recreational exercise at comparatively low intensities by increasing its stiffness. Active twins showed a 28% greater Achilles tendon stiffness than their inactive twin ($p < 0.05$). Further, our research extends existing ideas on sport-specific adaptation by showing that tendon stiffness seemed to respond more to exercise activities that included an aerial phase such as running and jumping. Interestingly, the comparison of twin pairs revealed a high variation of Achilles tendon stiffness (305.4–889.8 N/m), and tendon adaptation was only revealed when we controlled for genetic variance. Those results offer new insights into the impact of genetic variation on individual Achilles tendon stiffness, which should be addressed more closely in future studies.

Keywords: Achilles tendon, connective tissue adaptation, genetic variation, exercise activities, aerial phase, sports, tendon stiffness, twin study

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INTRODUCTION

In humans, the gastrocnemius muscle inserts together with the soleus muscle *via* a well-developed Achilles tendon onto the calcaneus (Swindler and Wood, 1973; Standring et al., 2016). Particularly the anatomical length of the human Achilles tendon is outstanding among extant great apes from a comparative perspective. In our closest relatives, the chimpanzees (*Pan troglodytes*) and gorillas (*Gorilla gorilla*), the gastrocnemius muscle inserts almost immediately onto the calcaneus, so that the Achilles tendon is barely visible (Swindler and Wood, 1973; Myatt et al., 2011). Several studies consider the well-developed Achilles tendon as an adaptation that fosters energy-efficient locomotion (Alexander, 1984, 1991), particularly during bipedal running (Bramble and Lieberman, 2004). Extensive research has shown that during running, the Achilles tendon acts like a spring

element, which is stretched and loaded with strain energy during the initial phase of stance and recoils during the late phase of stance to support the foot push-off (Hof et al., 2002; Arampatzis et al., 2006; Lichtwark et al., 2007). While the evolutionary advantage of this adaptation seems generally accepted (Bramble and Lieberman, 2004), debate continues about structural adaptations of the Achilles tendon in response to exercise-related mechanical loading.

Generally, the Achilles tendon can adapt to external mechanical loading by increasing its stiffness, elastic modulus, and cross-sectional area (for review see Wiesinger et al., 2014; Bohm et al., 2015). The adaptation to external mechanical loading seems to depend on frequency, duration, and intensity. Data from several longitudinal training studies suggest that high muscle contraction intensities (i.e., 70–90% of maximum voluntary contraction) endured over several seconds are most effective in inducing structural changes within the tendon (for review see Bohm et al., 2015). It has been speculated that longer durations of tension during strength training exercises result in a more efficient transmission of the tendon strain *via* the extracellular matrix to the cytoskeleton of the tendon cells (Bohm et al., 2014). Consequently, the strain triggers cellular and molecular responses, such as the synthesis of collagen and matrix proteins, thereby affecting the Achilles tendon's mechanical and morphological properties (Wang, 2006; Heinemeier and Kjaer, 2011; Galloway et al., 2013). The loading stimulus duration argument has recently been applied to explain why jumping results in less pronounced adaptive responses of Achilles tendon properties than an exercise protocol with longer loading duration (i.e., 3 s) (Bohm et al., 2014).

However, several cross-sectional studies on running and jumping report changes in the Achilles tendon's cross-sectional area and/or stiffness, despite much shorter strain durations (i.e., 0.2–0.25 s). For example, Rosager et al. (2002) and Magnusson and Kjaer (2003) detected a significantly larger Achilles tendon cross-sectional area in trained runners than non-runners. Similarly, elite runners and volleyball players had larger Achilles tendon cross-sectional areas than elite kayak athletes, whose training includes less frequent mechanical stimulation of the lower extremities (Kongsgaard et al., 2005). In the same vein, Wiesinger et al. (2016) showed greater Achilles tendon cross-sectional areas in highly trained endurance runners and world-class ski jumpers compared to national-level elite water polo players and sedentary individuals. Notably, within the same study it was found that differences in the cross-sectional area were not consistently mirrored by differences in stiffness. Here, tendon stiffness was only higher in ski jumpers than in sedentary individuals but did not differ between runners and water polo players. In contrast, Karamanidis and Epro (2020) measured a significantly increased Achilles tendon stiffness in elite high and long jumpers compared to age-matched controls. Those results, although partly inconclusive, suggest that chronic exposure to repetitive loading by running and jumping can result in tendon adaptation.

An essential characteristic of running and jumping movements is a repetitive aerial phase, during which the athlete lifts both feet off the ground and then freely falls under gravity before touching the ground again (Zaciorskij, 2000). With touchdown, at the end of an aerial phase, the impact exposes the Achilles tendon to mechanical loading that can be magnitudes greater than during activities with no aerial phase, such as walking (Lichtwark et al., 2007; Lai et al., 2015; Kharazi et al., 2021). Particularly the pioneering work by Komi et al. (1987, 1992) and Fukashiro et al. (1995) revealed that Achilles tendon forces are highest in activities with an aerial phase. Also, the Achilles tendon undergoes considerable length changes (i.e., 5.6% for running and up to 8.2% for single-leg hopping) (Lichtwark and Wilson, 2005, 2006) within stretch-shortening cycles. While numerous research indicates that exercise activities with a regular aerial phase, respectively, exercise including stretch-shortening cycles, such as running and hopping, do not appear to induce immediate mechanical or morphological changes in the Achilles tendon (Obst et al., 2013), data on long-term tendon adaptation are rare. Regarding long-term effects, it is reasonable to speculate that individuals who regularly engage in exercise activities with an aerial phase (e.g., running, basketball, or tennis) show more pronounced Achilles tendon adaptations than individuals who engage in exercise activities without an aerial phase (e.g., cycling, inline skating, swimming).

Although there is evidence for enhanced adaptations in runners and jumpers, the generalizability of the existing research is problematic because those studies are often limited to elite athletes. Additionally, those studies do not control for genetic variation, even though there is evidence that the genome of elite athletes varies in some alleles compared to non-elite athletes (for review see Macarthur and North, 2005). Currently, it cannot be excluded that genetic variation also affects baseline Achilles tendon mechanical properties. One promising approach to overcome the challenge of genetic variation might be studying identical (monozygotic) twins. Identical twins derive from a single fertilized egg and inherit identical genetic material (Boomsma et al., 2002). There is a growing body of literature on identical twins that highlights the critical role played by genetic variation on strength and fitness outcomes (Marsh et al., 2020), bone metabolism (Smith et al., 2003), and exercise-induced muscle damage (Gulbin and Gaffney, 2002). However, no twin study has investigated the effect of exercise activities on Achilles tendon properties.

In light of the controversial literature on Achilles tendon adaptation and the potential impact of genetic variation, this study attempted to find evidence that long-time regular exercise activities, even at a recreational level, can trigger Achilles tendon adaptation. Another objective was to determine whether exercise activities with a regular aerial phase have greater effects on tendon adaptation. To this end, the experiment was designed as an explorative cross-sectional twin study with recreationally active or non-active identical twins. The experimental design allowed us to control for genetic variation. We measured Achilles tendon stiffness to evaluate tendon adaptation in response to regular exercise activities at a recreational level. Research shows that, in addition to tendon morphological properties such as the cross-sectional area, tendon mechanical stiffness increases in response to mechanical loading (Arampatzis et al., 2007; Bohm et al., 2014). Thus, changes in tendon stiffness are indicative

of tendon adaptation. By comparing Achilles tendon stiffness among identical twin pairs, we aimed to test two hypotheses: (1) Regular recreational exercise activity leads to greater Achilles tendon stiffness compared to no regular exercise. (2) Exercise activities with an aerial phase lead to greater Achilles tendon stiffness than exercise activities without an aerial phase.

MATERIALS AND METHODS

Participants and Experimental Design

Forty identical twin pairs (19 female and 21 male pairs) participated in this explorative cross-sectional study. All participants (mean age: 40 ± 18 years; body mass: 61 ± 18 kg; height: 162 ± 17 cm) were required to be healthy, with no injuries of the lower limbs within the last 6 months. Each participant gave written informed consent to participate in the study. The study was approved by the local ethics committee of the Faculty of Behavioral and Social Sciences and conducted according to the Declaration of Helsinki.

All participants answered a questionnaire, including questions about regular physical activity ("Do you participate in regular physical activity?"), kind of physical activity ("If yes, what kinds of physical activities do you participate in?"), weekly training load ("How many hours do you spend exercising each week?"), and, if they remembered, total years of training ("For how many years have you participated in this sport regularly?"). Participants were considered physically active if they followed a regular training regime of 60 min/week for at least 1 year, regardless of intensity. The different exercise activities were divided into sports with and without an aerial phase. Sports with an aerial phase had to be characterized by regular movements with both feet off the ground. Three investigators (KL, NCK, and FS) evaluated all sports independently and agreed with their decision on regular aerial phases. Sports without an aerial phase include bouldering, cycling, hiking, horse riding, nordic walking, pilates, resistance training (excluding plyometric training), speed skating, swimming, water gymnastics, weightlifting, and yoga. Sports with an aerial phase include indiaca, running, soccer, tennis, and trampoline.

Once the questionnaire was answered, we determined and marked the middle of the free Achilles tendon in the twin pairs. Free Achilles tendon length is defined as the distance from the most distal insertion of the soleus muscle in the Achilles tendon to the distal insertion of the tendon at the calcaneus (Kongsgaard et al., 2005). According to Kongsgaard et al. (2005), the average free Achilles tendon length is about 10% of the lower leg length. Therefore, we measured the lower leg length in one of the identical twins in a standing position, as the distance from the palpated knee-joint gap (the gap between the femoral and fibular bone at the lateral side) to the floor. Then, we calculated the free Achilles tendon length (0.1 × lower leg length) and used that measure to draw a line from the palpated distal insertion of the tendon at the calcaneus toward the distal insertion of the soleus muscle (**Figure 1**). Next, we marked the middle of that line for the stiffness measurement. The same parameters were used to define the middle of the free Achilles tendon in the other twin, assuming anthropometric similarity in identical twins (Chatterjee et al., 1999). Within-pair variance in knee height of identical twins has been reported to be 0.24 cm (Chatterjee et al., 1999), suggesting that differences in Achilles tendon length are most likely equally small. Then, both twins had to sit on a chair with their knees bent at 90° and their feet positioned at a 20° dorsiflexion angle on a self-built construction (**Figure 1**). This position allowed us to measure the Achilles tendon stiffness in a relaxed state while applying a defined ankle angle (Davis et al., 1999; Orishimo et al., 2008; Hug et al., 2013; DeWall et al., 2014).

Stiffness Measurement

To measure tendon stiffness, we used a handheld oscillation device (MyotonPRO®, Myoton AS, Tallin, Estonia). The device applies an external mechanical impulse to the surface of a tendon and, thereby, compresses the underlying tissue. An accelerometer then registers the response of the tendon in the form of a damped oscillation curve. Consequently, the amplitude and frequency of the sinusoidal curve are used as a surrogate measure of tendon stiffness (Schneider et al., 2015). The easily accessible and cost-effective device has already been used in previous studies to test its validity and reliability and to detect changes in Achilles tendon stiffness non-invasively (for review see Sichting and Kram, 2020). Regarding the quality of stiffness assessment, several studies have demonstrated that the use of the handheld device produces valid (Pruyn et al., 2016; Feng et al., 2018) and reliable results. Data consistently suggest good to excellent reliability for repeated measurements with intra-class correlations (ICC) of 0.87 (95% confidence interval: 0.61–0.96) (Ko et al., 2018), 0.83 (0.67–0.91) (Pruyn et al., 2016), and 0.90 (0.76–0.96) (Liu et al., 2018). Further, Schneebeli et al. (2020) reported a standard error of 10.8 N/m and a minimum detectable change of 30.0 N/m for intra-rater measurements of the relaxed Achilles tendon.

In our setup, we measured the Achilles tendon stiffness approximately in the middle of the free Achilles tendon. During each measurement, the device applied five consecutive short-term mechanical impulses (force: 0.4 N, impulse time: 15 ms), each separated by 1 s to allow for the vibrations to dissipate before the next impulse began. The mean of the five consecutive impulses was used to calculate stiffness. It should be noted that tendon stiffness derived from oscillation-based measurements differ quantitatively from stiffness values derived from ultrasound supported dynamometry.

Statistics

We performed several analyses to test the hypothesis that regular recreational exercise activity leads to greater Achilles tendon stiffness than no regular exercise. We tested this hypothesis twice, (1) with and (2) without controlling for genetics. First, the unequal variance *t*-test of unrelated data (Welch's *t*-test) (Ruxton, 2006) was applied to compare Achilles tendon stiffness in physically inactive and physically active twin pairs (both twins inactive vs. both twins physically active). This analysis aimed to test Achilles tendon adaptation to exercise activities without controlling for genetic predisposition. To control for age, we applied the Welch's *t*-test to compare age in those twin pairs. Previous research has shown that age can affect Achilles tendon

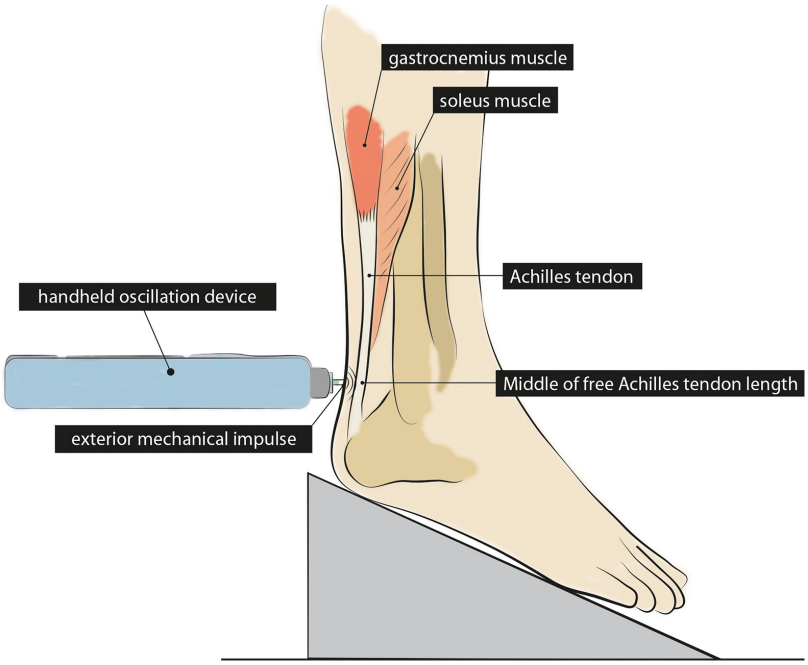

FIGURE 1 | Illustration of the Achilles tendon stiffness measurement with the foot placed in a 20° dorsiflexed position. The stiffness of the Achilles tendon was measured in the middle of the free Achilles tendon length using a handheld oscillation device. The device applied a mechanical impulse to measure the oscillation response of the Achilles tendon as a surrogate measure of stiffness.

stiffness (Waugh et al., 2012; Delabastita et al., 2018). Another Welch's *t*-test was applied to compare Achilles tendon stiffness in those twin pairs, in which twin A was regularly physically active while twin B was not (twin A vs. twin B). This analysis aimed to control for genetic predisposition. In addition to that, absolute differences in Achilles tendon stiffness were calculated for those twin pairs, in which both twins were physically inactive, and those twin pairs, in which twin A was regularly physically active, while twin B was not. The Welch's *t*-test was applied to compare the two twin pairs. Finally, we plotted Achilles tendon stiffness of twin A against Achilles tendon stiffness of twin B for inactive twins pairs and those twin pairs, in which twin A was regularly physically active while twin B was not. Pearson correlation coefficients (r) were calculated to establish relationships between stiffness measurements of twin A and twin B. Correlations below 0.4 were qualitatively interpreted as weak, between 0.4 and 0.75 as moderate, and above 0.75 as strong (Fleiss, 1981).

For our second hypothesis, we predicted that individuals who engage in an exercise activity with an aerial phase show greater Achilles tendon stiffness than individuals who engage in an exercise activity without an aerial phase. To test this hypothesis, we compared mean stiffness differences in those twin pairs, in which both twins participated in a sport without an aerial phase against those twin pairs, in which at least one twin participated in a sport with an aerial phase using the Welch's *t*-test. For all comparisons, we performed a power analysis to determine the effect size (Cohen's d) of the results ($\geq$ 0.8 = large; $<$ 0.8–$>$ 0.2 = medium; $\leq$ 0.2 = small) (Cohen, 2013). All statistical analyses were carried out using R Studio (R Foundation for Statistical Computing, Vienna, Austria). The level of significance was set at $\alpha = 0.05$.

RESULTS

Across all twin pairs (**Figure 2**), 17 different sports were performed for an average weekly duration of 4.0 ± 3.5 h. Thirty-eight participants were able to remember when they began their respective sport. Those participants performed their sport regularly for 16 ± 15 years (range: 2–45 years). Achilles tendon stiffness measures showed large variation between monozygotic twin pairs, ranging from 305.4 to 694.4 N/m in inactive twins, whereas in active twins measures ranged from 306.2 to 889.8 N/m (**Figure 3**).

Further comparison between physically active (both twins active) and inactive twin pairs (both twins inactive) did not reveal any significant differences (n_{active} = 25: 619.1 ± 121.5 N/m vs. $n_{inactive}$ = 8: 605.0 ± 46.1 N/m) (**Figure 4A**). There was also no significant difference in age between those twin pairs (active: 41 ± 20 years vs. inactive: 36 ± 19 years). However, for twin pairs, in which only one twin was regularly physically active ($n_{oneactive}$ = 7), Achilles tendon stiffness was significantly higher in the active (636.0 ± 115.5 N/m) compared to the inactive twin (496.8 ± 142.7 N/m), on average by 28.0% ($p < 0.01$) (**Figure 4B**). Cohen's d was 0.59, indicating a moderate effect. The correlation analysis revealed a strong correlation between twin A and twin B, both for inactive twin pairs ($r = 0.83$) and those twin pairs, in which twin A was regularly physically active while twin B was not

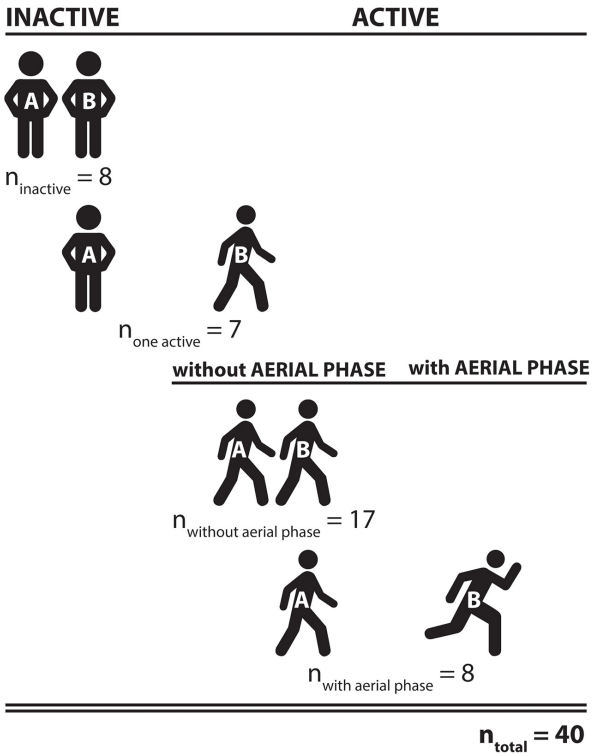

FIGURE 2 | Summary of identical twin pairs who participated in the study. Among all 40 twin pairs, both twins were physically inactive in eight pairs, activity differed in seven pairs (twin A was regularly physically active, twin B was not), and both twins were regularly physically active in 25 pairs. The regular exercise activity in those 25 twin pairs included aerial phases in at least one of the twins activities in 8 pairs and no aerial phases in 17 pairs.

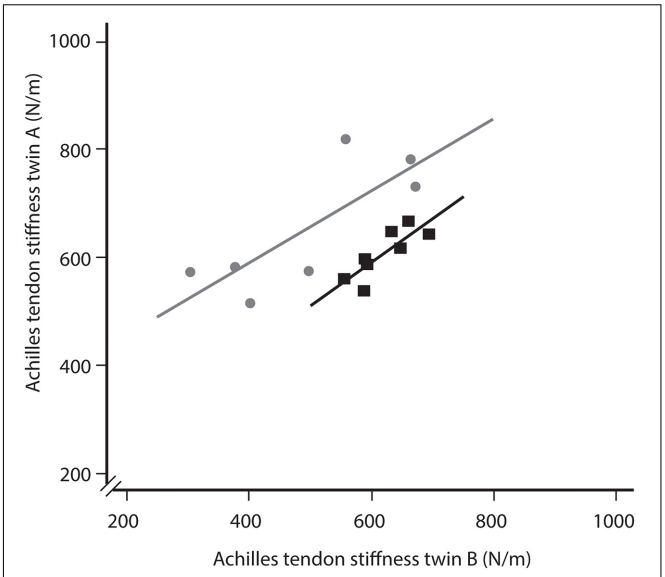

FIGURE 3 | Comparison of Achilles tendon stiffness between identical twins, in which either both twins were inactive (black squares) or one twin was active while the other was not (gray dots). For the latter comparison twin A represents the active twin and twin B represents the inactive twin. The offset between the two regression lines indicates a greater Achilles tendon stiffness in the active twins.

($r = 0.79$) (**Figure 3**). Notably, the was a clear offset between the regression lines, representing inactive twin pairs and those twin pairs, in which twin A was regularly physically active while twin B was not. The offset might reflect the greater Achilles tendon stiffness in active twins compared to their inactive siblings.

In terms of differences in Achilles tendon stiffness between identical twins, the mean absolute stiffness difference in inactive twin pairs was significantly smaller than in pairs with one active and one inactive twin ($n_{inactive} = 8$: 30.5 ± 26.1 N/m vs. $n_{oneactive} = 7$: 139.2 ± 86.6 N/m, $p = 0.02$) (**Figure 5A**). Cohen's d was 0.84, indicating a large effect. Further, the mean absolute difference in Achilles tendon stiffness between identical twins who both performed a sport without an aerial phase was significantly smaller than between twins, in which at least one twin performed in a sport with an aerial phase ($n_{noaerialphase} = 17$: 57.9 ± 47.7 N/m vs. $n_{aerialphase} = 8$: 160.6 ± 74.6 N/m, $p < 0.01$) (**Figure 5B**). Cohen's d was 0.96, indicating a large effect.

DISCUSSION

The present study was primarily designed to determine the effect of regular recreational exercise activity on Achilles tendon stiffness. To control for genetic variation, this study was conducted as an identical twin study. We identified a large variability in tendon stiffness among twin pairs, which seems at least partially genetically predetermined. When controlling for genetic preposition, the results of our study indicate that Achilles tendon stiffness was greatest in those individuals who regularly engaged in recreational exercise activities. Moreover, we found differences in Achilles tendon stiffness between individuals who engaged in an exercise activity with an aerial phase and those without an aerial phase. These findings add novel insights to the current understanding of tendon adaptation.

Our comparison of identical twin pairs indicates that Achilles tendon stiffness appears to be at least partially genetically predetermined. The correlation analysis of inactive twin pairs and twin pairs with one active twin revealed a strong linear relationship between identical twin pairs in Achilles tendon stiffness. Further, we found a considerable variation in tendon stiffness between twin pairs, ranging from nearly 300 N/m to a threefold of that tendon stiffness. Certainly, this variability between twin pairs would be expected if they were doing different levels of activity. However, against this prediction, we found a considerable overlap in tendon stiffness in inactive and active twin pairs (305.4–694.4 N/m vs. 306.2–889.8 N/m, respectively). This observation may support the hypothesis that Achilles tendon stiffness is partially genetically predetermined. Further, it lets us speculate about interindividual variation in Achilles tendon adaptation in response to mechanical loading. In this regard, data from genetic analyses indicate a significant interindividual variation in collagen fibril assembly and vulnerability to develop symptoms of chronic Achilles tendinopathy (Mokone et al., 2006; Hay et al., 2013). Related research by Passini et al. (2021)

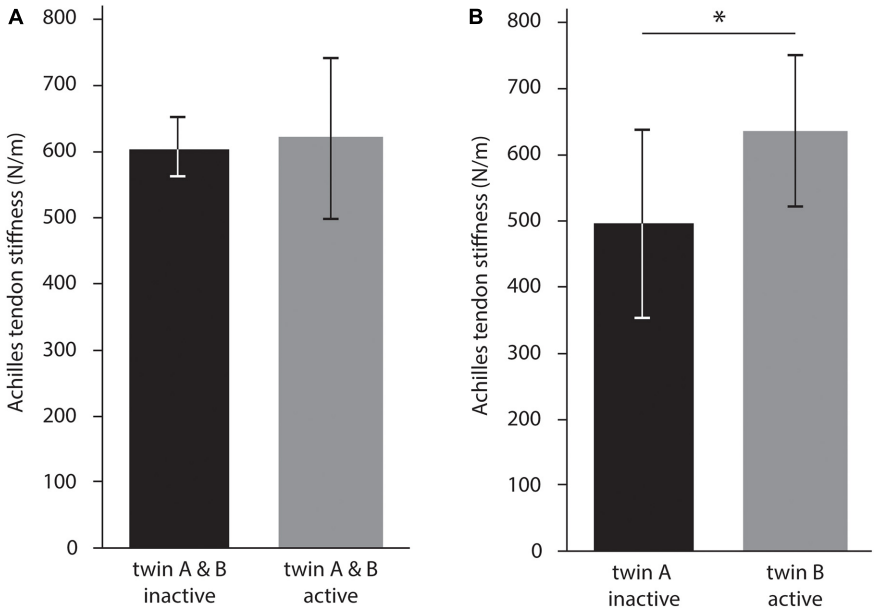

FIGURE 4 | Analysis of the impact of regular exercise activity on Achilles tendon stiffness. **(A)** Compares inactive and active twin pairs. In contrast, **(B)** compares identical twin pairs where twin A is inactive and twin B is active. The latter comparison allowed to control for genetic predisposition and showed a significant difference, indicated by the asterisk (*$p < 0.05$).

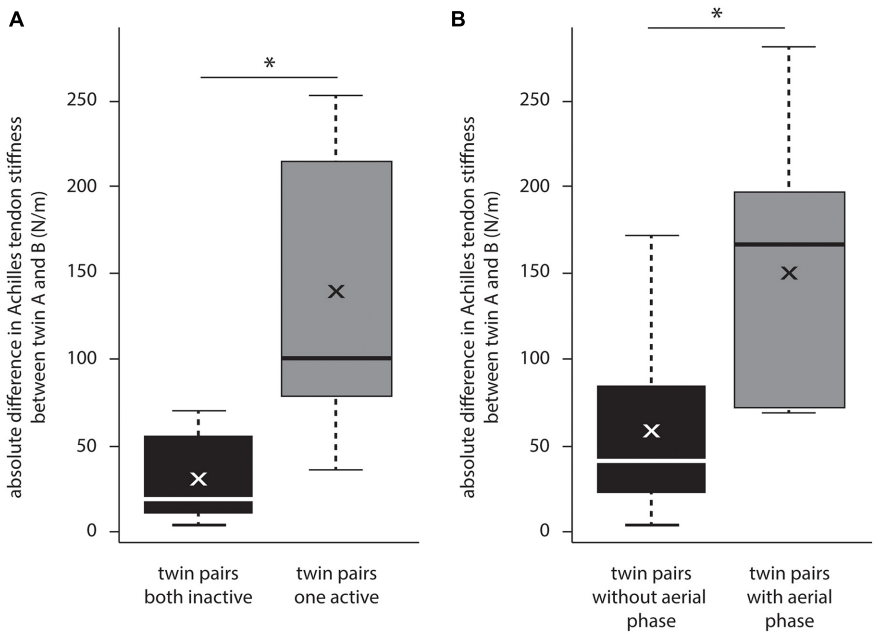

FIGURE 5 | Comparison of Achilles tendon stiffness within identical twin pairs, expressed as absolute difference in Achilles tendon stiffness. **(A)** Shows the significant impact of regular exercise activities. **(B)** Shows the significant impact of an aerial phase on differences in Achilles tendon stiffness. Significant differences ($p < 0.05$) are indicated by an asterisk.

further shows that genetic mutations in the ion channel *PIEZO1*, which regulates the mechanosensitive function of tenocytes, can affect tendon properties. Similar results of a potential genetic predisposition have also been found for bone properties (Smith et al., 2003) and muscle strength (Marsh et al., 2020). While considerably more work needs to be done to understand the genetic influence on Achilles tendon stiffness and adaptation, our findings suggest that genetic predisposition should be considered when investigating tendon adaptation to exercise. The need for such a consideration of genetics becomes also apparent

in our analyses of the impact of regular exercise activities on Achilles tendon stiffness. When we did not control for genetics by comparing physically inactive and active twin pairs (both twins inactive vs. both twins physically active), tendon stiffness was slightly but not significantly increased in active twin pairs (increased by 2.3%). In contrast, when we did control for genetics by comparing twin pairs, in which twin A was regularly physically active while twin B was not, tendon stiffness was significantly increased by 28.0% in the active twin. These findings on the role of genetics might help explain the controversial results on the effect of exercise activities in previously published cross-sectional studies.

Another important factor to consider when investigating tendon adaptation to exercise appears to be the presence of an aerial phase during movement. Our findings suggest that sports that include an aerial phase affect Achilles tendon stiffness more than sports without an aerial phase. Achilles tendon differences were greatest among twin pairs in which at least one twin participated in a sport with an aerial phase. In contrast, differences in Achilles tendon stiffness were relatively small when both twins participated in a sport with no aerial phase. This finding is consistent with data obtained by Wiesinger et al. (2016), who found that Achilles tendon stiffness in elite athletes with a regular aerial phase (ski jumpers) was considerably higher than in athletes without an aerial phase (water polo athletes). A potential influencing factor may be the stretch-shortening cycle of the Achilles tendon during exercise activities with an aerial phase. Previous research indicates that the stretch-shortening cycle in exercise activities with an aerial phase can be characterized by considerable stretching of the Achilles tendon during landing to store energy. Most of this energy is rapidly released during shortening to contribute to the power generated by the muscle-tendon unit during the propulsive phase (Maganaris and Paul, 2002; Ishikawa et al., 2005). Although there is evidence that those regular stretching stimuli have no immediate effect on Achilles tendon stiffness (Obst et al., 2013), there is much less information about long-term effects. However, research on long-term bone tissue adaptation might support the idea that exercise activities with an aerial phase have the greatest effect on Achilles tendon adaptation. A considerable body of literature shows that bone adaptation is greatest in high-impact sports, such as gymnastics, running, soccer, or rugby (e.g., Nordström et al., 1998; Daly et al., 1999; Lima et al., 2001; Morel et al., 2001; Scerpella et al., 2003; Yung et al., 2005; Maïmoun et al., 2013). While the underlying mechanism for the differences in bone adaptation is still part of an ongoing debate, it has been speculated that those high impacts are followed by bone deformation induced by high muscle forces pulling on the bone (Snow-Harter et al., 1990; Madsen et al., 1993; Nordström et al., 1998), which may result in larger adaptation. Large muscle forces pulling on the tendon during landing and propulsion may also help explain the observed differences in Achilles tendon stiffness in individuals who engaged in a sport with and without an aerial phase. However, our results also show large variation in Achilles tendon stiffness between twins being active in the same category of exercise. This variation might be explained by considerable differences in impact forces, acting muscle forces, and impact frequencies. For example, previous research has shown reasonable differences in Achilles tendon strain magnitude between participants who are forefoot or rearfoot strikers (Rice and Patel, 2017).

Interestingly, our findings on the impact of an aerial phase on Achilles tendon adaptation contrasts with an argument by Kharazi et al. (2021), who recently investigated Achilles tendon strain during walking and running. Their results let them speculate that submaximal running at up to 3.5 m/s does not provide sufficient tendon loading magnitude for triggering improvements of the Achilles tendon stiffness (Kharazi et al., 2021). This argument builds upon former studies that found no difference in Achilles tendon stiffness between runners and untrained individuals (Karamanidis and Arampatzis, 2006; Arampatzis et al., 2007). However, none of these studies controlled for genetic variation. Likely, those previous results were masked by a large natural variation in Achilles tendon properties. Our study allowed us to control for genetics. Particularly the comparison within inactive twin pairs supports the argument for a genetic predisposition. Here, we found a strong correlation and high similarity between twin A and twin B, with significantly smaller mean differences in Achilles tendon stiffness (30.5 $\pm$ 26.1 N/m) compared to twin pairs with one active and one inactive twin (139.2 $\pm$ 86.6 N/m). Also, there was a large variation between those inactive twin pairs, ranging from nearly 300 to almost 700 N/m. Most likely, the close similarity in Achilles tendon stiffness between twin A and twin B and the considerable variation between twin pairs can be explained genetically. That said, additional data from dizygotic twins could provide more definitive evidence for heritability in Achilles tendon stiffness (Boomsma et al., 2002).

Another finding that adds to the existing literature on exercise-related Achilles tendon adaptation refers to the activity level and response to habitual exercise. Currently, most research is limited to elite athletes (e.g., Rosager et al., 2002; Magnusson and Kjaer, 2003; Kongsgaard et al., 2005; Wiesinger et al., 2016; Karamanidis and Epro, 2020). In our study, however, all participants followed their exercise activity at a recreational level for an average weekly duration of 4.0 $\pm$ 3.5 h. Still, we found differences in Achilles tendon stiffness between active and non-active twins which could be attributed to habitual exercise. In contrast to this finding, Hansen et al. (2003) found no changes in Achilles tendon properties (i.e., cross-sectional area and stiffness) in previously untrained individuals after 9 months of habitual running (about 78 sessions and 43 h). Possibly, 9 months of habitual exercise were still not long enough to achieve measurable tendon adaptation, and tendons may need more time to adapt due to the generally low tissue turnover rate in tendons (Heinemeier et al., 2013). In our study, participants were considered active if they performed their sport regularly for at least 1 year. However, on average, they performed their sport regularly for more than 15 years (range: 2–45 years), which is a very long duration of regular mechanical stimulation of the tendon. The long exposure to regular activity raises the possibility that the Achilles tendon had sufficient time to adapt slowly to habitual exercise activities even at a recreational activity level, with structural adaptations accumulating and eventually becoming detectable. Thus, the investigation duration might be another factor besides genetic

predisposition that can help to explain the controversial results on changes in Achilles tendon stiffness. Further research is required to fully understand the factors that trigger Achilles tendon adaptation in response to habitual exercise activities.

To build further confidence in our results more twin pairs with different athletic profiles (active vs. non-active, or aerial phase vs. no aerial phase) would help. Our study shows that most identical twins share a very similar lifestyle, including similar exercise activities. However, we did not collect data on exercise activities during childhood and adolescence. It is reasonable to speculate that exercise activities during childhood and adolescence might have affected tendon properties in adult individuals. Research on the life-long turnover of human tendon tissue indicates that the tendon core is formed during height growth and is essentially not renewed after that (Heinemeier et al., 2013). Thus, exercise activities during childhood and adolescence might be another factor that impacts Achilles tendon properties in adults. It should further be noted that our interpretation of the role of an aerial phase is currently limited to a comparison of twin pairs in which at least one twin performed in a sport with an aerial phase. A comparison of twin pairs in which one had performed an aerial phase sport while the other did not would have been a more valid analysis. Unfortunately, our sample of twin pairs included only two pairs who fulfilled that criterion. For that reason, we decided to run the analysis as presented. Additional caution should also be taken when interpreting the classification approach of the different sports. Here, we used a qualitative approach to classify the various exercise activities. Although this approach allows a relatively simple classification, it does not control or account for the variation in tendon load history. Future studies should aim to quantify impact forces and frequencies, as well as Achilles tendon strain.

Being limited to the handheld oscillation device for stiffness measurements, this study does not provide information about morphological adaptation, such as the tendons' cross-sectional area or other relevant material properties, such as the elastic modulus. Those properties should be assessed to develop a more comprehensive understanding of tendon adaptation. That said, a study similar to this one could be carried out using ultrasound and dynamometry. Also, ultrasound would be a more precise method to determine free Achilles tendon length. Previous research indicates considerable variation in the length of the free Achilles tendon length (Drakonaki et al., 2021), but also between runners and non-runners (Devaprakash et al., 2020). Although we followed a standardized protocol to determine the middle of the free Achilles tendon, caution must be applied, as our approach does not guarantee that the stiffness measurements were acquired within the free portion of the tendon. Ultrasound could also help improving the definition of anatomical landmarks, like muscle-tendon or tendon-bone insertions. Advanced ultrasound technologies, like shear wave elastography, might be a consequent next step to measure stiffness properties along the complete free Achilles tendon, instead of using one measurement point as done in this study.

Notwithstanding these limitations, our study provides first evidence that the individual Achilles tendon stiffness is partially determined by genetic variation. In addition, the study has also indicates that exercise activities at a recreational level can trigger Achilles tendon adaptation if stimulated regularly for years. Our classification of exercise activities revealed that activities with a regular aerial phase seem to have the greatest impact on Achilles tendon adaptation. This finding extends an existing idea on sport-specific tendon adaptation presented by Wiesinger et al. (2016), which is currently limited to three sports activities (ski jumping, running, water polo) at an elite level. Our more general finding might also have implications for Achilles tendinopathy, an overuse injury of the Achilles tendon. When reviewing the literature on sports-related Achilles tendinopathy, the highest prevalence is reported for sports with a regular aerial phase, such as running, soccer, basketball, or rugby (Sobhani et al., 2013). Thus, it seems likely that regular exercise activities with an aerial phase can increase the risk of an overuse injury of the Achilles tendon. Consequently, individuals who participate in an exercise activity with an aerial phase should slowly increase their training intensity level to allow the Achilles tendon sufficient time to adapt to the high impact loading. Further, when performing a sport with an aerial phase, it might be helpful to include specific strength training sessions, as proposed recently by Radovanović et al. (2021). Such strategies could help to improve Achilles tendon properties and to prevent overuse injuries. That said, more research is needed to understand the impact of different exercise activities on Achilles tendon adaptation and the associated risk of injuries.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics committee of the Faculty of Behavioral and Social Sciences, Chemnitz University of Technology. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FS and KL conceived the experiment, interpreted the data, and drafted the manuscript. NK and KL performed the experiments and substantially contributed to data analysis. FS analyzed the data. NK made important intellectual contributions during revision. All authors approved the final version of the manuscript and agreed to be accountable for the content of the work.

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Insights in the Effect of Fluctuating Female Hormones on Injury Risk—Challenge and Chance

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It is time to take on the challenge of investigating the complex effect of fluctuating female hormones on injury risk as this offers a chance to improve female athletes' health and performance. During the recent decade, the body of knowledge on female hormones and injury risk has largely been increased. New insights have been offered regarding the association of certain phases of the menstrual cycle and injury prevalence as well as regarding relationships between hormone levels and musculoskeletal changes such as, for example, ligamentous stiffness and knee laxity. However, current research often follows the theme of a causal relationship between estrogen levels and musculoskeletal function or injury and thus—one might argue—further enhances a rather simplistic approach, instead of uncovering complex relationships which could help in establishing more nuanced ways of preventing female injuries. To uncover real effects and to truly understand the physiological responses, we suggest to reflect on potential bias regarding research questions and current approaches. It may enhance future studies to apply a more nuanced approach to causation, to include multidimensional perspectives and to implement an interdisciplinary methodology.

Keywords: estrogen, menstrual cycle, gender bias, injuries, women, sport, exercise, ligament

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INTRODUCTION

It is well accepted that females are currently less represented as research subjects in exercise physiology studies (Costello et al., 2014). Most probably, reasons for this imbalance are, that fluctuating female hormones are thought to generate less-controlled experimental conditions and to increase variation thereby reducing statistical power and making studies more complex and expensive (Uhl et al., 2007). As a result, specific knowledge on women's response to exercise is limited, hindering our understanding of female exercise physiology. This imbalance in research does not hold true for all fields of exercise physiology studies. Changes in bone health as a result of hormonal and metabolic changes due to insufficient energy intake and/or excessive exercise energy expenditure, known as the female athlete triad, have predominantly been investigated in women. Today, it is accepted that detrimental effects of energy deficiency, respectively, the relative energy deficiency syndrome are not limited to exercising girls and women and but are also occurring in men (de Souza et al., 2021). However, the overall imbalance in research cannot be neglected, which also raises ethically relevant questions about

whether women receive lesser chances of performance progression and about whether women are subjected to a higher risk of injury compared to men due to a limitation of specific knowledge. Indeed, it has been shown for injuries sustained in car accidents, that protective concepts, which have been based on the biomechanical behavior of male bodies only, critically impact the safety of women in everyday life (Linder and Svedberg, 2019).

Consequently, it can be argued that the specific relevance of studies about female exercise physiology which consider hormonal fluctuations lies within their potential of providing applied knowledge on how to prevent injuries and improve performance in exercising women. In light of this starting point, this article sheds light on the progress made in female exercise physiology, namely on how research and researchers deal with the menstrual cycle and injury risk and it furthermore identifies and discusses current and future challenges in this field of research. We will particularly focus on ACL injuries, using this type of injury as an example to demonstrate the specific achievements and challenges associated with menstrual cycle-related research in exercising women. In a first step, we will review current studies on this topic by portraying their research questions, results, and explanatory approaches. As a result, we will show that the body of knowledge about the menstrual cycle and injury risk has risen significantly. However, some challenges, which we will critically reflect and discuss in the second part of this paper, remain to be acknowledged and addressed. Secondly, as a result, we will unfold the thesis that we—as researchers—might not always be as objective as we often assume to be and that we furthermore tend to simplify the complex relationship between the menstrual cycle and injury risk. Having identified and discussed these shortcomings, we will consequently outline perspectives and strategies for future research in the conclusion of this article.

STATE OF RESEARCH

Female Hormones and Injury Risk

Exercise-associated injuries are accompanied by a variety of side effects. Besides generating costs for the healthcare system, they may affect the injured athletes' quality of life or their athletic careers (Palmer et al., 2021). This insight often leads sport scientists to the value-driven desire of developing strategies that reduce the prevalence of those injuries. In this context, one approach is to identify risk factors for specific injuries, which then could be addressed with corresponding interventions.

Some of the injuries frequently occurring during exercise activity are more prevalent in women than in men (Waldén et al., 2011; Lovalekar et al., 2020), leading some to suggest that female sex itself is a risk factor. As physiological differences between men and women are to a great extent a result of a differing hormonal profile, it suggests itself to take a closer look at the effect of female sex hormones. It has long been known and frequently been reported for female athletes, that permanently low levels of female sex hormones, accompanied by menstrual cycle perturbations such as amenorrhea, are detrimental for bone health and increase the risk for stress fractures (Olson, 1989). The incidence of menstrual cycle disturbances is thereby substantially higher in athletes than in the general population, particularly in aesthetic and weight-bearing sports (Redman and Loucks, 2005).

In eumenorrheic exercising women, the blood plasma concentration of female sex hormones naturally fluctuates during the menstrual cycle (Pirke et al., 1990). Thus, one line of research about the effect of female sex hormones focuses on the correlation between the menstrual cycle phase and injury risk, trying to establish if hormone level fluctuations lead to corresponding fluctuations in injury risk (Martin et al., 2021). Indeed, if we look at ruptures of the anterior cruciate ligament (ACL), not only will we observe, that ACL ruptures are much more prevalent in women than in men (Myklebust et al., 1998; Waldén et al., 2011), but also that they are associated with certain phases of the menstrual cycle (Myklebust et al., 1998; Wojtys et al., 2002; Ruedl et al., 2009; Lefevre et al., 2013).

Menstrual Cycle and ACL Ruptures

Due to the striking difference in sex-related injury prevalence, with a 2–3-fold elevated relative injury risk in women compared to men (Waldén et al., 2011), the ACL rupture may be the best investigated of all sex associated sport injuries. In addition, it can be argued that the high medical burden of ACL injuries makes it all the more urgent to develop strategies, respectively, recommendations that can effectively reduce injury prevalence specifically in women. Thus, many studies have investigated if the occurrence of ACL ruptures is related to the hormonal profile and hence differs between the follicular, the ovulatory and the luteal phase of the menstrual cycle. Several reviews and meta-analyses, surveying five (Herzberg et al., 2017), seven (Hewett et al., 2007), and nine (Somerson et al., 2019) studies, have since concluded that there is a decreased relative risk of an ACL tear in the luteal phase, respectively, that female athletes are more predisposed to ACL ruptures during the preovulatory phase of the menstrual cycle. The finding, that there is an association of ACL ruptures with the preovulatory phase, as detected in a study describing the prevalence of ACL ruptures along the menstrual cycle during skiing, may only allow for rather general recommendations such as "Female skiers should take special care during this period" (Lefevre et al., 2013). One might ask if this actually is a realistic point of action, suited to reduce injuries. To allow for more precise recommendations, which are derived from addressing the reasons (and not the outcomes) for variations in injury prevalence, we need to uncover the mechanisms behind the association of menstrual cycle phase and injury prevalence.

Hormonal Fluctuations, Ligamentous Properties, and Knee Laxity

The most popular explanation for the statistical correlation between preovulatory phase and injury peak is to directly link high estrogen levels to a mechanical weakness of ligaments, which would then—in turn—increase the likelihood for ACL injuries (**Figure 1**). It has been assumed, that female hormones, and in particular estrogen, make female ligaments susceptible

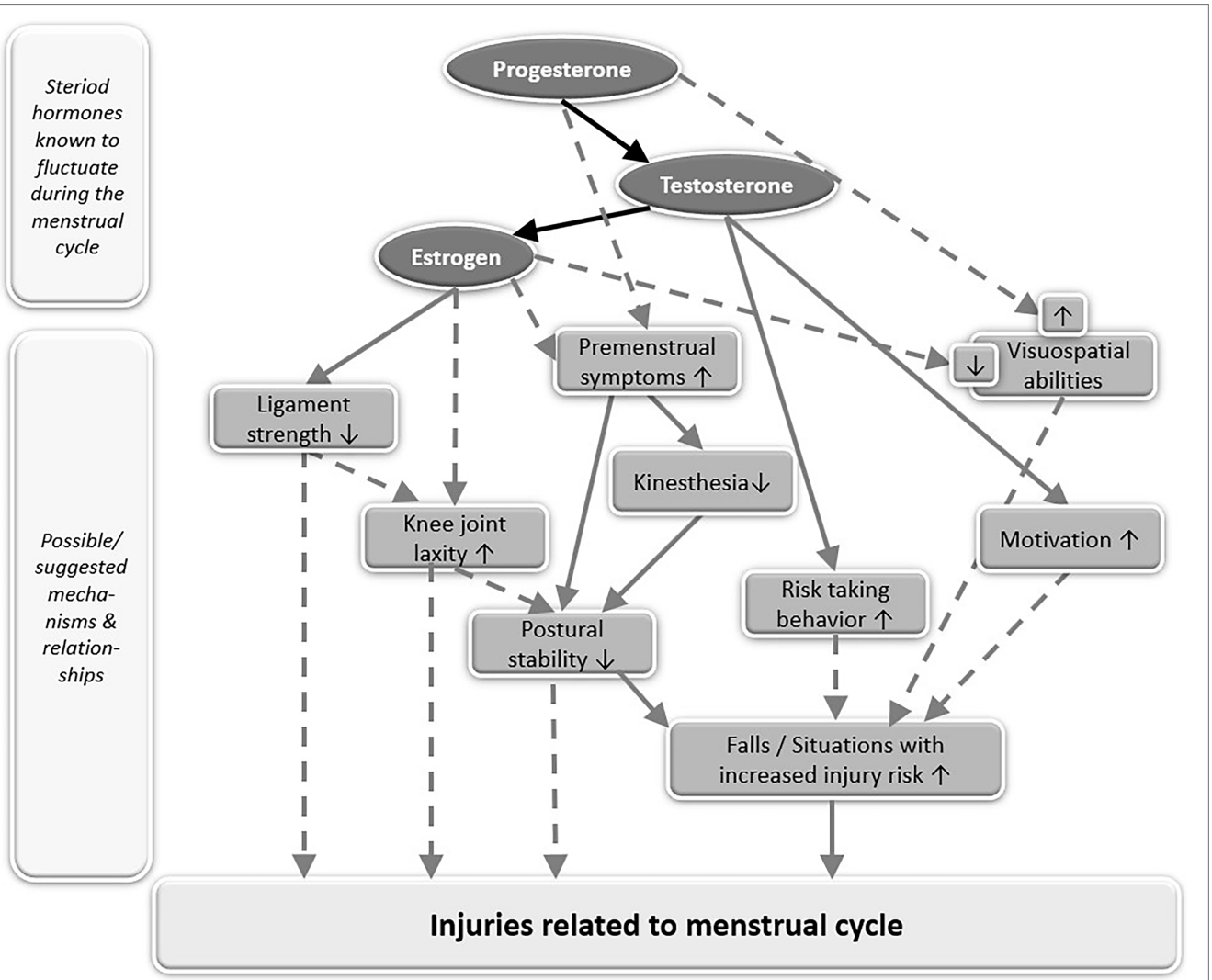

FIGURE 1 | Simplified model of known (solid grey arrows) or suggested (broken grey arrows) relationships between steroid hormones, fluctuating during the menstrual cycle, and their effects on the musculoskeletal and neurophysiological system, assumed to be related to injury risk. Upwards pointing arrows within the boxes symbolize an increase while downwards pointing arrows within the boxes symbolize a decrease. Black arrows connecting progesterone, testosterone, and estrogen symbolize the biosynthetic pathway of steroid hormones.

for injury by directly affecting ligamentous metabolism (Wojtys et al., 2002; Chidi-Ogbolu and Baar, 2018). It has also been suggested, that the ligamentous response to estrogen may be related to an evolutionary adaptation as decreased connective tissue stiffness and laxer joints would facilitate healthy childbirth (Chidi-Ogbolu and Baar, 2018). However, when taking a closer look at this assumed causal relationship between high estrogen levels and weak, more compliant or less stress-resistant ligaments, recent study results raise the question whether this causal explanation is too naïve. One might argue, that when pregnancy actually occurs and estrogen levels are much higher, an evolutionary advantageous decrease in connective tissue stiffness must be much more pronounced. However, there is no evidence to support this. In contrast, stiffness of the ligamentum patellae does not change during pregnancy and it neither differs between pregnant women and non-pregnant controls (Bey et al., 2019). Similarly, no difference in biomechanical properties of the ligamentum patellae, fibril characteristics, or collagen cross-linking was observed between the different phases of the menstrual cycle or between women using oral contraceptives and those who did not (Hansen et al., 2013). While these results do not necessarily or finally falsify the assumption about the causal relationship between estrogen levels and ligamentous stiffness, they at least raise serious concerns about its effect size.

The assumption, that female hormones make ligaments susceptible for injury, leads us to a second causal relationship, which has often been proposed and which links high estrogen levels to increased knee laxity, which would then—in turn—increase the likelihood for ACL injuries (**Figure 1**). The concept, that ligamentous stiffness of knee joint structures decreases

with rising estrogen levels, is related to the observation of increased knee joint laxity when estrogen levels are high during the menstrual cycle (Heitz et al., 1999) and during pregnancy (Schauberger et al., 1996). As knee joint laxity is thought to increase injury risk, several studies have been conducted to investigate the relationship of knee laxity, injury risk, and the menstrual cycle. Two meta-analyses reviewing 9 (Zazulak et al., 2006) and 19 studies (Somerson et al., 2019) have since concluded, that knee laxity indeed varies within the menstrual cycle, with the highest laxity occurring during ovulation, followed by the luteal phase and the lowest laxity occurring in the follicular phase. However, both meta-analyses also concluded, that periods of increased laxity were not associated with an increased prevalence of ACL ruptures (Zazulak et al., 2006; Somerson et al., 2019), suggesting that there is no direct link between hormonal effects on ACL injuries and hormonal effects on knee laxity. It may further be questioned if transient non-pathological changes in healthy women's knee laxity have a pronounced effect on injury risk, as a 3-year prospective cohort study with young female athletes could not detect a difference in knee laxity between those who subsequently teared their ACL and those who did not (Nakase et al., 2020). It also has to be considered, that tibial translation, commonly described as knee joint laxity, is no direct measure of the ACLs mechanical properties. While tibial translation has been found to inversely correlate with ultimate failure strength of ACL grafts in animal studies (Beynnon et al., 1994), other variables than ligamentous stiffness may also contribute to knee stability. Knee laxity might change without a change in ligamentous stiffness as an animal study with pregnant rabbits suggests (Hart et al., 2000). There, pregnancy was associated with increased laxity while it had no effect on the structural (stiffness and failure load), material (stress at failure and Young's modulus), or viscoelastic (cyclic and static relaxation) properties of the medial collateral ligament (Hart et al., 2000).

Menstrual Cycle and Postural Stability

While hormonal-induced increases in knee laxity may not directly be related to ligamentous stiffness and may not directly affect injury risk, they could have an indirect effect by impairing postural control (**Figure 1**). However, there is no consensus as to if and when postural control is impaired during the menstrual cycle, as studies found both improved (Emami et al., 2019) and impaired (Lee et al., 2017) postural control at ovulation or no change in postural control during the menstrual cycle at all (Legerlotz et al., 2018). One explanation for those varying results may be, that pronounced hormonally induced impairments in postural control only occur in a specific subpopulation of women: those who suffer from premenstrual symptoms (Fridén et al., 2003, 2005). These women showed increased postural sway in the luteal phase, when symptoms like discomfort and pain were particularly prevalent, while sway values stayed stable throughout the menstrual cycle in women without symptoms (Fridén et al., 2005). That postural stability in women with premenstrual symptoms is generally reduced compared to women without symptoms may be related to changes in kinesthesia, as women with premenstrual symptoms also displayed a greater threshold for detection of passive movement in the knee (Fridén et al., 2003).

Menstrual Cycle and Cognitive Functioning

Hormonal changes during the menstrual cycle can not only affect physical and functional variables, such as knee laxity, postural control or kinesthesia, which are related to movement control. They can also affect the main instance of movement control itself, our brain. Sex hormones may have a far-reaching impact on brain function, with both behavioral consequences as well as changes in neuropsychological processing (Hornung et al., 2020). It has been suggested that healthy women show small fluctuations in cognitive performance across the menstrual cycle, with low-performance scores in the luteal phase for visuospatial and motor skills, attention and concentration, verbal memory, visual memory, working memory, and reaction time (Souza et al., 2012). However, associations between prefrontal cognitive functioning and hormone levels across the female menstrual cycle detected in one cycle can not necessarily be replicated when analyzing a second cycle, suggesting the occurrence of false-positive findings attributable to random variation particularly in small samples (Leeners et al., 2017). Sex differences in visuospatial abilities have long been reported, with men usually performing better in the Mental Rotations Test, and women with low estrogen levels performing as strongly as males (Peragine et al., 2020). In contrast, a recent study with a large sample size of 528 women found no effect of estrogen on mental rotation performance, while high levels of progesterone, characteristic of the luteal phase, were instead associated with small performance increases (Shirazi et al., 2021). In general, the observed effects are small and not consistent and should thus be treated with caution.

Menstrual Cycle and Behavior

Hormone-associated changes in brain function may also change women's behavior during the menstrual cycle, which could as well have an impact on injury prevalence. The motivation to train and the motivation to compete have both been shown to be elevated around ovulation (Cook et al., 2018). It may be speculated, that increased motivation potentially changes the characteristics of movement, such as movement intensity, thereby affecting the risk of injury (**Figure 1**). In addition, behavioral experiments have indicated that risk-taking behavior also changes along the menstrual cycle, with women being willing to take higher risks around ovulation (Cook and Crewther, 2019). It seems likely, that increased risk-taking behavior may positively affect performance and competition outcome while at the same time increasing the risk of injury.

DISCUSSION

We acknowledge the great efforts that have been made to uncover relationships between female hormones, musculoskeletal properties, neurophysiological changes and injury risk, the fact that the body of knowledge has largely been increased, and

new insights have been offered. However, when taking a closer look at this particular field of research several questions remain to be unanswered, several contradictions yet to be solved, several correlations yet to be explained. Research often follows the theme of a causal relationship between estrogen levels and musculoskeletal function or injury and thus—one might argue—further enhances a rather simplistic approach, instead of uncovering complex relationships which could help in establishing more nuanced ways of preventing female injuries. With this specific evaluation of the state of research in mind, we will discuss possible reasons for these shortcomings and identify three themes, respectively, approaches that require some further elaboration:

1. **Deficit-oriented approach:** The effects of female sex hormones appear to be seen and valued in light of weakening the women's body, with females being—be it consciously or unconsciously—viewed as less functioning, weaker or less well-adapted men. In the context of the menstrual cycle research often appears to start with the underlying—yet often implicit—assumption that female hormones, the menstrual cycle or the menstrual bleeding are seen as something avoidable, as something "special" or as something undesirable. As a consequence, it is assumed that female hormones or the menstrual cycle are responsible for higher rates of specific injuries or reduced performance. This deficit- and problem-oriented approach is mirrored in the underlying assumptions that often initiate research and is reflected by the mechanistic approach that has been applied to link female sex hormones such as estrogen to mechanical or functional weakness (Chidi-Ogbolu and Baar, 2018), interpreting physiological changes observed during the menstrual cycle as negative, and associating those changes with an increased risk of injury. Research that focuses on possible, positively associated or protective effects of female hormones appears to be rare.
2. **Oversimplification:** Although the literature strongly suggests that there is an association of ACL ruptures with the preovulatory phase, this by itself does not explain, why the injury prevalence varies between phases, but it rather begs the question "why." It is crucial to not fall into the trap of mistaking a correlation for a causation, of overseeing the possibility that statistical effects can also be caused by a third variable, only hold true for some subgroups, or that further variables could be way more relevant than the one regarded in one's own statistical model. Thus, to uncover the mechanism behind peaks in injury incidence during the menstrual cycle it is important to understand, that it is not exclusively the estrogen level that varies with different phases of the menstrual cycle. Other variables, such as, for example, the testosterone level similarly vary with different phases of the menstrual cycle (Cook et al., 2018; Cook and Crewther, 2019). Further physiological, biomechanical, functional, but also psychological and behavioral variables need to be considered in interdisciplinary studies to uncover the complex relationship of injury risk in female exercise. In addition, it may be too simplistic to view women as one homogenous group, particularly in relation to hormonal variation. Hormone levels can vary greatly between female elite and non-elite athletes (Cook et al., 2018), and different subpopulations of women, for example, those suffering from premenstrual symptoms, may adapt or react differently (Fridén et al., 2003, 2005).
3. **Researcher bias:** We argue that current research approaches aiming to investigate the relationship of female hormones and musculoskeletal adaptation or injury risk indicate that we—as researchers—might not be as objective as we often claim to be. Besides from being researchers, academics or sport scientists we always also are and always will be members of societies in which we have been socialized and through which we have incorporated a specific set of norms and values that also imply gender-related issues. Methodological choices and criteria for evidential support may not involve value commitments—even though this too is a rather controversially discussed topic within science theory (Lacey, 2018). However, it has long been discussed and approved that whichever research question we claim is to be more relevant than another and whichever reasons we find for this relevance is a value-based decision that is likely to be correlated to our position in society (Homans, 1976; McMullin, 1982; Lacey, 2018). Furthermore, and knowing that our views on society and on specific topics as well as our experiences are also—yet not exclusively—shaped by the gender we identify with or that is ascribed to us, leads us to ask whether the deficit- and problem-oriented approach to female physiology might also be a result of a male-dominated research field.

CONCLUSION: ACCEPTING THE CHALLENGE

Shifting the focus from the status quo and its evaluation to perspectives for research, one could argue that studying and understanding the complex effects of the menstrual cycle on injury and performance remains a task yet to be fulfilled, yet also a task that can build on the achievements of prior research in this field. Following our line of argumentation leads us to the following, more nuanced recommendations:

- Self-reflection on potential bias and a more nuanced approach to causation can be seen as crucial points of action for future research. Raising research questions that help to uncover the supposedly multiple factors that contribute to injury risks and that lead researchers to also include positively associated or protective effects of female hormones in their studies, implementing approaches that acknowledge the great differences within the group of women and a more careful interpretation of results can be regarded as relevant tasks for future research.
- Multidimensional and interdisciplinary research is strongly needed. To uncover real causal effects and to truly understand

the direction of hormonal effects as well as the meaning and the significance of these effects, requires to put them into perspective. Interdisciplinary research that simultaneously—and not separately—considers multiple factors can help to achieve this. For example, postural stability variations during the menstrual cycle can be affected by a variety of variables such as neurophysiological or visuospatial abilities, psychological state (anxiety) and tissue mechanical properties, which all need to be considered within the same study.

- Accounting on sex and gender, not only regarding research subjects as the European Commission has recently requested (Nature Editorial, 2020), but also regarding the group conducting the research, makes for better science. Following the argument, that approaching the research field and defining the research questions is at least partly dependent upon our experiences in and our views on society leads us to assume that future research could benefit from diversly composed research groups and particularly the inclusion and promotion of women in this research.

DATA AVAILABILITY STATEMENT

The original contributions presented are included in the article, and further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

KL and TN interpreted the literature and drafted the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for the content of the work.

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Renal Function Recovery Strategies Following Marathon in Amateur Runners

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Long distance races have a physiological impact on runners. Up to now, studies analyzing these physiological repercussions have been mainly focused on muscle and cardiac damage, as well as on its recovery. Therefore, a limited number of studies have been done to explore acute kidney failure and recovery after performing extreme exercises. Here, we monitored renal function in 76 marathon finishers (14 females) from the day before participating in a marathon until 192 h after crossing the finish line (FL). Renal function was evaluated by measuring serum creatinine (sCr) and the glomerular filtration rate (GFR). We randomly grouped our cohort into three intervention groups to compare three different strategies for marathon recovery: total rest (REST), continuous running at their ventilatory threshold 1 (VT1) intensity (RUN), and elliptical workout at their VT1 intensity (ELLIPTICAL). Interventions in the RUN and ELLIPTICAL groups were performed at 48, 96, and 144 h after marathon running. Seven blood samples (at the day before the marathon, at the FL, and at 24, 48, 96, 144, and 192 h post-marathon) and three urine samples (at the day before the marathon, at the finish line, and at 48 h post-marathon) were collected per participant. Both heart rate monitors and triaxial accelerometers were used to control the intensity effort during both the marathon race and the recovery period. Contrary to our expectations, the use of elliptical machines for marathon recovery delays renal function recovery. Specifically, the ELLIPTICAL group showed a significantly lower ΔGFR compared to both the RUN group ($p = 4.5 \times 10^{-4}$) and the REST group ($p = 0.003$). Hence, we encourage runners to carry out an active recovery based on light-intensity continuous running from 48 h after finishing the marathon. In addition, full resting seems to be a better strategy than performing elliptical workouts.

Keywords: acute kidney injury, marathon, glomerular filtration rate, active recovery, passive recovery

INTRODUCTION

Given the increase of marathon running popularity, the physiological alterations caused by performing such a demanding effort have increased the interest of the scientific community (Scheer, 2019; Rojas-Valverde et al., 2021b; Scheer et al., 2021). Running a long-distance race demands a vigorous physical effort, which has been shown to generate transient elevation of biomarkers associated with pathological conditions such as muscle damage, inflammation, heart damage, and renal failure (Briviba et al., 2005; Khodaee et al., 2015; Belli et al., 2018; Knechtle and Nikolaidis, 2018; Nikolaidis et al., 2018; Bernat-Adell et al., 2019, 2020; Martínez-Navarro et al., 2020a,c; Scheer et al., 2021).

In the last few years, several studies have focused on studying acute kidney injury (AKI) after performing a physically demanding exercise (Lipman et al., 2014; Traiperm et al., 2016; Mansour et al., 2017; González et al., 2019; Rojas-Valverde et al., 2019; Poussel et al., 2020; Khodaee et al., 2021). Contrary to other acute pathological alterations, renal function has been shown to be normalized 24 h after running a long-distance race. As a result, the collection of biomarkers related to AKI have been usually restricted to the running phase (Lipman et al., 2014; Belli et al., 2018) or to the first 24 h of the recovery phase (McCullough et al., 2011; Traiperm et al., 2016; Mansour et al., 2017; Khodaee et al., 2021). However, we observed that glomerular filtration rate (GFR) significantly worsened 48 h after marathon running (González et al., 2019). Therefore, there is a need for monitoring renal function more than 48 h after performing a strenuous exercise.

The recovery of exercise-associated physiological damage has been a matter of concern for sport science researchers, coaches, and medical staff. Recent studies aimed at determining how long is needed, as well as what is the best strategy, for recovering from muscle damage and for normalizing neuromuscular performance after performing a long-distance race (Sherman et al., 1984; Wiewelhove et al., 2018; Martínez-Navarro et al., 2020b). These studies analyzed the effect of different recovery strategies usually followed by marathoners (massage, cold water immersion, total rest, light running, and elliptical machine workouts) on muscle damage recovery without obtaining any conclusive results. However, a study that comprehensively characterizes renal function normalization including a significant cohort of runners is lacking in the field.

Physical exercise increases body temperature and leads to peripheral vasodilatation and blood flow (Poortmans, 1984; Poortmans et al., 1988; Ferreira et al., 2019). This fact promotes the activation of renin-angiotensin-aldosterone system, which increases the filtration pressure and consequently the GFR. Given that physical exercise has shown a beneficial effect in patients with chronic kidney disease (Johansen and Painter, 2012; Heiwe and Jacobson, 2014; Viana et al., 2014; Greenwood et al., 2015; Santana et al., 2017), we hypothesized that performing a low-impact physical activity will accelerate the recovery of marathon-induced acute kidney damage faster than resting during the whole post-marathon week.

Here, we present a research study focused on exploring the effects of exercise within a proposed strategy to optimize kidney function recovery following the completion of a marathon race. Our large cohort of 76 marathon finishers allowed us to compare the effect of three different recovery strategies (resting, running, and elliptical workout) on renal function after a marathon race.

MATERIALS AND METHODS

Sample Set and Subsampling

All participants of the Valencia Fundación Trinidad Alfonso EDP 2016 Marathon received an invitation by email to participate in this study. Three informative seminars were organized to fully explain the study design to those individuals who accepted the invitation (n = 456). A total of 98 recreational marathon runners were selected to participate in this study, according to the following inclusion criteria: (1) being between 30 and 45 years old; (2) having a previous marathon experience, with a marathon personal best between 3 and 4 h for males and between 3:30 and 4:30 h for females; (3) having a body mass index (BMI) between 16 and 24.99; and (4) being free from cardiovascular disease, renal dysfunction, and dyslipidemia. All individuals selected were fully informed and gave their written consent to participate. The research was conducted according to the Declaration of Helsinki, and it was approved by the Research Ethics Committee of the Jaume I University of Castellon. This work is part of a project aiming at finding the best strategy to recover from marathon-induced physiological damage. This project is enrolled in the https://clinicaltrials.gov/ct2/show/NTC03155633 database, with the code number NCT03155633.[1] Therefore, the same study population was used in previous publications (Martínez-Navarro et al., 2020a,b,c).

Ninety-five out of 98 volunteers started the Valencia Marathon on 22 November 2016. From them, 88 (74 males and 14 females) crossed the finish line (FL) and were thus randomly included in the three intervention groups, which were monitored until 192 h after crossing the FL. Although 78 runners completed the entire study, two of them were discarded from the analysis because they consumed non-steroidal anti-inflammatory drugs (NSAIDs) during the recovery phase. Therefore, a total of 76 runners were finally used to explore the hypotheses of this study.

Each group of runners followed one of the three strategies to test during the recovery phase. The first group (N = 32; six females) did not perform any physical activity (REST group). The second group (N = 22; four females) performed a 40-min run (RUN group). The third group (N = 22; four females) performed a 40-min workout on an elliptical machine (Synchro excite 500, Technogym, Cesena, Italia; ELLIPTICAL group). All participants who used the elliptical machine in the recovery period had previously used this tool, although it was not their usual training method. In any case, we allowed runners to get used to the elliptical machine during 5 min before each session of the recovery phase. Since there are biomechanical differences between the three possible positions (using the handles, holding onto a central bar and free-hand) of using an elliptical machine (Jackson et al., 2010; Moreside and McGill, 2012), participants were not allowed to perform workout sessions holding the central bar.

[1] www.clinicaltrails.gov

We selected three biomechanically similar activities because: (1) all three activities generate equivalent movements in the Cartesian coordinate axes allowing to have comparable accelerometry-based estimation of energy expenditure (Cordero et al., 2014; Rowlands et al., 2014; de Almeida Mendes et al., 2018; Hernando et al., 2018, 2020), (2) the intervention of gravity is not significantly disturbed when performing the activity movement (unlike in cycling or swimming), (3) the activity movements are similar to those performed in marathon training sessions and racing, and (4) participants can be continuously controlled by researchers when doing the two active strategies.

Runners performed three times the same workout in the recovery phase (at 48, 96, and 144 h after finishing the marathon). The physical intensity required was between 95 and 105% of their ventilatory threshold 1 (VT1). To control the physical intensity at which runners were performing the physical activity, each runner wore a heart rate monitor (Polar M400 HR monitor, Kempele, Finland). All interventions were supervised by experts to guarantee that workouts were correctly performed by runners. Although preventive strategies can be applied for accelerating recovery from marathon-related AKI (Juett et al., 2020), no intervention was performed to prevent AKI. No participant expressed physical limitations for performing workouts during the recovery period. No control of food and liquid intake was performed during the whole study.

In addition, the physical activity done by each runner throughout the study was monitored using accelerometer devices. Each participant wore a GENEActiv accelerometer (Activinsights Ltd., Kimbolton, Cambridgeshire, United Kingdom) on the non-dominant wrist as a watch from the day before the marathon until 192 h post-race. Applying our validated approach (Hernando et al., 2018), we calculated the relative caloric consumption ($kcal \cdot kg^{-1} \cdot min^{-1}$) every 8 h following the circadian rhythm criteria (Vitale et al., 2015). For comparison of the relative caloric consumption, we added the caloric consumption of three different moments: (1) the time elapsed from arrival at finish line to 48 h post-race (six 8-h segments), (2) the three 8-h segments where the activity was performed (24 h in total), and (3) the time of the intervention phase where runners are not performing the activity (13 8-h segments).

Data Collection

Training- and Competition-Related Data

A suitable questionnaire (Hernando et al., 2018) was used to collect demographic, sociographic, and medical information, the training plan, and competition history.

Cardiopulmonary Exercise Test

Prior to running the marathon, all individuals selected for this study performed a cardiopulmonary exercise test on a treadmill (pulsar® 3p, h/p/cosmos Sports and Medical GmbH, Nussdorf-Traunstein, Germany) until exhaustion. Breath-by-breath gas exchange was measured by the Jaeger MasterScreen® CPX gas analyzer to identify the first ventilatory threshold (VT1), the second ventilatory threshold (VT2), and the maximal oxygen consumption ($\dot{V}O_{2max}$; Skinner et al., 1980; McLellan and Skinner, 1985).

Blood Samples

Seven blood samples (at the day before the marathon, at the finish line, and at 24, 48, 96, 144, and 192 h post-marathon) were collected per participant. These samples were taken from runners' antecubital veins by venipuncture using BD Vacutainer PST II tubes, centrifuged at 3,500 rpm for 10 min, and transported to the Vithas NISA Hospital in Valencia at 4°C for biochemical analysis. In the recovery phase, blood samples were collected prior to performing the active recovery workouts. The impact of dehydration on plasma volume alterations was taken into account in the analysis of biochemical parameters collected at post-marathon time points. Adjustments were performed by applying the method of Dill and Costill (1974), which uses hematocrit and hemoglobin to determine the magnitude of plasma volume changes after the race in each participant (Alis et al., 2015).

Urine Samples

Three urine samples (at the marathon day prior to run, at the finish line and at 48 h post-race) were also collected per participant to evaluate hematuria and hydration status. Samples were taken by the participant himself using sterilized recipients. Except at the finish line, participants were informed to collect the first-morning-void urine sample. The presence of blood in urine was firstly tested by using a dipstick (Aution Sticks 10EA, Arkray, Shiga, Japan), and only positive cases were then explored at microscopic level in order to count the number of red blood cells per field. Hematuria was considered when more than five erythrocytes per field were found in the urine sample (Grossfeld et al., 2001; Loo et al., 2009). Given that no morphological evaluation was performed, we could not determine the tissue origin of erythrocytes in the urine sediment. The urine's specific gravity (USG; sediMax conTrust, 77 Elektonika Kft, Budapest, Hungary) was used to calculate hydration status (Casa et al., 2000; Kavouras, 2002).

Body Mass

For evaluating dehydration, we calculated the percentage of differences between the body mass before and after the completion of the marathon race (Noakes et al., 2005; Hoffman et al., 2017). Body mass was measured using a calibrated electronic scales (Seca 813, Vogel and Halke, Hamburg, Germany) on a firm surface, and runners wore their running clothes and shoes. Participants were not allowed to take a large meal 4 h prior to the prerace evaluation. At the finish line, participants were allowed to drink but not eat before measuring their body mass.

Assessment of Renal Function

Acute kidney injury was evaluated according to the acute kidney injury network (AKIN) criteria (Mehta et al., 2007). Grades of AKI were defined as previously (González et al., 2019). The estimation of AKIN grades required the measurement of serum creatinine (sCr) levels and the GFR (Mehta et al., 2007; McCullough et al., 2011; Hodgson et al., 2017; Rojas-Valverde et al., 2021b).

Although sCr is the classic biomarker for monitoring renal function in healthy individuals at baseline (Rojas-Valverde et al., 2021b), we are aware that its kinetics can be influenced by the acute phase of muscle damage after long-distance running (Hodgson et al., 2017). However, we are specifically interested in its values through the recovery phase where muscle damage is limited (Bernat-Adell et al., 2019).

The GFR was estimated using the equation defined by The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI; Levey et al., 2009). We also calculated the relative increase of GFR at each time point with respect to their basal level (ΔGFR), by applying the following equation: fold increase (Δ) = (post-race value – pre-race value)/pre-race value.

We assessed the evolution of renal function through the entire study in each intervention group. In addition, we also compared the renal function between intervention groups in the intervention phase of the study to evaluate the impact of each recovery strategy on normalizing the different parameters measured.

Statistical Analyses

Statistical analyses were carried out using the SPSS software v27, and two-sided values of $p < 0.05$ were considered statistically significant. The Kolgomorov-Smirnov test was used for testing data normality. Since variables were not normally distributed, non-parametric statistical tests were applied. To describe data collected, we used median and interquartile range (IQR) for continuous variables, and sample size and frequency (%) for categorical variables. The Friedman test was used to analyze the evolution of parameters over time in each intervention group. The Kruskal Wallis test was used for comparing parameters among groups at the post-intervention moments. Pairwise comparisons were performed using the Bonferroni method. Chi-square test was used for comparison of categorical variables among groups. ANOVA Levene test was used for comparison of quantitative variables among groups.

TABLE 1 | Description of study cohort.

Variable		RUN *N* = 22 (four females)	ELLIPTICAL *N* = 22 (four females)	REST *N* = 32 (six females)
Physiological characteristics*	Age	38.73 ± 3.92	37.86 ± 3.72	38.94 ± 3.26
	BMI	22.71 ± 1.27	23.49 ± 2.05	22.73 ± 1.74
	% body fat	14.74 ± 3.25	13.81 ± 3.67	19.54 ± 4.16
	Weight	67.88 ± 7.87	72.77 ± 10.86	71.03 ± 8.93
	Height	171.32 ± 8.47	173.73 ± 9.75	174.91 ± 7.84
	$\dot{V}O_{2max}$ (ml·kg^{-1}·min^{-1})	55.08 ± 6.04	53.96 ± 5.42	54.06 ± 6.21
	VT1 (ml·kg^{-1}·min^{-1})	38.75 ± 3.80	38.48 ± 4.71	37.11 ± 4.45
	VT2 (ml·kg^{-1}·min^{-1})	46.54 ± 4.35	45.87 ± 4.91	45.06 ± 4.49
Training indicators*	Sessions per week	4.73 ± 1.08	5.05 ± 0.67	4.84 ± 0.81
	Kilometers per week	61.82 ± 14.27	63.33 ± 11.33	65.16 ± 12.21
	Hours per week	7.00 ± 2.74	7.83 ± 2.90	7.36 ± 2.00
History as marathoner*	Marathons finished	3.41 ± 2.94	3.29 ± 3.02	2.69 ± 2.40
	Marathon per year	1.36 ± 0.90	1.14 ± 0.48	0.91 ± 0.39
Work intensity#	High intensity	4.50%	13.60%	6.30%
	Medium intensity	40.90%	18.20%	34.40%
	Low intensity	54.50%	68.20%	59.40%
Levels of study#	School graduate	4.50%	4.80%	6.30%
	High school graduate	4.50%	4.80%	9.40%
	Professional certificate	13.60%	23.80%	15.60%
	Undergraduate degree	77.30%	66.70%	68.80%

N, number of samples; F, female; BMI, body mass index; SD, standard deviation; $\dot{V}O_{2max}$, maximal oxygen consumption; and VT, ventilatory threshold.

**Values are presented as mean ± SD.*

#Values are presented as percentage of all individuals.

RESULTS

Assessment of Renal Function Prior to Intervention

Race conditions (15.6°C on average temperature, 50% of humidity, and relatively short and flat race with non-significant elevation changes) limited the impact of well-known factors influencing AKI levels (Junglee et al., 2013; Hou et al., 2015; Rojas-Valverde et al., 2019, 2021a). In general, runnewrs were not dehydrated at the finish line (USG < 1.02 g/ml; Casa et al., 2000; Kavouras, 2002) and percentage of body loss was estimated around 3% (Noakes et al., 2005; Hoffman et al., 2017 in all groups; **Supplementary Table S1**). No significant differences in marathon performance were observed across AKI grades (Kruskal Wallis test, $p = 0.262$). No significant differences were observed between the three subsets of runners prior to intervention (**Tables 1** and **2**; **Supplementary Table S1**). Thus, we considered them to be sufficiently homogeneous for comparison.

Kidney damage is normally observed after performing a highly demanding physical activity, such as running a marathon (McCullough et al., 2011; Traiperm et al., 2016; Mansour et al., 2017; González et al., 2019). According to levels of sCr and GFR collected at the finish line, 37 runners (48.68%) presented AKI immediately after running the marathon, being Grade I in 97% of cases and Grade II in the remaining 3% of cases (**Table 2**). The frequency of runners with kidney damage was similar across groups ($p = 0.341$). Biomarkers related to renal function (sCr, GFR, and ΔGFR) progressed similarly over time in the three groups (**Table 3**; **Figure 1**). All runners normalized the levels of these three biomarkers, and thus recovered from AKI, 24 h after finishing the marathon. However, an alteration of these parameters (increase of sCr and decrease of GFR and ΔGFR levels) was observed again at the 48 h post-marathon (**Table 3**; **Figure 1**). Our results are in concordance with previous studies (Irving et al., 1986, 1990; González et al., 2019).

Hematuria was observed in urine samples collected from 28 runners (36.85%) at the finish line. No differences were

observed in the frequency of runners with hematuria across subsets ($p = 0.886$). Except for two runners included in the REST group (6.2%) and one runner included in the ELLIPTICAL group (4.5%), hematuria disappeared in the urine samples collected 48 h after finishing the marathon.

TABLE 2 | Comparison of data collected prior to intervention.

Variable	RUN *N* = 22 (four females)	ELLIPTICAL *N* = 22 (four females)	REST *N* = 32 (six females)	*p*
Marathon time (min)	213.59 ± 20.24	216.40 ± 19.63	215.85 ± 21.87	0.869*
Absence AKI at the finish line	11 (50%)	9 (40.9%)	17 (53.1%)	0.341#
Presence AKI at the finish line	11 (50%)	11 (50%)	15 (46.9%)	
Grade 1 at the finish line	10 (45.5%)	10 (45.5%)	15 (46.9%)	
Grade 2 at the finish line	1 (4.5%)	1 (4.5%)	0 (0.0%)	
Presence of hematuria at the finish line	9 (40.9%)	8 (36.4%)	11 (34.4%)	0.886#
Presence of hematuria 48 h after marathon	0 (0%)	1 (4.5%)	2 (6.3%)	0.503#

N, number of subjects; F, female; AKI, acute kidney injury; and p, p-value. Data is presented as mean ± SD for continuous variables, and sample size (percentage) for categorical variables.
**ANOVA Levene test.*
#Chi Square test.

Impact of Recovery Strategy on Renal Function Normalization

We then explored the effect of three different workouts on kidney damage recovery following the completion of a marathon by monitoring three different biomarkers.

From the first intervention (performed 96 h after finishing the marathon), the evolution of both sCr and GFR were similar in the three intervention groups (**Table 3**). However, we observed significant differences in the evolution of ΔGFR over time among groups (**Figures 1, 2**). Both RUN and REST groups normalized ΔGFR values 96 h after finishing the marathon, having a significantly better filtration rate than the basal level at the two last time points measured (144 and 192 h after finishing the marathon). Therefore, both resting and running seem to be convenient strategies for recovering from acute kidney injury.

Conversely, except at the 192 h post-marathon time point, the ELLIPTICAL group presented a lower relative filtration rate in all time points measured during the study (**Figure 1B**). Contrary to what we observed in the other two intervention groups, the ELLIPTICAL group did not show significantly better filtration rates after finishing the recovery phase of the study. We are aware that the ELLIPTICAL group did not show improvement in filtration rates 24 h after finishing the marathon with respect to baseline, and this group also showed the lowest ΔGFR values prior to intervention (48 h post-marathon). However, the lack of significant differences in filtration rates observed prior to intervention between groups supports that such workout is not optimal for renal damage recovery.

Finally, we compared the ΔGFR levels between groups at each time point of the intervention phase (**Figure 2**). Significant differences in ΔGFR were only observed in samples collected

TABLE 3 | Evolution of serum creatinine (sCr) and the glomerular filtration rate (GFR) in the three groups during the whole study.

	Start line (1st time point)	Finish Line (2nd time point)	24 h post (3rd time point)	48 h post (4th time point)	96 h post (5th time point)	144 h post (6th time point)	192 h post (7th time point)	Friedmann *p* value
Serum creatinine (mg/dl)								
RUN *N* = 22 (four females)	1.00 [0.88–1.10][2,6]	1.34 [1.15–1.49][1,3,4,5,6,7]	0.90 [0,80-1,03][2,4]	1.00 [0.90–1.10][2,3,5,6,7]	0.90 [0.80–1.03][2,4]	0.90 [0.80–1.00][1,2,4]	0.90 [0.80–1.00][2,4]	**3.80×10^{-14}**
$ELIPTICAL *N* = 22 (four females)	0.90 [0.80–1.00][2,4]	1.26 [1.15–1.36][1,3,4,5,6,7]	0.90 [0.85–1.00][2,4]	1.00 [0,90–1.10][1,2,3,7]	1.00 [0.80–1.10][2]	0.90 [0.88–1.10][2]	0.90 [0.80–1.00][2,4]	**3.56×10^{-9}**
REST *N* = 32 (six females)	0.90 [0.80–1.08][2,6]	1.31 [1.13–1.44][1,3,4,5,6,7]	0.90 [0.80–1.00][2]	1.00 [0.90–1.10][2,6,7]	0.90 [0.80–1.08][2]	0.90 [0.80–1.00][1,2,4]	0.90 [0.80–1.00][2,4]	**0.00**
Kruskal-Wallis *p* value	0.448	0.712	0.832	0.526	0.534	0.183	0.996	
Glomerular filtration rate (ml/min/1.73 m²)								
RUN *N* = 22 (four females)	72.91 [66.24–99.25][2,6]	51.69 [44.17–61.03][1,3,4,5,6,7]	94.64 [67.95–104.22][2,4]	69.91 [62.95–90.27][2,3,5,6,7]	95.69 [68.06–102.92][2,4]	100,46 [73.30–105.26][1,2,4]	98.06 [73.30–104.44][2,4]	**1.10×10^{-13}**
$ELIPTICAL *N* = 22 (four females)	94.92 [71.65–100.50][2,4]	54.60 [48.93–61.16][1,3,4,5,6,7]	81.37 [68.93–98.76][2,4]	69.41 [63.84–92.19][1,2,3]	69.90 [61.53–101.20][2]	80.52 [63.84–101.74][2]	97.03 [69.17–105.12][2]	**1.42×10^{-9}**
REST *N* = 32 (six females)	85.87 [66.54–97.88][2,6]	51.10 [45.85–61.74][1,3,4,5,6,7]	94.62 [69.29–102.74][2]	71.90 [63.62–97.06][2,6,7]	95.25 [64.72–100.62][2]	97.37 [72.53–104.01][1,2,4]	95.58 [72.15–102.02][2,4]	**0.00**
Kruskal-Wallis *p* value	0.415	0.704	0.759	0.636	0.514	0.168	0.695	

Data is presented as median and interquartile range (IQR). Values of blood biomarkers in finish line (FL) were adjusted according to the method of Dill and Costill (1974).
GFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (Levey et al., 2009). N, number of participants; F, female; and p, p-value.
[1,2,3,4,5,6,7]Significant differences between the different time points where data was collected after applying Bonferroni correction method. Bold font indicates statistical significance across all time points after applying Bonferroni correction method.
$Blood samples from two participants (one female) included in the ELLIPTICAL group were not collected in the FL (2nd time point) because of logistic problems.

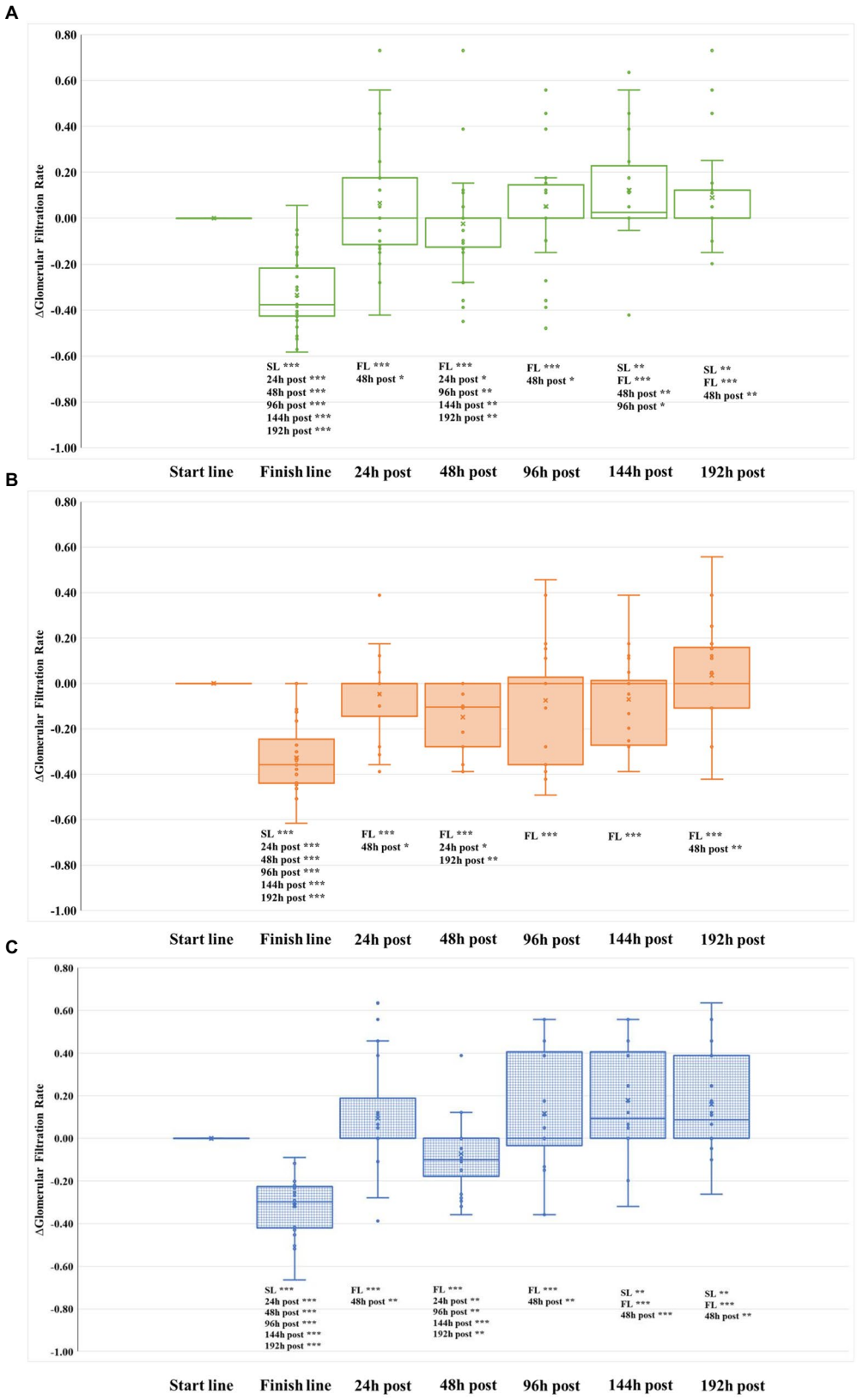

FIGURE 1 | Evolution of glomerular filtration rate relative to baseline (ΔGFR) in the three groups during the whole study. **(A)** REST group. **(B)** ELLIPTICAL group. **(C)** RUNNING group. SL, start line; and FL, finish line.

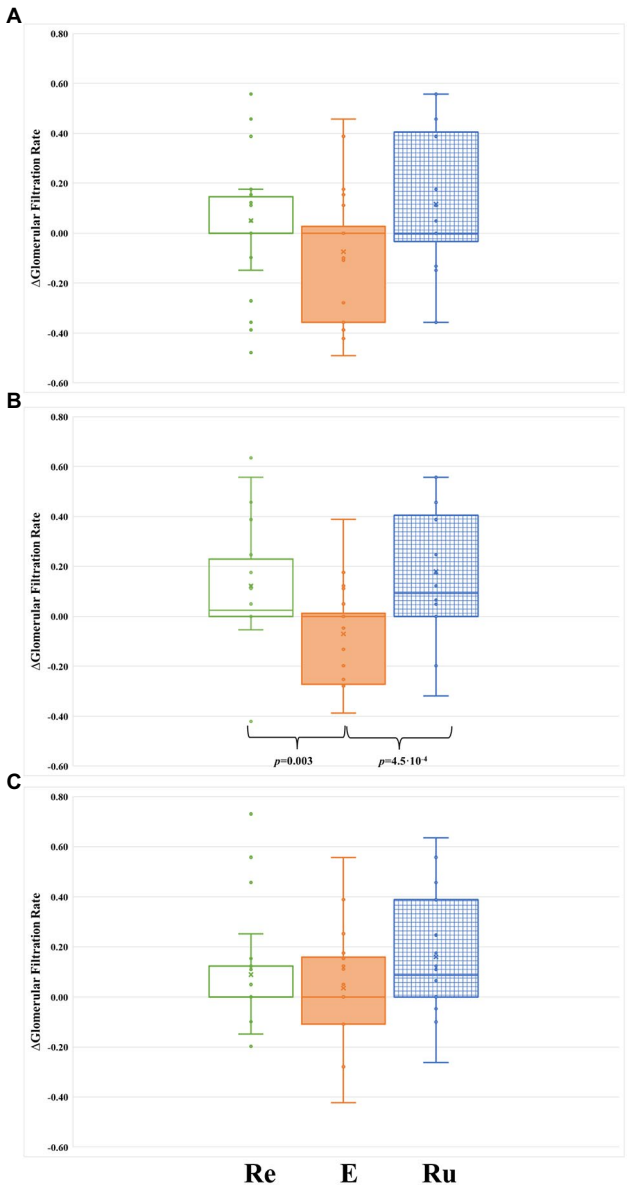

FIGURE 2 | Comparison of ΔGFR between the tree groups during the intervention phase of the study. **(A)** After 48 h from the first intervention (at 96 h post-marathon). **(B)** After 48 h from the second intervention (at 144 h post-marathon). **(C)** After 48 h from the third intervention (at 192 h post-marathon). Re, participants included in the REST group (green-rimmed boxes); E, participants included in the ELLIPTICAL group (orange-filled boxes); and Ru, participants included in the RUN group (blue-dashed boxes).

immediately before starting the second recovery workout (144 h post-marathon: $p=0.001$). Specifically, the ELLIPTICAL group showed a significantly lower ΔGFR compared to both the RUN group ($p=4.5\times10^{-4}$; **Figure 2B**) and the REST group ($p=0.003$; **Figure 2B**).

To control that runners strictly followed the activity proposed, we measured the caloric consumption of each participant through the entire intervention phase of the study. No differences in caloric consumption were found between the three groups when they were not performing the recovery activity ($p=0.354$; **Figure 3B**), confirming that runners did not perform any extra physical exercise apart from the one controlled by us. However, when only the time of the recovery activity was considered, we observed highly significant differences in the caloric consumption between groups ($p=1.22\times10^{-11}$; **Figure 3A**). As expected, the REST group consumed significantly less calories than the RUN group ($p=1.40\times10^{-12}$) and the ELLIPTICAL group ($p=0.008$). We also observed that the RUN group consumed significantly more calories than the ELLIPTICAL group ($p=4.4\times10^{-5}$). Thus, running seems to be the physical activity that requires the highest caloric consumption (**Figure 3A**).

DISCUSSION

Here, we present a robust study focused on exploring acute kidney failure and its recovery after running a marathon, one of the most physically demanding activities. As far as we are aware, this is the first interventional study testing three strategies for accelerating AKI recovery following a strenuous exercise. Our approach not only complements previous studies looking for optimal recovery strategies (Kellmann et al., 2018; Wiewelhove et al., 2018; Martínez-Navarro et al., 2020b; Kwiecien et al., 2021), but also presents a novel experimental design to continuously monitor participants through the whole study, limiting thus the uncontrolled factors that may influence results. In addition, unlike previous studies (Sherman et al., 1984), the intensity of the recovery activity was defined and thus not selected by the individual him/herself so that all participants performed workouts at an equivalent relative intensity. Our approach, together with the substantial number of participants included in the study, allowed us to comprehensively define the best strategy for recovering from acute renal injury.

To monitor renal function, we decided to measure levels of a classical renal function biomarker (sCr). This biomarker is well established for monitoring renal function in healthy individuals, but can be influenced by acute muscle damage (Hodgson et al., 2017). As previously shown (Irving et al., 1986; González et al., 2019), the depression of GFR seems to be biphasic. This observation could be influenced by the evolution of muscle damage biomarkers – a similar recovery pattern of a well-known muscle damage biomarker (LDH) have been previously described (Bernat-Adell et al., 2019). Therefore, analyzing novel biomarkers [i.e., neutro-phil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), or Cystatin (C)] would be relevant to assess acute kidney damage caused by high-intensity physical activities in order to avoid their overestimation (McCullough et al., 2011; Hodgson et al., 2017). In addition, these new biomarkers reflect intracellular alterations being more sensitive and specific to evaluate acute glomerular and tubular damage (Panizo et al., 2015). However, these novel biomarkers are still not validated for long-term renal function evaluation and they are not cost-effective diagnostic markers (Poortmans et al., 2013; Hodgson et al., 2017; Rojas-Valverde et al., 2021b). In addition, we measured renal function at the recovery phase where muscle damage is limited (Rojas-Valverde et al., 2021b).

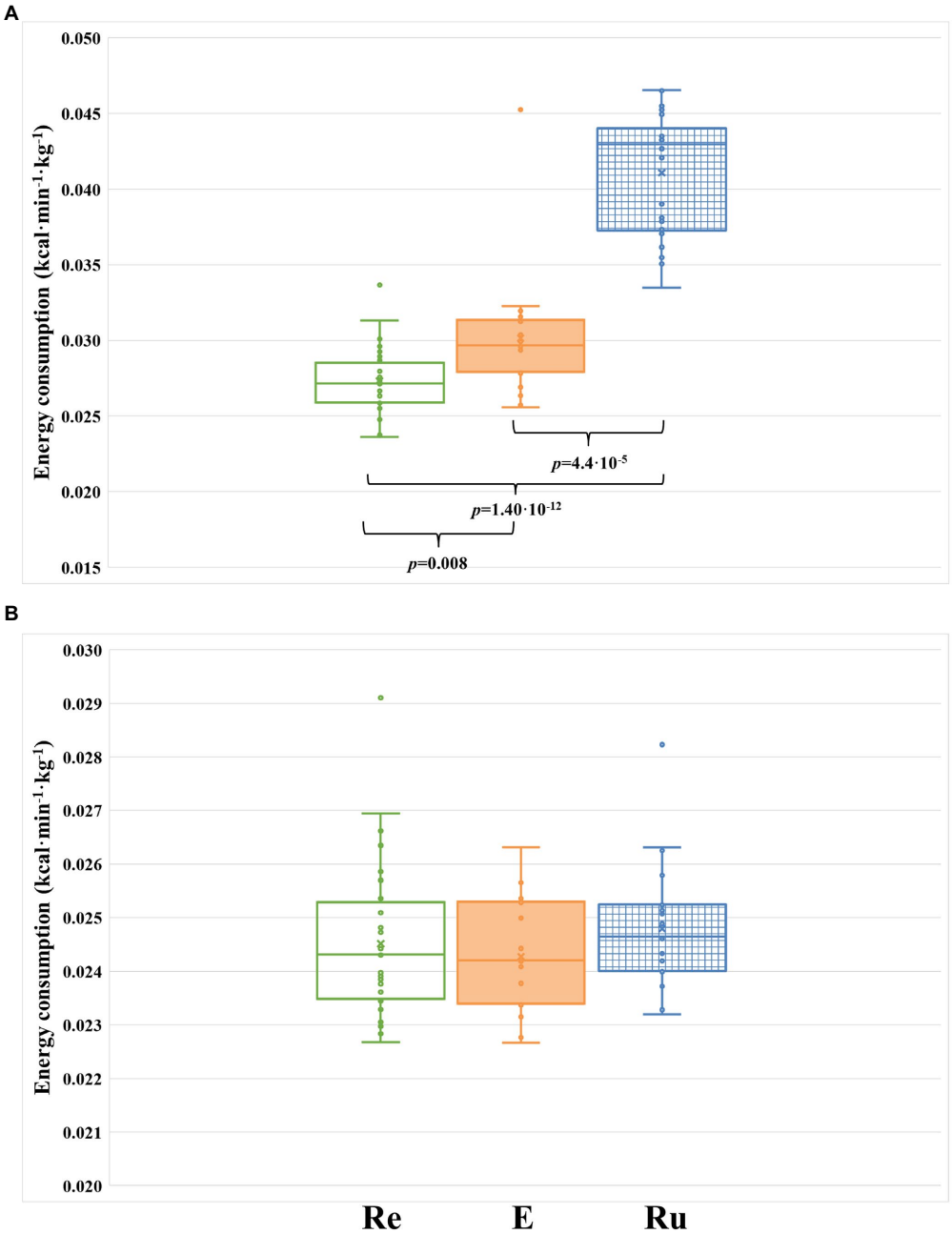

FIGURE 3 | Comparison of energy consumption (kcal/kg/min) between the three groups during the intervention phase of the study. **(A)** Energy consumed by each group during the three 8-h segments where the recovery activity was performed (24 h in total). **(B)** Energy consumed by each group during the time of the intervention phase where runners are not performing the activity (13 8-h segments). Re, participants included in the REST group (green-rimmed boxes); E, participants included in the ELLIPTICAL group (orange-filled boxes); and Ru, participants included in the RUN group (blue-dashed boxes).

Further work is required to explore renal function recovery after strenuous exercise using novel biomarkers.

The biphasic depression of GFR observed in our study, apart from being correlated with muscle damage levels (Bernat-Adell et al., 2019), could also be related with the hydration status (Poortmans, 1984; Poortmans et al., 1988). Runners tend to increase their fluid intake after marathon running, which may promote glomerular filtration; but it usually backs to normal after 48 h post-marathon running. Similar adequate hydration levels were observed 48 h after completing the marathon race compared to those measured at the finish line. The observed transient GFR recovery at the 24 h post-marathon time point may be due to overhydration. However, since there is no information about both the hydration status at this time point and the rehydration strategy followed by runners, this hypothesis should be tested and validated in future studies.

Previous studies in the field have generally focused on measuring biomarkers of muscle and cardiovascular damage. Up to now, only two studies monitored renal function for more than 48 h after finishing a long distance race to evaluate how long of full resting is needed for renal function recovery (Irving et al., 1986, 1990). According to their results, levels of serum creatinine remained significantly elevated up to 72 h post-race. The inconsistency with our observations may be due to the limited sample size of these studies (less than 10 individuals analyzed) and the daily activities performed by participants during the recovery period (they were not continuously monitored as in our study).

Recently, in the same study population, we reported data on muscle damage recovery after running a marathon (Martínez-Navarro et al., 2020b). We observed that both active and passive recovery had similar effects in muscle damage recovery, which was supported by a previous study (Sherman et al., 1984). However, the RUN group showed a faster recovery in neuromuscular function compared to REST and ELLIPTICAL groups. Therefore, we concluded that running at 95–100% of VT1 seemed to be the optimal strategy for muscle function recovery 48 h after finishing the marathon, as long as pain did not prevent exercise from being properly performed. In case of muscle pain, we recommended runners to perform elliptical workouts during the week after marathon racing (Martínez-Navarro et al., 2020b).

However, results achieved in this study lead us to strongly advise against the use of elliptical machines for marathon recovery because of its negative impact on renal function recovery. Hence, we encourage runners to carry out an active recovery based on light-intensity continuous running from 48 h after finishing the marathon. This will promote both muscular and renal function recovery. Our results also suggest that full resting is a better strategy than using an elliptical machine (its use should be delayed until at least 1 week after marathon running). This observation is contrary to our initial hypothesis, and further work is also required to understand why runners who perform elliptical workouts (a lower-impact exercise for lower-limb joints) recovered later from renal damage. Note that our observations were not affected by the dehydration status, which has been shown to limit renal recovery (Poortmans, 1984; Panizo et al., 2015; Rojas-Valverde et al., 2021b), since all participants had a correct hydration prior to the recovery phase.

The main weakness of our study is the need of selecting comparable recovery strategies in terms of biomechanical movements for being able to monitor participants using triaxial accelerometers. This limitation is caused by the lack of validation of accelerometer activity-level specific cut-offs for cycling and swimming in a cohort with a substantial level of physical activity compared to normal population. Moreover, being able to swim during 40 min at 95–105% VT1 requires a previous adaptation impeding to randomly include participants in an intervention group using swimming as recovery strategy. The fact that the elliptical machine is not normally used by participants for training can also be a limitation, since it may be uncomfortable and hard to coordinate leg and arm movements. In addition, it is not well-known whether elliptical workout requires a greater physical effort at the muscular (not joint) level compared to running, which may in fact delay renal recovery. As discussed above, another limitation of our study is the impact of muscle damage on classical renal injury biomarkers.

In summary, our results show the beneficial impact of light-intensity continuous running on marathon-induced physiological damage recovery. Our study is an important resource to guide runners, coaches, and medical specialists in their search for the most optimal recovery strategy after running a marathon.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of the Jaume I University of Castellon. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CarlosH and BH contributed to conception and design of the study, article drafting, and critical revision of the article. CarlosH and CarlaH contributed to data curation, analysis, and interpretation. CarlosH, IM-N, EC-B, AF-A, and NP contributed to data collection and critical revision of the article. CarlosH, IM-N, and EC-B contributed to funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.812237/full#supplementary-material

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REVIEW
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Physical Exercise in the Context of Air Pollution: An Emerging Research Topic

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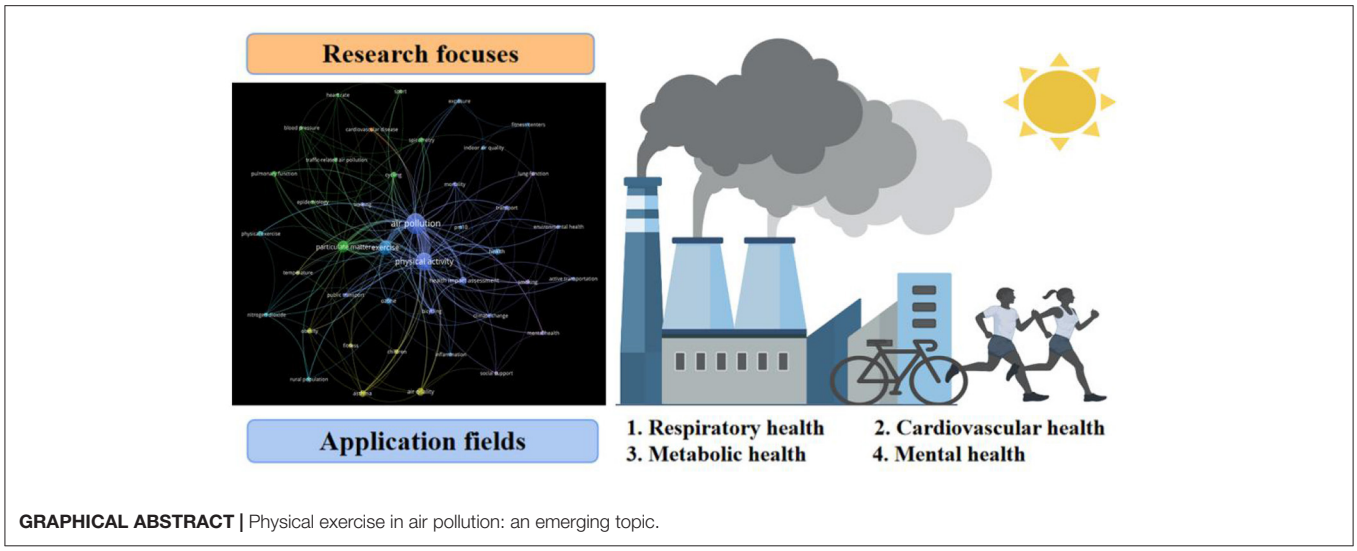
Physical exercise (PE) brings physiological benefits to human health; paradoxically, exposure to air pollution (AP) is harmful. Hence, the combined effects of AP and PE are interesting issues worth exploring. The objective of this study is to review literature involved in AP-PE fields to perform a knowledge-map analysis and explore the collaborations, current hotspots, physiological applications, and future perspectives. Herein, cluster, co-citation, and co-occurrence analysis were applied using CiteSpace and VOSviewer software. The results demonstrated that AP-PE domains have been springing up and in rapid growth since the 21st century. Subsequently, active countries and institutions were identified, and the productive institutions were mainly located in USA, China, UK, Spain, and Canada. Developed countries seemed to be the major promoters. Additionally, subject analysis found that environmental science, public health, and sports medicine were the core subjects, and multidimensional communications were forming. Thereafter, a holistic presentation of reference co-citation clusters was conducted to discover the research topics and trace the development focuses. Youth, elite athletes, and rural population were regarded as the noteworthy subjects. Commuter exposure and moderate aerobic exercise represented the common research context and exercise strategy, respectively. Simultaneously, the research hotspots and application fields were elaborated by keyword co-occurrence distribution. It was noted that physiological adaptations including respiratory, cardiovascular, metabolic, and mental health were the major themes; oxidative stress and inflammatory response were the mostly referred mechanisms. Finally, several challenges were proposed, which are beneficial to promote the development of the research field. Molecular mechanisms and specific pathways are still unknown and the equilibrium points and dose-effect relationships remain to be further explored. We are highly confident that this study provides a unique perspective to systematically and comprehensively review the pieces of AP-PE research and its related physiological mechanisms for future investigations.

Keywords: knowledge map, visualized analysis, air pollution, physical exercise, physiological mechanisms



GRAPHICAL ABSTRACT | Physical exercise in air pollution: an emerging topic.

INTRODUCTION

Air pollution (AP) occurs in sync with the progress of industrialization and urbanization. The global burden of disease assessment shows that AP has become the world's largest environmental health problem (Cohen et al., 2017) and is associated with mortality among different populations (Di et al., 2017a,b; Burnett et al., 2018). Six pollutants are designated as criteria air pollutants: particles with aerodynamic diameters under 10 and 2.5 um, ozone, sulfur dioxide, nitrogen dioxide, carbon monoxide, and lead (Suh et al., 2000). Among them, the specific particulate matter (PM) has attracted extensive attention due to its impact on several diseases, such as asthma, hypertension, diabetes, and depression (Polichetti et al., 2009; Kramer et al., 2010; Guarnieri and Balmes, 2014; Kim et al., 2016). Indeed, there is also evidence that AP has caused 4.2 million premature deaths worldwide and will become the seventh most lethal factor in the world in the next two decades (Foreman et al., 2018). As an emerging strategy to mitigate the detriment of AP to the body, exercise and physical activities play irreplaceable roles in promoting health, preventing chronic diseases, reducing disease complications, and delaying disease progressions (Haskell et al., 2007; Colberg et al., 2010; O'Donovan et al., 2010; Booth et al., 2012; Lavie et al., 2019). In this condition, a rarely known but increasingly crucial issue is the relationship between AP and its impact on exercise and physical activities. Given that physical inactivity and sedentary lifestyle itself can induce multiple chronic diseases, while outdoor exercise is regarded as a unique solution for sustaining physiological balance, improving a metabolic cycle, and enhancing immunity. These facts, in this sense, lead to a dilemma whether the positive effects of exercise could be suppressed by the ambient AP.

It is of great significance to examine the risk-benefit relationship between health benefits of physical exercise (PE) and the potential risks of the increased air pollution. First, for people living in the long-term polluted regions, they have no choice but to commute during the hustling traffic or maintain a certain intensity of physical activities no matter how serious the pollution is, so exploring the effects of exercise on health in the context of air pollution is meaningful to provide exercise suggestions (e.g., mode, duration, and intensity) and health guidelines for this targeted group. Second, with the springing up of global outdoor sports represented by major marathons, people participating in outdoor activities are also at higher risk of exposure to pollutants. Studying the safety threshold of exercise during AP exposure can not only guide city governments to organize various events, such as the Olympic Games, world, or nationwide sporting events, etc., but also help sports enthusiasts or professional athletes to prepare for these games. Finally, in view of the fact that AP itself can lead to an increase in the prevalence and mortality of chronic diseases, probing into the prevention and rehabilitation of related disease using exercise strategy during the intermittent period of AP can help to determine whether exercise can mitigate the health detriment caused by pollutant exposure.

Concerning the research on the AP, PE, and public health, a comprehensive analysis containing these three fields is lacking. In response to the increasing volume of articles on the relationship among these issues, this study aims to synthesize the accumulating knowledge in the field and provide a bird's eye view of the research using bibliometric and visualized approaches. Comparing to traditional content analysis of the literature, this strategy can provide an objective and holistic overview to discover the spatial and temporal distributions of study status, identify the major and highly-cited scholars, reveal emerging thematic frontiers, and ultimately, contribute to moving the research field forward. However, to the best of our knowledge, the systematic analysis based on the review of PE in the context of AP has not been made yet. To fill these research gaps, this study employs bibliometric mapping and the visualized method to address the following key issues: (i) to identify countries, institutions, categories, and journals that have played important roles in global AP-PE research; (ii) track current concerns and

physiological fields by reviewing the nowadays' status and new efforts; and (iii) to summarize the frontiers that need to be more concerned in the AP-PE context.

Collectively, this paper's innovation lies in the following points. It is the first attempt at a knowledge-map review of AP-PE, which provides an innovative perspective to comprehensively examine the basic features of the literature and identify the hotspots. Simultaneously, building on the selection of the authoritative database, this manuscript systematically elucidates the latest international cooperation status, evolution trends, and the multi-level research on the AP-PE domain. Eventually, several priority directions of the AP-PE field are highlighted to advance the knowledge progress in this area.

In summary, this study is structured as follows: Section Methodology introduces the methodology. Subsequently, based on the knowledge map and bibliometric strategies, the characteristics of documents are presented in section Results and Discussion. In light of the reference co-citation analysis and keywords co-occurrence analysis, section Research Hotspots and Application Fields Analysis discusses the application fields and reviews the new efforts and research implications. Section Conclusion and Future Perspective concludes the study and suggests the future work.

METHODOLOGY

Data Acquisition and Search Strategy

Four indexes, the Science Citation Index Expanded (SCI-Expanded); the Social Sciences Citation Index (SSCI); the Arts & Humanities Citation Index (A&HCI); and the Emerging Sources Citation Index (ESCI), were selected from the Core Collection of Web of Science as the data source. Several reasons justified the selection of the WoS database in this research. As noted, the WoSCC remains as the standard database for knowledge-map-based analysis (Meho and Yang, 2007), which has been applied in a number of bibliometric studies (Mao et al., 2018; Martinho, 2019; Yan et al., 2020; You et al., 2021a,b). In addition, the WoSCC is a multidisciplinary database and includes literature on environmental science and public health emerging from distinctive research areas and disciplines published in more than 20,000 journals. Applying specialized databases such as PubMed may result in biases into the search strategy favoring medical domains. Still, it is important to note that, although other interdisciplinary databases like Scopus can provide similar coverage, it has complete citation information only from 1996 (Li et al., 2010), so the results from Scopus are still imperfect. Additionally, in terms of classification, WoS is also one step higher than Scopus in representing metadata lists. Actually, search results in WoS are not only classified by author, year, subject, and document types like Scopus but also by institutions and countries (Chadegani et al., 2013), which are more convenient for scholars to analyze the distribution of organizations. Thus, in view of the above factors, we chose the four indexes of WoSCC as the database for further analysis.

The following methods were conducted for search publications: [TS = ("exercise" OR "fitness" OR "sport" OR "physical exercise" OR "physical activity" OR "exercise training" OR "physical fitness" OR "aerobic exercise" OR "non-aerobic exercise" OR "interval exercise" OR "resistance exercise" OR "strength exercise" OR "breathing exercise" OR "muscle stretching exercise"] AND TS = (["air pollution" OR "polluted air" OR "pollution air" OR "air quality" OR "indoor air pollution" OR "outdoor air pollution" OR "air pollution health" OR "air pollution cardiovascular" OR "air pollution lung" OR "air pollution asthma" OR "air pollution respiratory" OR "household air pollution" OR "ambient air pollution" OR "particulate matter" OR "ozone")] AND [AK = ("exercise" OR "fitness" OR "sport" OR "physical exercise" OR "physical activity" OR "exercise training" OR "physical fitness" OR "aerobic exercise" OR "non-aerobic exercise" OR "interval exercise" OR "resistance exercise" OR "strength exercise" OR "breathing exercise" OR "muscle stretching exercise"] AND AK = ["air pollution" OR "polluted air" OR "pollution air" OR "air quality" OR "indoor air pollution" OR "outdoor air pollution" OR "air pollution health" OR "air pollution cardiovascular" OR "air pollution lung" OR "air pollution asthma" OR "air pollution respiratory" OR "household air pollution" OR "ambient air pollution" OR "particulate matter" OR "ozone")], time span = 2001–2021. To avoid bias, all hits were retrieved as "full-record and cited-references" files from WoSCC on August 12, 2021 for further analysis. Referring to the previous pieces of research (Yan et al., 2020; You et al., 2021a,c), we only selected "articles or reviews" for analysis, and the language was limited to "English"; other document types and non-English articles were excluded. The reason for setting the time range in the new century was that few publications related to AP-PE were produced before year 2001. Basic information for each document was gathered into text documents, such as countries, institutions, journal sources, authors, and references. A total of 213 papers were initially found; then, the document type and language were filtered, resulting in a final number of 181 publications. The research framework is summarized in **Figure 1**.

Research Methods and Analysis Tools

The research methods used in this paper were the knowledge map and bibliometrics. The knowledge-map-based methods can be used to perform visualized analysis on a certain research field, and its execution mostly involves the applications of data mining, scientific measurement, information analysis, and drawing. By mapping literature distribution and overall efforts, the knowledge map has been used widely to get more refined information related to the research itself (Rodriguez and Moreiro, 1996; Chen et al., 2005). Bibliometric overview is another widely recognized approach to evaluate topics from a library and an information science perspective (Oelrich et al., 2007; Bornmann and Leydesdorff, 2014), which can help discover knowledge flows and patterns in the structure of a field, demonstrate its scientific roots, reveal emerging thematic areas (Skute et al., 2017), and ultimately present an in-depth analysis of the knowledge domain. Combining these two strategies can provide audience with visual graphics to clearly understand the study status, frontiers, and hotspots, thereby suggesting perspectives to guide future directions.

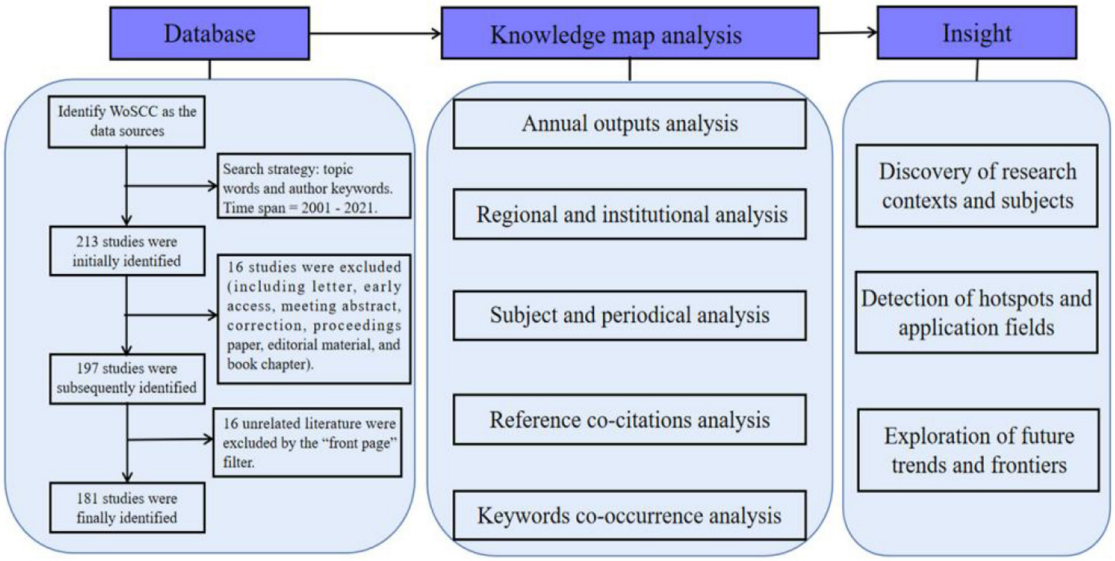


FIGURE 1 | The summary of the flowchart and study design.

Two java-based visualization tools, CiteSpace and VOSviewer, were used to characterize and reveal the results from bibliometric analysis. CiteSpace was developed by Professor Chen Chaomei of Drexel University, and it is mainly used to build the knowledge map (Chaomei, 2006); the software has been iterated to version 5.8.R1. In this study, the CiteSpace software was applied to visualize the network of countries and institutions, collaboration network among authors and cited authors, relationship of categories and journals (a dual-map overlay) involved in this field, and the timeline view map of references with co-citations. Noticeably, a co-citation is defined as two publications share a citation from the same third study (Small, 1973; Merig et al., 2019). VOSviewer (version 1.6.16) was applied to generate the co-occurrence of related keywords in special issues.

Additionally, a knowledge map typically features a set of points and lines to elucidate collaborations among publications (Chaomei, 2006). Different nodes are used to represent indexes like a country or region, author, institution, journal, reference, or keyword. The size of the nodes speaks on behalf of the betweenness centrality of publications, and nodes with larger size indicate higher occurrence or citation frequency (Chen et al., 2014). The betweenness centrality index in this study can be regarded as a bridge extending from earlier to more recent viewpoints, which further reflects the extent to which paths in the network go through a certain node. The values of centrality are usually standardized to the unit interval [0, 1]. Simultaneously, lines represent connections among the nodes, with stronger connections indicated by wider lines. The knowledge map represents the keywords and references with citation bursts. Occurrence bursts describe the strength of a certain theme frequency, whereas citation bursts are used to express the frequency of the reference (Chaomei, 2006). The burst of nodes was calculated with a given network through Kleinberg's algorithm (Jon, 2003). Through burst index and this kind of maps, scholars can better understand emerging trends and grasp the research hotspots.

RESULTS AND DISCUSSION

Annual Outputs Analysis

A total of 181 candidate publications were finally retrieved from database WoSCC. Based on the statistical analysis, the details of annual publications since the new century are presented in **Figure 2**. The dynamic change in the number of publications can be used as an indicator to reveal the potential research trends. The outputs tendency can be briefly divided into the following three stages: 2001–2010, 2010–2016, 2016-present. Although the hazards of air pollutants to health have been widely reported, no further study participated in exploring the exercise activities in polluted air. In 2001, Carlisle and Sharp (2001) paid attention to exercise in the outdoor ambient air pollution, which aroused more interest in this field. While the annual outputs never exceeded five before 2010, which indicated that there are still few scholars paying attention to PE under the background of AP and the research on this domain remained stagnant in this stage. In 2010, with the enhancement of people's active health awareness, Johan de Hartog et al. (2010) proposed the question whether the health benefits of cycling outweighed the risks exposed in pollutants, and this called for more follow-up pieces of research about this issue. Subsequently, Tainio et al. (2016) in 2016 found that benefits of physical activity and active travel outweighed the harm caused by air pollution in most cases, which further strengthened people's confidence in maintaining physical activity even in a polluted environment. The number of publications on this field increased from 5 in 2010 to 16 in 2016 and has kept an overall growth trend up to now. We next drew the above results

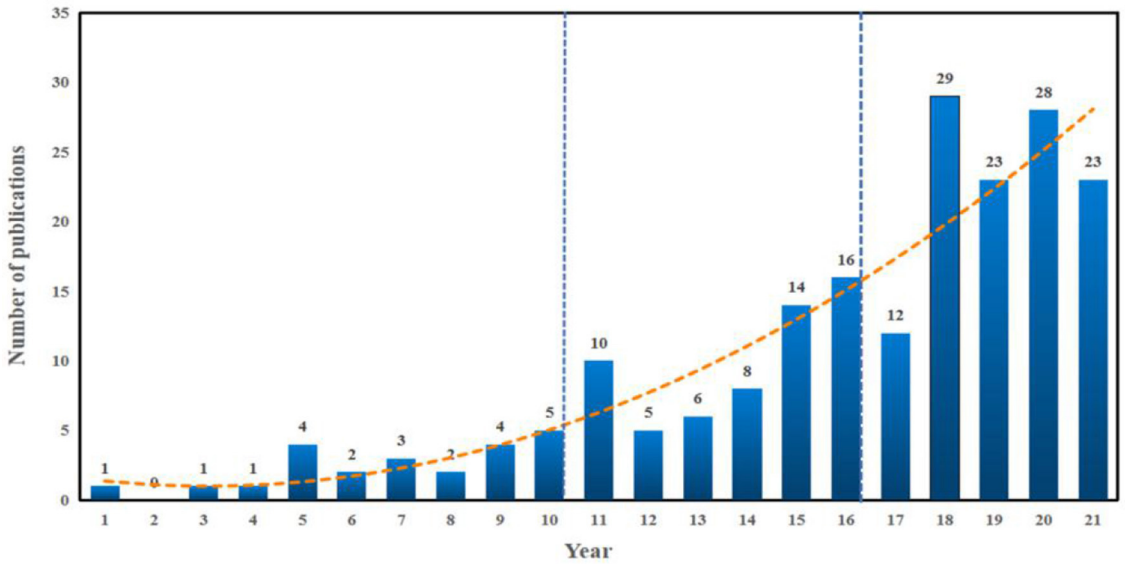


FIGURE 2 | The output of publications and growth trends of pieces of air pollution-physical exercise (AP-PE) research.

TABLE 1 | Ranking of top 10 countries and institutions of pieces of air pollution-physical exercise (AP-PE) research.

Rank	Country	Publications	Centrality	Rank	Institution	Publications	Centrality
1	United States	61	0.35	1	Ctr Res Environm Epidemiol CREAL	19	0.18
2	China	30	0.09	2	Centro de Investigacion Biomedica en Red	14	0.13
3	United Kingdom	28	0.20	3	UPF	8	0.03
4	Spain	22	0.05	4	Univ British Columbia	8	0.03
5	Canada	20	0.01	5	Zhengzhou Univ	6	0.01
6	Germany	16	0.07	6	Flemish Inst Technol Res VITO	6	0.01
7	Switzerland	16	0.06	7	Univ London Imperial Coll Sci Technol & Med	6	0.01
8	Brazil	14	0.01	8	Univ São Paulo	6	0.06
9	Australia	13	0.03	9	Univ Basel	5	0.01
10	Italy	12	0.04	10	US EPA	5	0.02

into a fitting curve and used the curve fitting method to obtain the change trend formula of the number of international AP-PE literature, as shown in formula $y = -4E\text{-}06\ x^3 + 0.0843\ x^2 - 0.5183\ x + 1.7978$. Among them, y represents the number of papers, and x represents the year. It can be estimated that the publication amount related to this topic will continue to increase in the following years.

Regional and Institutional Analysis

Scholars from more than 40 regions and 280 institutions contributed to publications on the domain of AP-PE research. The details of the top 10 countries and institutions are shown in **Table 1**. The cooperation network among different countries is illustrated in **Figure 3A**, which describes the research status of AP-PE in multiple areas. Each point in the figure represented a country, and the size of the point reflected the number of documents in that country. The international academic collaborations were generated by CiteSpace software with 41 nodes and 228 links, which means that the total documents collected were published in 41 countries or regions, and 228 connections were built among these countries. We can observe that the mainstream cooperation relationship was divided into three groups, and the United States, the United Kingdom, and China occupied the dominant position, which conducted the majority of pieces of AP-PE research. The United States took the lead and had the highest amount of literature, 61, also the highest centrality (0.35). China and the United Kingdom were the second productive countries with 30 and 28 publications, respectively, followed by Spain (22 publications) and Canada (20 publications). The top five countries' contributions were all above 20 publications, which implied that they contributed chiefly in research achievements.

When it comes to institutional participation, **Figure 3B** reflects the collaborations among institutions with 281 nodes and 613 links. According to the definition of links and nodes, these organizations had close ties with each other and have

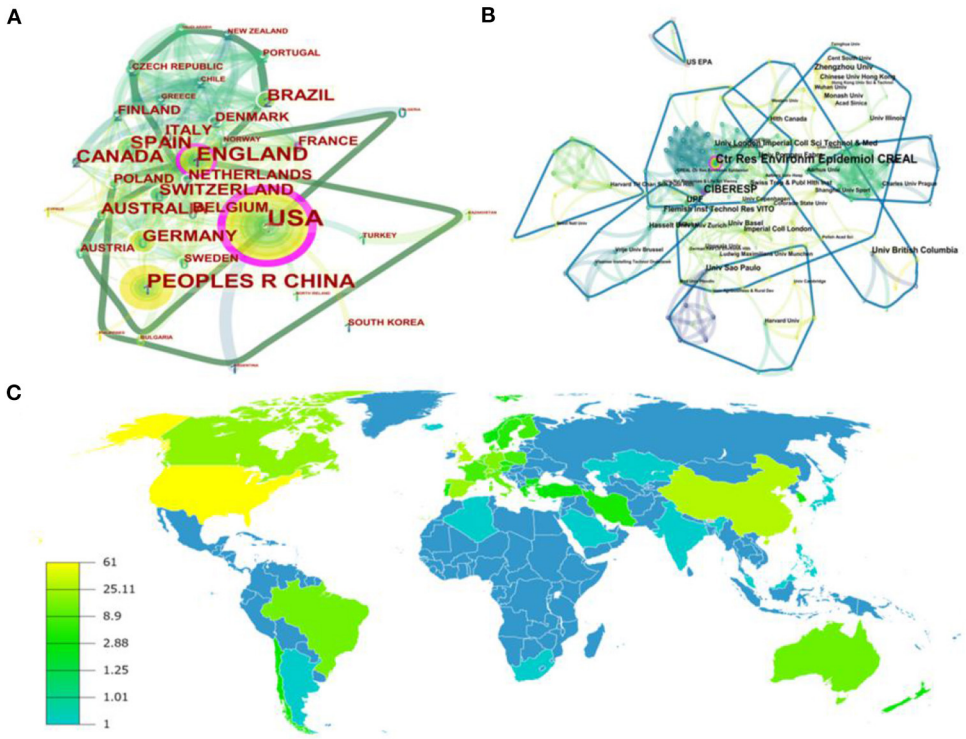


FIGURE 3 | The map of countries **(A)**, institutions **(B)**, and world distribution **(C)** of publications in the AP-PE field.

a considerable academic impact. Ctr Res Environm Epidemiol CREAL engaged in the most studies with 19 documents, followed by Centro de Investigacion Biomedica en Red, UPF, Univ British Columbia, with 14, 8, 8 literature, respectively. In terms of centrality, these top 10 institutions were also in a leading position. In addition, we can detect that over half of the institutions were universities, which indicated that colleges and universities played important parts in the innovation of this field. However, from the perspective of clustering, some non-university institutions seemed to have weak connections with others, while universities tended to maintain a large number of cooperative relationships.

Furthermore, the distribution of countries and regions is presented in **Figure 3C**. From this figure, we can find that countries and institutions in North America, western Europe, Australia, and East Asia led the way in the AP-PE domain. Consistently, most of the top 10 institutions referred in **Table 1** belonged to Western nations, which also helped explain that Western nations occupied the first echelon position in the AP-PE field. It seemed that developed regions might input richer resources into environmental and health-related topics and pay more attention to physical activities in air pollution. However, as the largest developing country, China also conducted significant contributions in this research field. China faces severe challenges in controlling air pollution and preventing its potential hazards. In view of the vast territory of China, it is difficult to change the fact that some areas have suffered from industrial pollution for a long time in the short term. Hence, it is more meaningful to study the relationship between air pollution, physical activities, and their impacts to body health in these regions. In brief, western countries and institutions seemed to be the major promoters; participants from different regions all made contributions to the AP-PE field; and different organizations should strengthen cooperation to win higher achievements.

Subject and Periodical Analysis

Subjects involved in publishing pieces of AP-PE research are displayed in **Figure 4A**. Referring to the description of centrality, the discipline "Ecology," "Public, Environmental, and Occupational Health," and "Environmental Sciences" are surrounded by the purple rings, which indicated that they played important roles in AP-PE studies. The merged network was consisting of 71 nodes and 145 links, which illustrated that 71 sub-disciplines were involved in pieces of AP-PE research and 145 cross-paths, and collaborative networks have been established within the disciplines. Compared to previous AP literature (Sweileh et al., 2018; Dhital and Rupakheti, 2019), the disciplinary cooperation networks in the fields of AP-PE were more complex and comprehensive. It was clear that all subjects made contributions to the scientific research. Environmental science and public health seemed to be core subjects, while sports science, medicine, and toxicology worked together to enrich the horizons of the disciplines and broaden the depth of research. Importantly, as the variety of disciplines tended to be diversified,

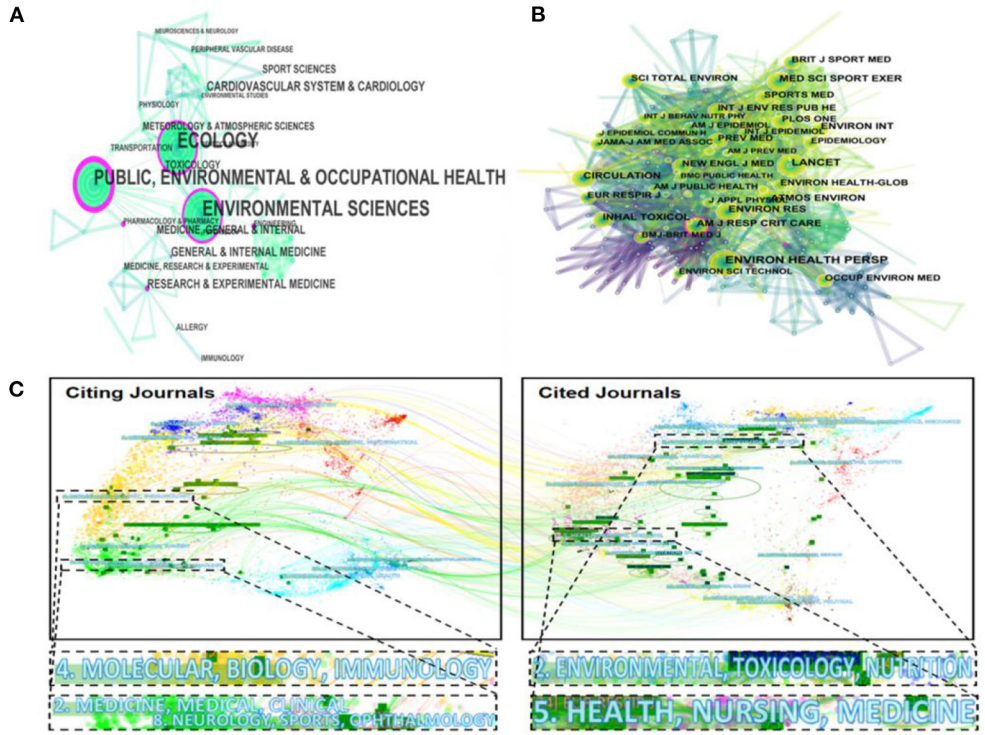


FIGURE 4 | The map of disciplines **(A)**, cited-journals **(B)**, and a dual-map overlay **(C)** of publications in the AP-PE field.

it can be seen that the AP-PE field has developed rapidly in the past decades.

Building on the analysis of subjects, we next discussed about the cited journal in the AP-PE domain. The term citing journal is defined as the journal that publishes AP-PE papers, and the cited journal is defined as the journal where the references cited in AP-PE papers are published. **Figure 4B** describes a panorama of cited journals. In this figure, 471 nodes and 2,939 links were built simultaneously. Among the nearly 500 journals, there were Sci Total Environ, Environ Int, Environ Res et al. in environmental science; Med Sci Sport Exer, Brit J Sport Med, Sports Med et al. in sports science; the medical field represented by Lancet, New Engl J Med, and Circulation. Several comprehensive journals, such as Plos One, were also among the abundant research scopes. Given that cited journals provided a theoretical basis for the citing journals, these diverse trajectories illustrated that the disciplinary center of the journals shifted from a single subject to multidisciplinary clusters.

Figure 4C presents a dual-map overlay of the research themes between citing journals and cited journals in the AP-PE field. This approach uses two graphs at the same time, with the citing journals on the left and the cited journals on the right. From this figure, we can find two major citation paths. The yellow paths showcased that research published in "molecular, biology, immunology" journals preferred to quote journals mostly in the domains of "environmental, toxicology, and nutrition." The paths colored with green demonstrated that studies published in "medicine, medical, clinical," and "neurology, sports, and ophthalmology" journals tended to cite journals primarily in the domains of "health, nursing, and medicine." It is to be noticed that AP-PE fields are actually a comprehensive interdisciplinary as well as an emerging realm worthy to be explored. For promoting the development of physical activities in air-polluted environments, there is an urgent need for more multidimensional communications.

Analysis of Co-cited References

Co-cited references refer to the citations of two scientific documents by one article (Chaomei, 2006). The more occurrences of co-cited documents, the closer the relationship between the two scientific documents. As time evolves, a huge structure of citation relationships has been formed among published scientific documents. In this study, we used the CiteSpace software to concretely express the relevant citation relationship network structure in the AP-PE field through the scientific knowledge map. By analyzing the clusters composed of co-cited references, we can have access to trace the roots of the scientific development and on this basis, to explore the themes and trends of AP-PE fields.

There are three options that can be applied to create label clusters: Log-Likelihood Ratio (LLR), Latent Semantic Indexing (LSI), and Mutual Information (MI). We used the Log-Likelihood Ratio algorithm since this strategy can cover the "uniqueness and coverage" of all labels created. The research

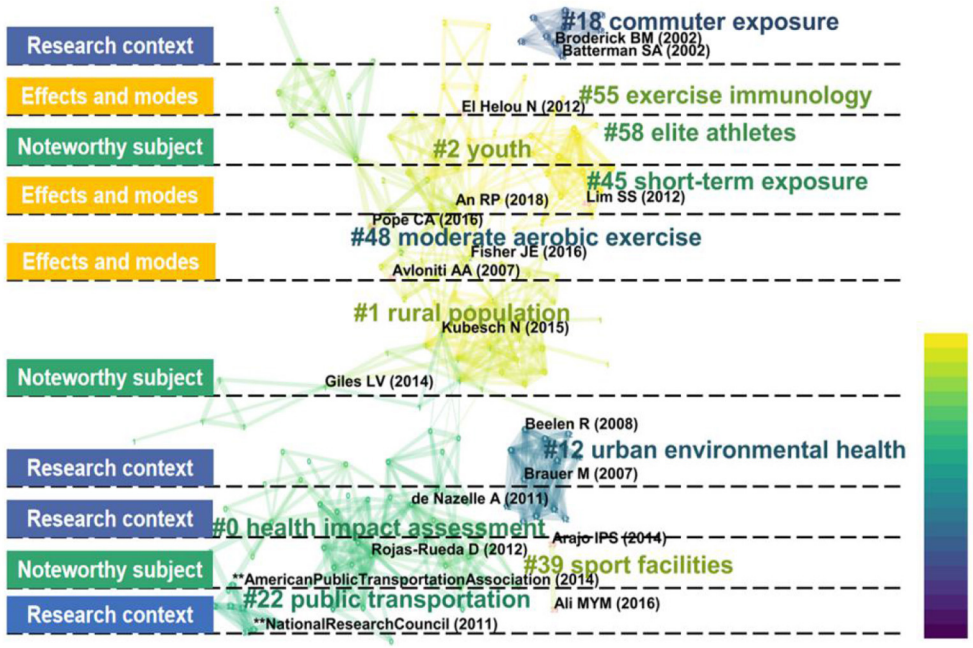


FIGURE 5 | The research topics and clusters of the co-cited references in the AP-PE field.

topics were divided into several clusters, which were labeled by "#," and then their time development trajectory was described, as shown in **Figure 5**. The darker the clusters' color, the later the literature appeared. Here, we concluded several noteworthy topics into three classifications: "research context," "effects and modes," and "popular subject." The modularity value (Q value) and weighted mean silhouette value (S value) were used to evaluate the rationality of clustering, and it is generally accepted that a $Q > 0.5$ cluster and $S > 0.7$ means that the cluster is convincing. In our clustering, the Q equaled to 0.885 and S equaled to 0.893, which further verified the rationality of the strategy.

Research context is mainly composed of four sub-clusters: commuter exposure, urban environmental health, public transportation, and health impact assessment. In recent years, "active transportation" (AT) has gradually become a popular new model, represented by walking and cycling for transportation. Motivations for the AT mode not only included traffic pollution mitigation, urban climate improvement, but also provided substantial health benefits. Unfortunately, the discussions on the benefit-risk and benefit-cost ratios of active transportation have never stopped so far. The current consensus is that AT under a lesser extent AP may be beneficial (de Nazelle et al., 2011), and, in this situation, AT can help people get rid of car dependence and increase activity levels (Lindsay et al., 2011). It is well-known that there is a dose-response relationship among pollution exposure, physical activity, and health. The greater the amount of physical activity and the lower the pollution concentration, the more likely it is to reduce the risk of morbidity and death. Therefore, there seems to be emerging interest in health impact assessment (HIA) as a strategy to evaluate health consequences. Despite the impact of potential publication bias, Mueller et al. (2015) applied the HIA method to systematically evaluate the AT mode and found that the benefits of increased PA surpassed detrimental influence of traffic incidents and air pollution exposure. In addition, Woodcock et al. (2009) estimated the relationship between AT and greenhouse gas emission reductions and suggested that mode shifts toward AT were beneficial to public health issues. Rather than traditional assessment, de Nazelle et al. (2011) first proposed a conceptual framework to further evaluate the pertinence and quantify relevant impacts. However, in view of the complexity of the active travel mode, there are still several factors, which cannot be fully quantified. Meanwhile, although modeling tools can be used to predict the relationship between vehicle emissions reduced by physical activity and AP, there are few real-world examples and lack of evidence from different regions.

In relation to research effects and modes, it can be divided into exercise immunology, short-term exposure, and moderate aerobic exercise. Immunity enhancement is one of the significant benefits that physical exercise brings to the body. A growing number of studies have proved that exercise induces considerable physiological changes in the immune system (Hoffman-Goetz and Pedersen, 1994; Nieman, 1997; Gleeson and Pyne, 2000; Pedersen and Hoffman-Goetz, 2000). Cytokines (IL-6, IL-8, IL-10, and TNF-α) (Normando et al., 2013; Bos et al., 2014; Lu et al., 2015a; Trnjar et al., 2017), inflammation-relate proteins (CRP, FeNO, CC16, CD62P, CD63, and CD40) (Rundell et al., 2007; Cutrufello et al., 2011; Normando et al., 2013; Trnjar et al., 2017), and stress hormones (including epinephrine, norepinephrine,

growth hormone, b-endorphins, insulin, and cortisol) (Volek et al., 1997; Molina-Sotomayor et al., 2020) are most common indexes to estimate the variety of immune function influenced by exercise. As for the duration of the study, in view of the potential ethical approval difficulties of long-term air pollution exposure, most of the existing studies focus on the impacts of exercise and physical activities in short-term exposure to the body. Current evidence tends to show that short-term exercise on non-regular basis trainers induces a short-term inflammatory response (Kasapis and Thompson, 2005), but if with the long-term regular exercise, habits can lead to an anti-inflammatory effects, including reductions in C- reaction protein (CRP) levels (Dufaux et al., 1986). An exercise mode is another variable factor, also a quantifiable and reproducible strategy that can be modified experimentally, while the most classic and common paradigm used in current research is moderate aerobic exercise (MAE), which is also known as moderate intensity aerobic exercise (MIAE). At present, there are a number of studies that have adopted the contrast mode between high and low exercise intensities or adopted emerging exercise modes such as interval training or sprint training or the mix of both like high-intensity interval training (HIIT) (Giles et al., 2012, 2014; Marmett et al., 2021), but the overall impact of changes in the exercise mode on air pollution exposure is relatively small. Since the new exercise mode seems to be difficult for beginners or amateurs, pieces of research on the effects of different exercise patterns remain to be excavated.

When it comes to a popular subject, it is important to highlight youth, elite athletes, sports facilities, and rural population. Youth groups are in the growth period, and their respiratory system is not yet mature; hence, they are more susceptible to atmospheric particulates than adults (Gauderman et al., 2004; Chen et al., 2015; Ghozikali et al., 2018). Evidence showed that changes in respiratory tract inflammation, FeNO, etc. can be observed in school children on just 1 day of short-term exposure to air pollutants (Idavain et al., 2019). Recently, several studies have discussed the relationship between adolescent physical activities and air pollution. Results proved that outdoor activity time duration was associated with air pollution, and the longer the outdoor activity time might lead to lower lung function (Yu et al., 2017; Lovinsky-Desir et al., 2021). Except for young people, elite athletes are also the targeted groups. Different from the general population, athletes have to perform year-round training, and the climate of the training or competition sites is not selectable. It is inevitable that athletes may encounter different air pollution conditions. Early studies have been conducted on the status of particulate matter inhaled by athletes during the Athens Olympic Games and Beijing Olympic Games (Florida-James et al., 2004; Lippi et al., 2008; Fitch, 2016). Not surprisingly, several results pointed to the fact that several problematic pollutants, including oxides of nitrogen (NO_x), PM_{10}, $PM_{2.5}$, and ozone, have a potentially deleterious impact on top-class athletes' health and athletic performance (Florida-James et al., 2004; Donnelly et al., 2016; Reche et al., 2020). Moreover, swimmers, skaters, skiers, or other groups practicing at specific sports venues are at greater risk of exposure to air pollution. Breathing airborne Teflon particles from fluorinated waxes in the confines of the indoor ski-slope may be risks to skiers (Rundell and Sue-Chu, 2013). Likewise, serious swimmers are exposed to high concentrations of trichloramines at the pool-surface level, and this kind of exposure may be continuous (Bougault and Boulet, 2012). However, it is still challenging to disentangle confounding air pollutants' effects on athletic performance under different sports circumstances. As for rural individuals, when AP is viewed in the context of low income, confounding factors can detrimentally affect their health, including physical inactivity, none medical insurance, poor nutrition, and other health risks. In relatively underdeveloped rural areas, the use of inefficient fuels and lack of sufficient ventilation led to worse air quality than other regions. Studies showed that communities with high proportions use of household solid fuel and the polluted emissions from inefficient stoves may cause ambient AP and place regions at risk for adverse health outcomes (Ward and Lange, 2010; Lim et al., 2012; Johnston et al., 2013). In a nutshell, youth groups, elite athletes, enthusiasts of special indoor sports activities, and rural population are emerging research subjects in AP-PE fields and deserve special attention.

Analysis of Co-occurrence Keywords

A map of keywords can present major research interests and hot topics of one certain field. CiteSpace software was applied to conduct the keywords co-occurrence map (**Figure 6**) in this study. The merged network in this figure consisted of 420 nodes and 2,045 links. Among the more than 400 keywords, the highest frequency belonged to the "air pollution," "physical activity," "exercise," which appeared 126, 90, and 61 times, respectively. This indicated that these three terms were the core subject words in the domain. It was easy to identify that the keywords "public health," "inflammation," and "particulate matter" have also received extensive attention.

To better understand the research hotspots, the keywords of AP-PE field were divided into four clusters: "respiratory health," "cardiovascular health," "metabolic health," and "mental health" for further analysis. The network distribution of keywords and its related mechanisms and factors are presented in **Figure 7**. Cluster "respiratory health" studied the impact of exercise on the respiratory system under air pollution conditions and the related risks it may cause, mainly about chronic obstructive pulmonary disease (COPD), asthma, and lung cancer. Cluster "cardiovascular health" focused on the related research of circulatory system as heart and blood vessels, which were represented by "cardiovascular diseases," "myocardial infarction," "arrhythmia," and other risk factors. Cluster "metabolic health" introduced reports on the interaction factors of air pollution and exercise on glucose, endocrine, and insulin metabolism, which was mostly related to metabolic problems, such as type 2 diabetes (T2D), obesity, hyperlipidemia, and hypertension. The "mental health" cluster was an emerging topic that emphasized the AP-PE factors on brain and nervous systems. In this topic, "depression," "anxiety," and "brain-derived neurotrophic factor (BDNF)" were highly referred keywords. Based on the above analysis results, the research hotspots in the AP-PE field are discussed in depth in section Research Hotspots and Application Fields Analysis.

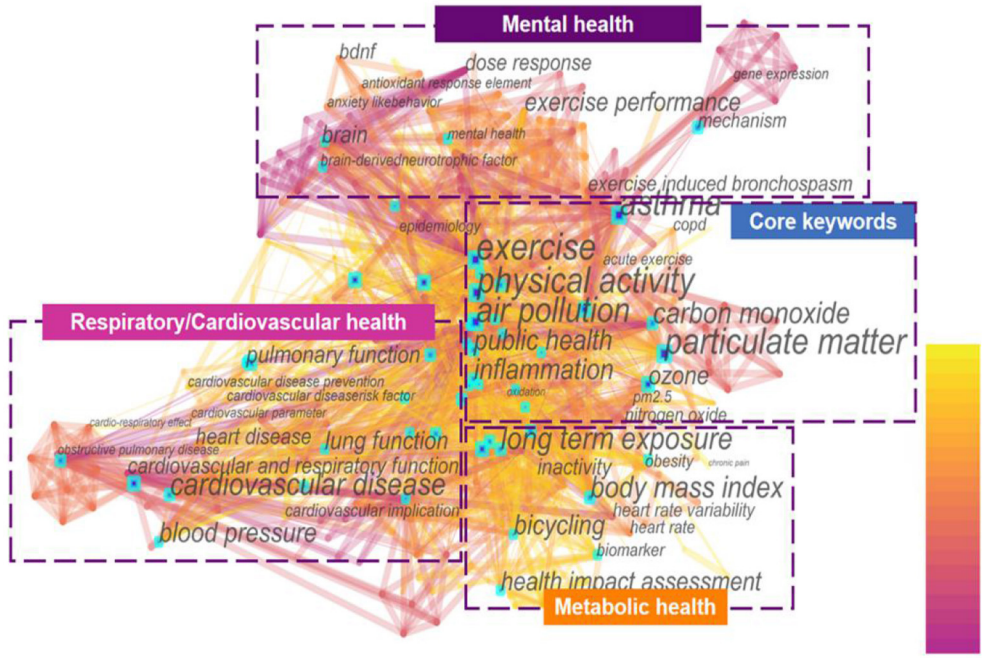


FIGURE 6 | The distribution of the co-occurrence network of keywords in the AP-PE field.

RESEARCH HOTSPOTS AND APPLICATION FIELDS ANALYSIS

Respiratory Health

Obviously, the organ directly affected by inhalation of polluted air is the respiratory system. In view of this fact, we understood that the research focusing on respiratory system is the basis of the whole AP-PE domain. **Figure 7A** is a keyword co-occurrence presentation drawn by keywords of respiratory health-related literature from the total 181 references included.

As AP is often accompanied by slower air velocity and higher concentrations of a particulate matter, people may unconsciously inhale more the deposited particulate matter and other harmful substances when they exercise in an air-polluted environment. In addition, the humidity in the AP environment is relatively high, which hinders the alveolar gas exchange to a certain extent, resulting in insufficient oxygen supply. The airway and the respiratory system, as the body's first barrier, are exposed to higher risks and always the first to be attacked. Studies showed that 9% of inhaled fine PM was deposited in the lung with 6%, reaching the alveolar region (Venkataraman and Kao, 1999). More specifically, one report further proved that a moderate-intensity exercise (minute ventilation of 38 L/min) in air pollutants can result in a 4.5-fold increment of the fractional deposition of a particulate matter (Daigle et al., 2003). In this section, research topics about airway inflammations, asthma, chronic obstructive pulmonary disease (COPD), lung cancer, and other symptoms (Berend, 2016; Bowatte et al., 2017; Schultz et al., 2017) associated with AP-PE fields are discussed in detail.

Airway inflammation is a sign of the body's response to AP exposure (e.g., ozone, nitrogen dioxide, and PM $<2.5\,\mu m$ in diameter) in the early stages (Seltzer et al., 1986; Aris et al., 1993; Solomon et al., 2000; Frampton et al., 2002). Similarly, airway hyper-responsiveness is another body's defensive response to AP stimuli and damage (Seltzer et al., 1986; Poynter et al., 2006). Pollutants are inhaled through the mouth and nose, and enter the lungs along each branch of the main bronchus. During this process, air pollutants may deposit in various parts of the respiratory system, such as nasal mucosa, respiratory tract, and alveoli, and stimulate macrophages and epithelial cells to induce inflammation.

Moreover, exposure to AP has been considered as one of the main causes of asthma. Researchers found that there was a correlation between the level of atmospheric particulate matter and the hospitalization of asthmatic patients (Malig et al., 2013; Tian et al., 2017). The current mainstream held the point urbanization seemed to be the most common contributor to asthma due to the rapid growth of roadway traffic and outdoor AP (Brunekreef et al., 2009; Robinson et al., 2011; Perez et al., 2013). Gowers et al. (2012) concluded a mechanism framework of the process that AP leads to asthma, which maintained oxidative stress, inflammation, airway remodeling, and immunological genes. As shown in **Figure 7B**, at first stage, air pollutants cause oxidative stress, and then it could further activate the expression of inflammatory factors and awaken the immunological responses; subsequently, genes related to airway development and repair, including remodeling, regulate the process of asthma and rehabilitation, and, in this way, a

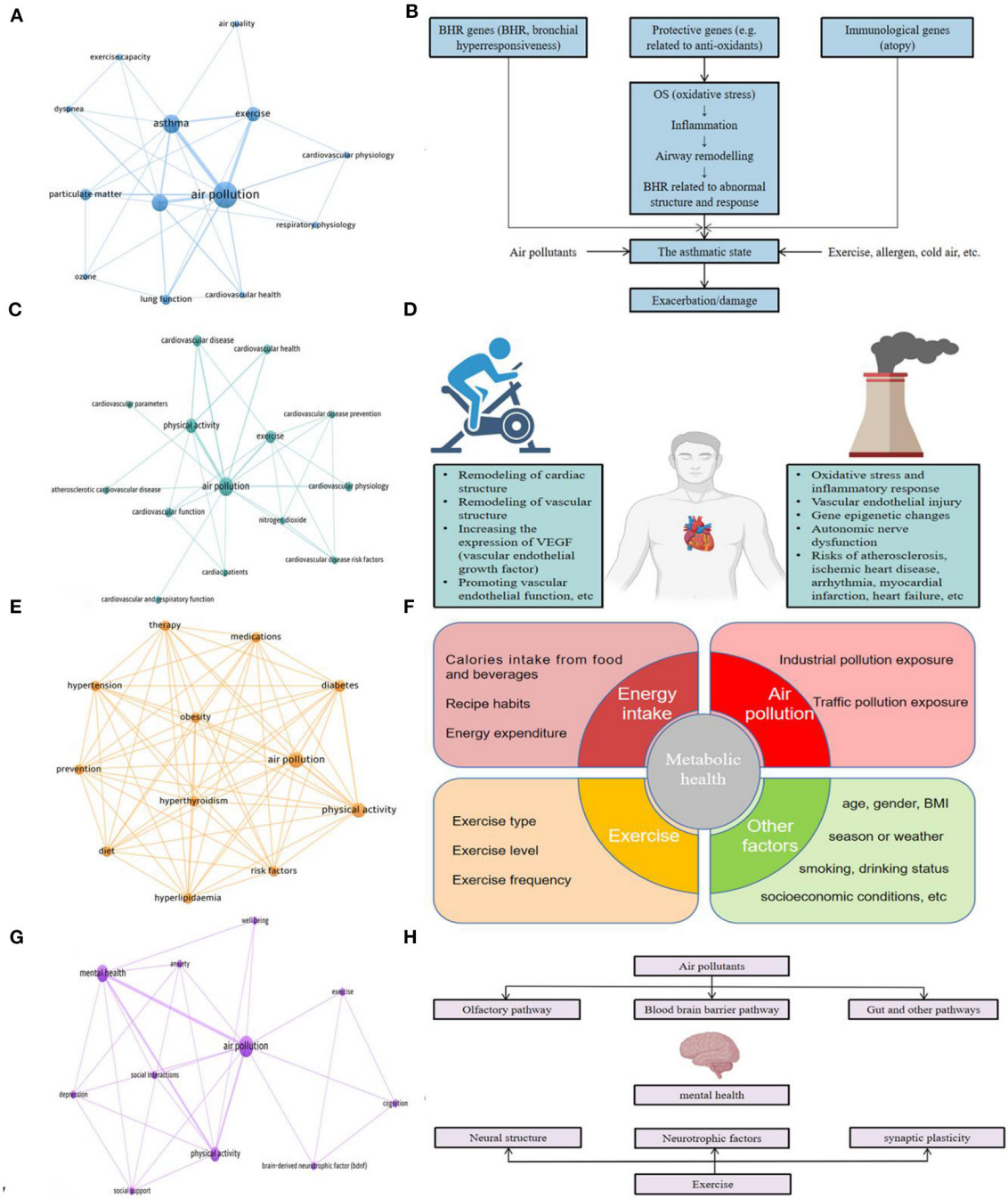


FIGURE 7 | (A) Network connections among keywords in respiratory health-related research in the AP-PE field; **(B)** the mechanistic process for air pollutants leading to asthma **(B)** was adapted from reference Gowers et al. (2012); **(C)** network connections among keywords in cardiovascular health-related research in the AP-PE field; **(D)** mechanisms and relationships between exercise and air pollutants to the cardiovascular system; **(E)** network connections among keywords in metabolic health-related research in the AP-PE field; **(F)** major factors affecting metabolic health; **(G)** network connections among keywords in mental health-related research in the AP-PE field; **(H)** pathways and factors of air pollutants and exercise on mental health.

wider range of pathways and biological mechanisms is involved in the interactions.

Long-term physical activities in air-polluted environment could further lead to chronic COPD and even lung cancer. COPD is a chronic airway inflammatory disease and has been a major public health problem worldwide (Lopez-Campos et al., 2016). With the increasing incidence rate and mortality of COPD, there is growing evidence that proved that AP might increase the

incidence rate of COPD (Hansel et al., 2016; Xia et al., 2016). As for the lung cancer, it is one of the most common malignant tumor, which has become the leading cause of death in great number of regions. Particulate matter (PM) contains a variety of carcinogenic or cancer-promoting components, including polycyclic aromatic hydrocarbons, cadmium, and mercury. One epidemiological research also supported the associations between PM and cancer, indicating that the mortality of lung cancer increased by 8% for every 10 pg/m^{-3} increase in PM concentration (Pope et al., 2002).

In short, due to the fact that exercise in AP can induce airway and lung inflammation, and even cause asthma, chronic obstructive pulmonary disease, and lung cancer, the injury to respiratory system in AP context cannot be underestimated. Hence, in this condition, the benefits of exercise to respiratory system may be offset.

Cardiovascular Health

Cardiovascular health is closely related to quality of life. In 2015, the global disease burden estimated that all forms of pollution combined were responsible for 21% of all deaths from cardiovascular disease (Landrigan et al., 2018). **Figure 7C** represents the highly referred keywords of pieces of AP-PE research on the special issue of cardiovascular health. From this figure, we can obtain that the keyword with cardio as the root appears frequently indicates that cardiovascular-related research is another interest in the AP-PE field.

A meta-analysis involved in 34 studies found that most air pollutants were significantly associated with a near-term increase in myocardial infarction risk (Mustafic et al., 2012). Another meta-analysis, which contained 59 studies, further demonstrated that every 10 $\mu g/m^3$ increase in PM_{10} or $PM_{2.5}$ can lead to an upward risk of cardiovascular disease by 0.36 and 0.63%, respectively (Lu et al., 2015b). Considering that repeated and uninterrupted exposure to air pollutants is regarded as one important risk factor in atherosclerosis, several regions faced greater cardiovascular health risks due to long-term exposure in air pollution (Wang et al., 2016). When it comes to the biological effects, the mechanisms of cardiovascular system injury caused by air pollutants have been partially studied, including oxidative stress, inflammatory response, vascular endothelial injury, gene epigenetic changes, and autonomic nerve dysfunction (Meng et al., 2016; Pope et al., 2016; Hamanaka and Mutlu, 2018; Rao et al., 2018). However, exercise training has been used as a supplementary therapy to treat cardiovascular diseases (Lavie et al., 2009; Fagard, 2011). It is self-evident that regular exercise and physical activities are safe, feasible, acceptable approaches to effectively improve cardiopulmonary function and reduce the risk of cardiovascular mortality (Nocon et al., 2008; Lee et al., 2014). The main mechanism of exercise improving the structure and function of cardiovascular system lies in remodeling of cardiac structure (cardiac physiological hypertrophy); improving the vascular structure (making the diameter of arterial vessels larger and the vessel wall thinner); increasing the expression of vascular endothelial growth factor (VEGF) in skeletal muscle and myocardium; and promoting vascular endothelial function, etc. (Green et al., 2008; Golbidi and Laher, 2011; Thijssen et al., 2012). A summary of the effects of exercise and air pollutants on the cardiovascular system is shown in **Figure 7D**.

The difficulty is that whether the benefits of exercise to cardiovascular system can be offset. Unfortunately, although there were still several doubts, the accessible evidence tended to point to this fact. An early research on traffic pollutants pointed out that exercise while commuting has an influence on inhaled PM, which was significantly associated with acute declines in heart rate variability, especially in pedestrians and cyclists (Nyhan et al., 2014). From the same perspective of traffic air pollution, another study further proved that whether exercising or not, every additional 1 $\mu g/m^3$ of particle matter (2.5–10 μm) can reduce forced expiratory volume in 1 s (FEV1) and forced vital capacity (FVC). Moreover, one recent study in young and healthy males has also found that exposure to ambient air pollution during short-term submaximal exercise is associated with a decrease in airflow (FEV1/FVC) and goes one step further to state that the decrease is more apparent when the exercise takes place under particularly high exposure conditions (Kocot and Zejda, 2021). Recently, scholars using an animal model have further explored the effects of exercise in the air pollution context; their results showed that, despite a reduction in proinflammatory markers and an increase in markers of the anti-inflammatory pathway have been detected, these benefits were not enough to prevent the damage of particles to cardiovascular events (Olivo et al., 2021). Last but not least, for people with cardiovascular disease or lack of physical activity, reducing exercise may lead to a greater risk of aggravating related diseases (Kim et al., 2021; Raza et al., 2021). Therefore, it may also be beneficial to maintain a certain amount of exercise under low-concentration air pollution.

In brief, a large increase in physical activities in a high-pollution environment may adversely affect cardiovascular health, whereas exercise in the context of low-to-moderate levels of air pollutants seemed to be beneficial as well.

Metabolic Health

Metabolism and the endocrine system jointly regulate human physiological activities and play an important role in human body. As shown in **Figure 7E**, we can discover that diabetes, obesity, and insulin metabolism are research hotspots in the metabolic-related topics in the AP-PE field. Overweight, obesity, and diabetes (especially T2D) are also leading risk factors and global public health burdens (Collaborators et al., 2017; Saeedi et al., 2019; Ampofo and Boateng, 2020). It should be noted that metabolism is a long-cycle process and can be affected by many factors. Here, we summarized the major factors that affect metabolic health, including physical activity, environment, diet habits, and other aspects (as shown in **Figure 7F**), including age, gender, body mass index (BMI), smoking/drinking status, season/weather, and socioeconomic conditions, etc. Based on these facts, there are few studies specifically targeting at physical activity or air pollution, and more are the comprehensive effects of complex factors on metabolism.

A global survey in 2016 showed that $PM_{2.5}$ led to 3.2 million new diabetes cases worldwide (accounting for 14% of the total number of new added reports) (Bowe et al., 2018). The relationship between air pollution and metabolic diseases has been widely studied, and pieces of evidence from many countries and regions in China, the United States, and Italy confirmed that air pollutants can lead to increased risk of diabetes and obesity (Dales et al., 2012; Solimini et al., 2015; Yang et al., 2018; Cao et al., 2021). The mechanism between air pollution and the blood glucose level has been studied in physiological mechanism. It is suggested that a series of pathways may be involved in the pathogenesis and development of diabetes and its related disease through disturbance of glucose metabolism, increase of the blood insulin level, elevation of insulin resistance index, induction of oxidative stress, and inflammation (Sun et al., 2005; Anderson et al., 2012; Fleisch et al., 2014). Physical exercise has been proved to improve insulin sensitivity and systematic metabolism *via* multiple adaptations. These adaptations are underpinned by inter-tissue communications (including skeletal muscle, liver, fat, and other major target organs of insulin action), which can ultimately prevent metabolic derangement (Hawley and Lessard, 2008; Saotome et al., 2019; Grunewald et al., 2020; Thyfault and Bergouignan, 2020; Iaccarino et al., 2021). On the other hand, another mechanism that exercise promotes metabolic health is that skeletal muscle can secrete a variety of bioactive cytokines (also known as myokines) during or after the training process. These myokines can act on various tissues and organs of the whole body through endocrine or paracrine ways. Taking irisin as an example, it can promote the browning of white fat so as to promote heat production and energy consumption (Bostrom et al., 2012). In addition, exercise-induced mediators can adjust the cross-talk between many tissues (including liver, heart, pancreas, gut microbiota, and brain), and these adaptions are beneficial to prevent excessive inflammation and oxidative stress (McGee and Hargreaves, 2020).

At present, most studies on physical activity and metabolic health in the AP context are longitudinal studies. We assume that the possible reason is that, unlike the respiratory and cardiovascular systems, the process of human metabolism is usually an evolutionary process rather than immediate changes and results that can be observed. One longitudinal cohort study in adults showed that a high level of physical activities and low $PM_{2.5}$ exposures were associated with a lower risk of hypertension and recommended regular PE can prevent hypertension for people residing in relatively polluted regions (Guo et al., 2020). On this basis, a recent study has been surprised to find that certain levels of physical activities can counteract the telomere length (TL) relative shortening caused by long-term exposure to NO_2 and PMs in impaired fasting glucose (IFG) participants as well as patients with T2D mellitus (T2DM) (Li et al., 2021a). Another study focused on elder groups also suggested that engaging in 5 or more times of moderate to vigorous physical activity (MVPA)/week was associated with decreased risk of diabetes within groups with both high and low/moderate levels of exposure to air pollutants, such as PM_{10} or $PM_{2.5}$, and concluded that MVPA may be inversely associated with the risk of diabetes with particulate matter exposure (Kim et al., 2020). In addition to longitudinal intervention, there are still a few studies on the impact of short-term exercise in air pollution on the metabolic system. A novel research (Wang et al., 2021) conducted in college students suggested that intermittent exercise in acute ozone exposure may trigger autonomic nervous system (ANS) imbalance and activate the hypothalamic-pituitary-adrenal (HPA) and sympatho-adrenomedullary (SAM) axes, and the metabolomics assay detected that associated pathways were in relation to steroid hormone metabolism, acute inflammatory response, oxidative stress, as well as energy metabolisms.

To sum up, compared with AP-PE's research on the respiratory and cardiovascular systems, the effects and mechanisms of metabolism are more complex, and the reasons for these changes need to be further revealed. In view of the fact that metabolism is a long-term and cyclic process, and it is difficult to observe substantial changes in short-term research, studies on this topic should pay more attention to the influence of possible confounding factors on the results.

Mental Health

Central nervous system (CNS) is the high-level center that regulates human activities, and mental health has attracted more attention than ever in the new century. Surprisingly, the microscopic particles sifting from freeways and power plants not only harm the heart and lungs but also attack the brain (Underwood, 2017). **Figure 7G** demonstrates the hot topics of pieces of AP-PE research on the mental health. From this figure, we can acquire that co-occurrence words represented by cognition, anxiety, and depression are frequently referred themes.

Figure 7H presents the pathways and factors of air pollutants and exercise on mental health. Evidence from epidemiological perspective has shown that air pollution exposure is associated with CNS-related diseases, including Alzheimer's disease (AD) (Calderon-Garciduenas et al., 2004; Younan et al., 2020; Chen et al., 2021; Rhew et al., 2021), Parkinson's disease (Chen et al., 2017; Jo et al., 2021; Yu et al., 2021), stroke (Tsai et al., 2003; Kettunen et al., 2007; Lu et al., 2021), and depression (Zijlema et al., 2016; Li et al., 2021b; Shih et al., 2021). How do these air pollutants affect the brain system? Early studies found that there are two main ways for the transport of air pollutants into the central nervous system (Oberdorster et al., 2004; Lampron et al., 2013): one way is that air pollutants precipitate in the respiratory tract and transport to the blood circulation system, and then penetrate the blood-brain barrier (BBB) into the brain; another approach is to pollutants deposit on the nasal and enters the CNS through the olfactory bulb of the brain. Despite the above two pathways, recent studies have also detected that air pollutants might affect the intestinal microbiome and interfere with the brain-gut axis pathway, resulting in neuro inflammation (Mutlu et al., 2011, 2018; Shou et al., 2019). Furthermore, pollutants entering the brain can cause nerve damage, and its main mechanisms include oxidative stress (Calderon-Garciduenas et al., 2015; Verma et al., 2015) and inflammatory response (Ku et al., 2017). In addition, studies have shown that particulate matters can lead to neurotoxicity

by mediating epigenetic regulation (Gondalia et al., 2019). However, previous studies have shown that exercise is not only beneficial to improve the brain function of healthy people but also has a protective and preventive effect on the patients with neurological diseases such as AD and depression (Heyn et al., 2004; Lautenschlager et al., 2008; Wegner et al., 2014; Kvam et al., 2016). The major effects of exercise on mental health can be divided into three aspects: remodeling of neural structure (Voss et al., 2013; Bonavita and Tedeschi, 2017), generation of neurotrophins (Dishman et al., 2006; Liang et al., 2021), and improvement of synaptic plasticity (Dishman et al., 2006; Petzinger et al., 2013; Liang et al., 2021). It should be noted that brain-derived neurotrophic factor (BDNF) is one of the key factors in improving cognition and mental health mediated by exercise. Studies have shown that impaired cognitive function and psychiatric disorders are closely related to the decline of the BDNF level (Connor et al., 1997; Shimizu et al., 2003; Angelucci et al., 2005; Cramer and Riley, 2008), and exercise can significantly upregulate the expression of BDNF and glial cell-derived neurotrophic factor (GDNF) (Aguiar et al., 2014; Soke et al., 2021). Besides, there are also studies that show that exercise can promote the enhancement of mitochondrial adaptability, and then improve the brain oxidative stress environment (Navarro et al., 2004; Gomez-Pinilla, 2008; Gusdon et al., 2017; Gan et al., 2018) so as to prevent or rehabilitate related neuropsychiatric diseases.

So far, there is shortage of in-depth studies on the interactive effects of AP and exercise on brain function. We preliminarily suppose that the reasons for this are not only the complexity of basic exploration in brain science itself but also the fact that changes in brain structure and function usually take time to accumulate. One short-term study explored the effects of exercise in different environments, while the results indicated that one 15-min bout of walking or jogging with or without air pollutants did not appear to affect participants' emotions (Han, 2020). There are also studies using aerobic and resistance training under the air pollutants conditions on cognition status (Molina-Sotomayor et al., 2019, 2020), and their findings tended to show that exercise may be serve as a protective factor against the effects that pollution has on cognition. However, most of these studies use evaluation methods such as blood parameters or mental scales, and there is little research on the mechanism of cognitive function changes. On the basis of these facts, several studies tried to answer the questions with the application of animal models, and it seemed that ultrafine particles exposure might mitigate the benefits of the exercise-induced upregulation of BDNF gene expression in the rats' hippocampus (Bos et al., 2012a,b). Additionally, changes in gene or mRNA levels are not necessarily consistent with protein levels (Soya et al., 2007), so these findings need to be confirmed on further dimensions.

In a nutshell, brain research and mental health have been emerging topics, also the crucial and difficult issues, in recent AP-PE studies. The balance between neurotoxicity caused by air pollutants and neurotrophic mechanism induced by exercise is not clear. What is certain is that BDNF is a mediator connecting air pollution and exercise-induced changes in brain function. Taken BDNF and its related neurotrophic factor as a bridge, further research can explore more molecular events and specific pathways under the AP-PE context.

CONCLUSION AND FUTURE PERSPECTIVE

Summary

For the first time, the research distribution, major topics, and relevant hotspots of the AP-PE field have been described by the knowledge-map strategy. Despite previous articles that have been conducted on the cohort design, associated mechanisms, interaction factors, etc., the understanding of total exercise and physical activities in an air-polluted environment is still limited. One reason is that exercise, as an emerging active living style, has mushroomed in recent years, and environmental health has attracted increasing attention since the new century. Another may be the development history of AP-PE interdisciplinary is relatively short, although the analysis of annual outputs and subjects suggests that it is currently in the prosperity era of the research field.

In this study, a total of 181 studies in the AP-PE field were retrieved from the WoSCC. The trend of annual publications displayed notable growth overall, especially from 2016 to present. Most of the related pieces of research were published in the journals with a focus on environment, medicine, and sports. The research contexts were mainly involved in commuter exposure, urban environmental health, public transportation, health impact assessment; the effects and modes are mostly connected with exercise immunology, short-term exposure, and moderate aerobic exercise, and the noteworthy subjects included youth, elite athletes, sports facilities, and rural population. In addition, on the basis of the AP and exercise's effects on different aspects of the body, we further discussed the four application fields in depth. Respiratory system, as the first barrier of the body to defense against air pollutants, has been widely studied, and it seems that the benefits of exercise to respiratory system may be offset under AP conditions. There are debates on the AP-PE condition to the cardiovascular health, and the AP level is an important factor mediating the beneficial effects of physical exercise. Metabolic pieces of research in AP-PE fields are closely related to public health issues, including diabetes, obesity, hypertension, etc. Since metabolism is a mixed result regulated by multiple axes, the metabolic studies need to be further explored with confounding factors included. Brain science, as the focus and emerging field of pieces of AP-PE research, is springing up nowadays. BDNF seems to be a link between exercise benefits and brain impairments caused by air pollution, while more mechanisms at the molecular and pathway levels remain to be discovered in the upcoming years.

Future Perspectives

According to the knowledge map and visualized results in our study, we consider that future research should pay more attention to the following concerns:

Overall, the AP-PE studies are still in the development stage and remained to be further standardized and systematized. In current studies, the differences in the research paradigm, sample size, exposure method, model selection, and outcome detection may lead to inconsistent research conclusions and difficult to integrate. To provide a greater scope for improvement, it is urgent to establish a scientific standard and conclude biomarkers and major indicators to guide future scholars through the directions to more explicitly explore the field. In this study, we summarized that the current mainstream research strategies are mostly related to the five aspects: (1) longitudinal studies on physical exercise exposed to long-term air pollution, typically represented by traffic pollution context. This type mainly focuses on the mitigation of benefits induced by exercise under air pollution. (2) Short-term intervention under one acute exercise experience under air pollution condition. This mode is mostly used to evaluate the exercise performance of elite athletes in special situations, such as Olympic Games and other sporting occasions. (3) Long-term empirical research that contains pre-exercise training in a non-exposed environment, and then comparing the trained groups' and non-trained groups' health status in air pollution. This study design is generally applicable in regions with intermittent air pollution and aims to observe whether the protective effects of exercise (including the improvement of immunity) can reduce the damage to the body caused by air pollution. (4) Different from the subjects selected in the first three modes, the fourth type of research tends to select patients living in air-polluted areas with basic chronic diseases as the control group, and explore if exercise can rehabilitate or improve air pollution-related diseases to some extent. (5) Pieces of research combined with two or more of the above four modes.

Although numerous AP-PE studies have detected the oxidative stress and inflammatory response on health system, few have focused on underlying mechanisms. When the focus is the composition of air pollutants, varied sources of pollutants in different regions and seasons are complex; thus, the components inducing toxicological effects are not well-understood. The molecular mechanism research on the components of major pollutants (such as PM) will help researchers to explain the impact of specific pollutants on body health, and, as well, has important guidelines for AP control. Additionally, previous reports mainly focus on the partial effect of one organ, and the research on the integration mechanism of AP, exercise, and health is still missing. Actually, the effects of pollutants and exercise on the body are systematic. For example, the stress reaction is related to the HPA axis induced by brain, and intestinal brain carries out negative feedback regulation of intestinal flora *via* the brain-gut axis, etc. Therefore, with the establishment and maturity of hybrid research strategy and multi omics technology, the research based on phenotype-function and upstream-downstream effectors will provide a new horizon for scholars to understand the profound mechanism under the AP-PE context.

Furthermore, one of the main challenges for pieces of AP-PE research regarding the dose relationship is to explore the dose effect of exercise under air pollution. There are two equilibrium points in the yield curve of AP-PE activities. One is the intensity and the mode of the exercise; another is the level and the component of air pollutants. Although there are some ongoing studies on the dose response between exercise and AP, the evidence level is not sufficient to support the explanation of the associated phenomenon. Up to now, the consensus on exercise is that low-to-medium intensity has a positive effect on reducing metabolism risks and improving the aerobic capacity, while high-intensity exercise has a better effect on the enhancement of cardiopulmonary fitness. Hence, it needs to be further clarified which exercise mode and intensity will bring better health effects under the AP context. Based on the acquired knowledge, it seems that doing one-bout aerobic exercise (a power bicycle) during an air quality index (AQI) ranking of "yellow" will not diminish exercise performance in healthy adults, nor has a negative effect on pulmonary function or biological health markers (Wagner and Clark, 2018). There is also evidence that the beneficial effects during exercise will be mitigated in $PM_{2.5} > 100\ \mu g/m^3$ (Tainio et al., 2016; Pasqua et al., 2018), which indicates that it should not be encouraged to exercise if $PM_{2.5}$ is greater than or equal to this value. Simultaneously, when the existing research cannot provide accurate physical activity guidelines under light or mild air pollution conditions, our recommendation for the exercise strategy is "listening to the body," since the intensity of activity that allows the body to adapt to air pollutants might be safe in these cases.

Totally speaking, even with increased amount of AP-PE publications in recent years, further research is still required to (i) establish the framework and standard to clarify the key issues and answer the major concerns; (ii) strengthen the interdisciplinary collaborations, and explore the systematic impact of molecular mechanisms and specific pathways on the whole life cycle and multi-organ crosstalk; (iii) find out the equilibrium points as well as the aspects of dose-effect relationships between exercise and the AP level.

Strength and Limitations

The greatest strength of our study lies in the extensive analysis of global publications on PE and AP conditions from an interdisciplinary perspective and novel strategy. The combination of the knowledge map and visualized analysis can quantitatively reflect research status and practical applications simultaneously, and demonstrate the distribution of collaborations among countries, regions, disciplines, etc. Our study applies the structured information, including clusters of co-cited references and keywords, while the use of unstructured knowledge and qualitative approaches is limited. Subsequently, we only use the four indexes of Web of Science Core Collection for analysis while the document included in one database may not be comprehensive. However, the realistic dilemma is that the existing knowledge map software does not support the extraction and meta-analysis of multi-database records. In addition, since the research included in this paper is not comprehensive enough on specific components of air pollutants, the relationship between multiple pollutants and physical activities remains to be further discovered. Based on our work, future scholars can use artificial intelligence combined with multiple-database analysis to further explore a research development path and promising

aspects in the field of physical exercise or activity under the air pollution conditions.

AUTHOR CONTRIBUTIONS

Conceptualization was contributed by YY, WL, and XM. Methodology was contributed by YY, DW, and YC. Validation was contributed by YY, DW, and JL. Formal analysis was contributed by YY, JL, and YC. Writing—original draft preparation was contributed by YY. Writing—review and editing were contributed by YY and DW. Supervision was contributed by WL and XM. Project administration was contributed by WL and XM. Funding acquisition was contributed by XM. All the authors have read and agreed to the published version of the manuscript.

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Resistance Training of Inspiratory Muscles After Coronary Artery Disease May Improve Obstructive Sleep Apnea in Outpatient Cardiac Rehabilitation: RICAOS Study

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Background: Obstructive sleep apnea (OSA) affects 5% of the adult population and its prevalence is up to 13 times higher in coronary artery disease (CAD) patients. However, OSA in this population is less symptomatic, leading to lower adherence to positive airway pressure (CPAP). While oropharyngeal exercise showed a significant decrease in apnea-hypopnea index (AHI) in patients with moderate OSA, there have been no studies testing the impact of specific inspiratory muscle training (IMT) for these patients. The aim of our study was to assess the effectiveness of IMT on AHI reduction in CAD patients with moderate OSA.

Methods: We included patients with CAD involved in a cardiac rehabilitation program and presenting an AHI between 15 and 30. Patients were randomized in a 1:1 allocation to a control group (CTL – classic training) or an IMT group (classic training + IMT). IMT consisted in 60 deep inspirations a day, 6 days a week, into a resistive load device set at 70% of the maximum inspiratory pressure (MIP). After 6 weeks, we compared AHI, neck circumference, Epworth Sleepiness Scale, Pittsburgh Sleep Quality index, and quality of life with the 12-item Short Form Survey before and after rehabilitation.

Results: We studied 45 patient (60 ± 9 y, BMI = 27 ± 6 kg.m^{-2}). The IMT group ($n = 22$) significantly improved MIP ($p < 0.05$) and had a significant decrease in AHI by 25% (−6.5 ± 9.5, $p = 0.02$). In the CTL group ($n = 23$), AHI decreased only by 3.5% (−0.7 ± 13.1; $p = 0.29$). Between groups, we found a significant improvement in MIP ($p = 0.003$) and neck circumference ($p = 0.01$) in favor of the IMT group. However, we did not find any significant improvement of AHI in the IMT group compared to CTL ($p = 0.09$).

Conclusion: A specific IMT during cardiac rehabilitation contributes to reduce significantly AHI in CAD patients with moderate OSA. Magnitude of the decrease in OSA severity could be enhanced according to implementation of specific IMT in this population.

Keywords: resistive inspiratory muscle training, obstructive sleep apnea, coronary artery disease, apnea-hypopnea index, cardiac rehabilitation, oxygen desaturation index

INTRODUCTION

Prevalence of moderate to severe obstructive sleep apnea (OSA; apnea plus hypopnea index >15 events.h^{-1}) is thought to affect up to 23% of women and 49% of men (Heinzer et al., 2015). OSA is characterized by recurrent episodes of complete (apneas) or partial (hypopneas) upper airway collapse during sleep. It always stems from obstruction of the pharynx during the inspiratory phase (Fogel et al., 2005).

Several studies have shown that OSA is an independent risk marker and probably risk factor of coronary heart disease (BenAhmed et al., 2014; Tietjens et al., 2019). It is also a major risk factor for cardiovascular morbidity and mortality (Peker et al., 2000; Mooe et al., 2001; Coughlin et al., 2004; Bradley and Floras, 2009). In comparison with other OSA patients, coronary artery disease patients with OSA are characterized by relatively poor diurnal symptoms (Javaheri et al., 1998, 2017; Sin et al., 2002; Arzt et al., 2006). The absence of clinical repercussions may induce a higher withdrawal rate of standard continuous positive airway pressure (CPAP) treatment. Also, for those who have accepted CPAP, long-term adherence remains an important issue (Anandam et al., 2013; McEvoy et al., 2016; Rotenberg et al., 2016; Libman et al., 2017; Askland et al., 2020). Alternative treatments, such as mandibular advancement or surgery, can be proposed for moderate OSA or for patients with limited daytime symptoms but with limited tolerance by some patients over time (Fleury et al., 2010; Giralt-Hernando et al., 2019). Therefore, alternative treatments are needed to reach better acceptance and adherence.

Excessive relaxation of the tongue and soft palate evidenced by a decrease in the EMG pattern of the genioglossus and tensor of the palate muscles (Fogel et al., 2005; Oliven et al., 2019) partly explains the OSA pathogenesis. While physical activity has shown relative effectiveness in improving apnea-hypopnea index (AHI; Kline et al., 2011; Aiello et al., 2016; Berger et al., 2018, 2021; Hupin et al., 2018), specific exercises of the oropharyngeal area could have a more targeted impact. Thus, Guimarães et al. (2009) have shown that strengthening oropharyngeal muscles by mouth, tongue, and pronunciation exercises reduce AHI in patients with moderate OSA by 8.7 events.h^{-1}. An improvement of subjective quality of sleep and a reduction in neck circumference were also found in the "strengthening oropharyngeal muscles" group. In the last decade, several other studies have investigated the effect of respiratory muscle training to decrease the impact of OSA (Vranish and Bailey, 2016; Souza et al., 2018; Ramos-Barrera et al., 2020). Indeed, the resistance strengthening of the inspiratory muscles would have a positive impact on the tone of the oropharyngeal muscles (How et al., 2007). However, these studies proposing inspiratory muscle training were carried out on small sample sizes and none of them were performed with asymptomatic coronary artery disease patients.

The objective of our study was to assess the effects of strengthening inspiratory muscles on AHI in coronary patients with moderate OSA engaged at the same time in a post-infarction cardiac rehabilitation program (CR) using a randomized controlled design. We assessed the benefit of adding an inspiratory resistance muscle training to OSA patients undergoing cardiac rehabilitation (IMT group) compared to a control group undergoing only cardiac rehabilitation (CTL group). We analyzed ventilatory polygraphy data and respiratory muscle strength before and after cardiac rehabilitation.

MATERIALS AND METHODS

Study Design

This was a 6 week randomized controlled trial, including on the one hand a control group with cardiac rehabilitation only, and on the other hand an interventional group admitted for CR and IMT. This study was conducted in the cardiac rehabilitation department at Saint-Etienne university hospital (France). Eligible patients were randomly assigned in a 1:1 allocation.

Patient Characteristics and Eligibility Criteria

During the initial evaluation, patients had a 24-h Holter ECG monitoring to assess cardiac arrhythmia and to evaluate their very low frequency power spectral density of heart rate increment (VLFI) which is a predictor of sleep disorder (Roche et al., 2002; Sforza et al., 2007). Patients with a high risk of OSA, attested by VLFI>4%, had a respiratory polygraphy recording to assess OSA severity more accurately. Adult patients initiating a cardiac rehabilitation, under the age of 80, with an AHI $\geq$15 and $\leq$30 events h^{-1} were eligible to participate.

Exclusion criteria were recent thoracic surgery by sternotomy (<12 weeks), obstructive ventilatory disorder with an FEV-1/FVC ratio <70%, already treated for OSA.

Ethics Approval and Consent of Participants

The study was performed from May 2015 to July 2021. Approval and ethical clearance were obtained from an institutional review board (CPP Sud Est I 1408189-2015-A00030-49) which was in accordance with the principles embodied in the Declaration of Helsinki. Prior to study initiation, the objectives of the study were clearly explained to subjects in order to obtain written informed consent. The study was *a priori* registered at ClinicalTrial.gov (NCT02494648).

Cardiac Rehabilitation CR

The CR at the Saint-Etienne University Hospital was a 7-week interdisciplinary program that combines interventions performed by cardiologists, nurses, rehabilitation physicians, physiotherapists, dieticians, and psychologists. At the time of enrollment, the patient's functional status was assessed by a cardiopulmonary exercise test (CPET). Exercise training consists of 20 sessions, three times a week (Monday, Wednesday, and Friday) for 1.5 h. Each session starts with a 10-min warmup period followed by 25 min of cycling or treadmill (power output at ventilatory threshold defined during the initial CPET) and a 5-min cool down period. Resistance

muscle training of upper and lower limbs is also offered. The workout was divided into 80% aerobic and 20% resistance exercises. The first week of CR, continuous endurance exercises were preferred, and from the second week, interval exercises were added. An expert physiotherapist and an adapted physical activity monitor supervised sessions with continuous heart rate and pulse oximetry monitoring. The workload progression was adjusted weekly according to the patient's tolerance (Borg Perceived Effort Scale). Patients attended weekly hour-long group sessions with healthcare professionals aimed at reinforcing their health education. Particular emphasis is provided on understanding the pathophysiology of coronary artery disease, the role of cardiovascular risk factors, and its management, mainly through physical activity, anxiety control, smoking cessation, nutritional balance, and adherence to guidelines for recommended drugs.

Interventional Group (IMT)

The interventional group followed the CR program described above, as well as a 6-week IMT. To strengthen the inspiratory muscles, we used a resistive loading device (POWERbreathe® Plus medium resistance, Southam, United Kingdom) with a flow-independent one-way valve to ensure consistent resistance and an adjustable specific pressure setting (from −23 to −186 cmH_20). IMT consisted of performing 6 days a week, two sessions of 30 inspirations against a load of 70% MIP, during the entire duration of the CR period, i.e., nearly 40 sessions in 6 weeks. On days when the patient was present at the CR center, the IMT sessions were carried out under the supervision of the physiotherapist (PL). The third week, the MIP was reevaluated in the same conditions as during inclusion in order to adjust the training load. Patients were asked to keep a training notebook with indication of the difficulty in performing the session on a 0–5 scale.

Control Group

Patients randomized in the control group participated only in the CR and performed the same evaluations as the IMT group at baseline and at follow-up.

Outcome Measures

All outcome measures described were performed at baseline and at 6-week follow-up.

Respiratory polygraphic data. AHI was the main outcome measured from respiratory polygraphy. Ambulatory polygraphy was performed at home using either a Nox-T3 (Nox Medical, Reykjavik, Iceland) or a $Vista_2$flux (Novacor, Rueil Malmaison, France). For both devices, the signals acquired were the following: nasal flow with pressure transducer and oxygen saturation with a digital pulse oximeter (Nonin, United States). For Nox-T3, respiratory signals were recorded with chest and abdominal respiratory inductance plethysmography belts. For $Vista_2$flux, respiratory signals were evaluated by thoracic impedance sensors embedded in a multimodal ECG Holter recorder (Roche et al., 1999; Poupard et al., 2008; Chouchou et al., 2013). For each patient, the second polygraphy at the end of the study was carried out with the same device as baseline in order to be more consistent. In accordance with the 2012 American Academy of Sleep Medicine guidelines (Berry et al., 2012), an apnea was defined as a cessation in airflow (≥90% baseline) for ≥10 s, and a hypopnea was defined as a reduction in airflow (≥30% baseline) for ≥10 s which resulted in a ≥4% oxygen desaturation. Sleep apnea severity was rated in accordance with the individual's AHI, defined as the total number of apneas and hypopneas per hour of sleep and oxygen desaturation per hour of sleep; where an AHI of 5–15 events.h^{-1} was defined as mild, 16–30 moderate, and >30 designated as severe OSA. Secondary polygraphic outcomes included oxygen desaturation index (ODI), central apnea index, the mean and the lowest oxyhemoglobin saturation (SpO_2), and the percentage of time with a SpO_2 under 90% (SpO2 < 90%).

Respiratory function assessment. Lung function was assessed by standard spirometry and included forced expiratory volume in 1 s (FEV-1), forced vital capacity (FVC), and peak expiratory flow in accordance with the guidelines of the European Respiratory Society (Laveneziana et al., 2019). Maximal inspiratory pressure (MIP) was obtained with a specific device (POWERbreathe KH2, POWERbreathe International Ltd., Southam, United Kingdom). The maneuvers were carried out with the patient seated and knees flexed to 90° and were performed using the residual volume to realize a forced inspiration. Maximal inspiratory pressure was determined as the highest average pressure over 1 s reached during exercise. We considered three valid maneuvers (coefficient of variation below 10%) and the highest value was used for IMT.

Surveys. We analyzed questionnaires evaluating subjective sleep quality and quality of life. Subjective daytime sleepiness was measured by the Epworth Sleepiness Scale (ESS) which evaluates the propensity to sleep from "no" (scored 0) to "intense" (scored 3) in eight different situations. A total score > 10 indicates excessive daytime sleepiness (Johns, 1991). Quality of sleep was evaluated with the Pittsburgh Sleep Quality Index (PSQI), which is a questionnaire that evaluates seven sleep components on a scale from 0 (no difficulty) to 3 (severe difficulty). The results are expressed as a global score (ranging from 0 to 21). A total score > 5 indicates poor sleep quality (Buysse et al., 1989). The 12-item Short Form Survey (SF-12) was used to assess self-reported health-related quality of life, which evaluates physical health with a range of 23.99 (poor) to 56.58 (good) and mental health with a range of 19.06 (poor) to 60.76 (good; Ware et al., 1996).

Statistical Data Analysis

Descriptive and inferential analyses were performed using GraphPad Prism 6 (GraphPad, San Diego, United States). Characteristics of the sample and treatment effect between groups were assessed using Student's *t*-test for independent samples. Pre-versus post-CR differences were examined using paired *t*-tests, and chi-square test corrected by Fisher's exact test was used for the categorical variables. The results are shown as mean ± standard deviation, number and percentage, and as differences of means and confidence interval (95%). Statistical significance was set at a two-tailed $p < 0.05$.

The sample size calculation was based on the Puhan et al. (2006) study results. For an expected difference of 7 points of AHI between the two groups, with a statistical power of 90%, we calculated a sample size of 44 patients. To consider dropouts before the end of the rehabilitation program, we decided to include 48 patients.

RESULTS

Demographics and Baseline Characteristics

During the inclusion period, 636 patients were admitted to our cardiac rehabilitation center and 191 patients (40%) were assessed for suspected OSA. After respiratory polygraphy, 44.5% ($n = 85$) had AHI > 30 events.h^{-1} and 29.8% ($n = 57$) had AHI < 15events.h^{-1}, and 48 patients with an AHI between 15 and 30 events.h^{-1} were included in our study. The flowchart of the study is depicted in **Figure 1**. Baseline clinical characteristics of the study population are summarized in **Table 1**. Analyses of characteristics showed no significant differences at baseline.

Change in Characteristics

The post-intervention change in BMI was not significantly different between groups (**Table 2**). In the IMT group, neck circumference decreased by 0.5 ± 0.9 cm ($p = 0.02$), and the

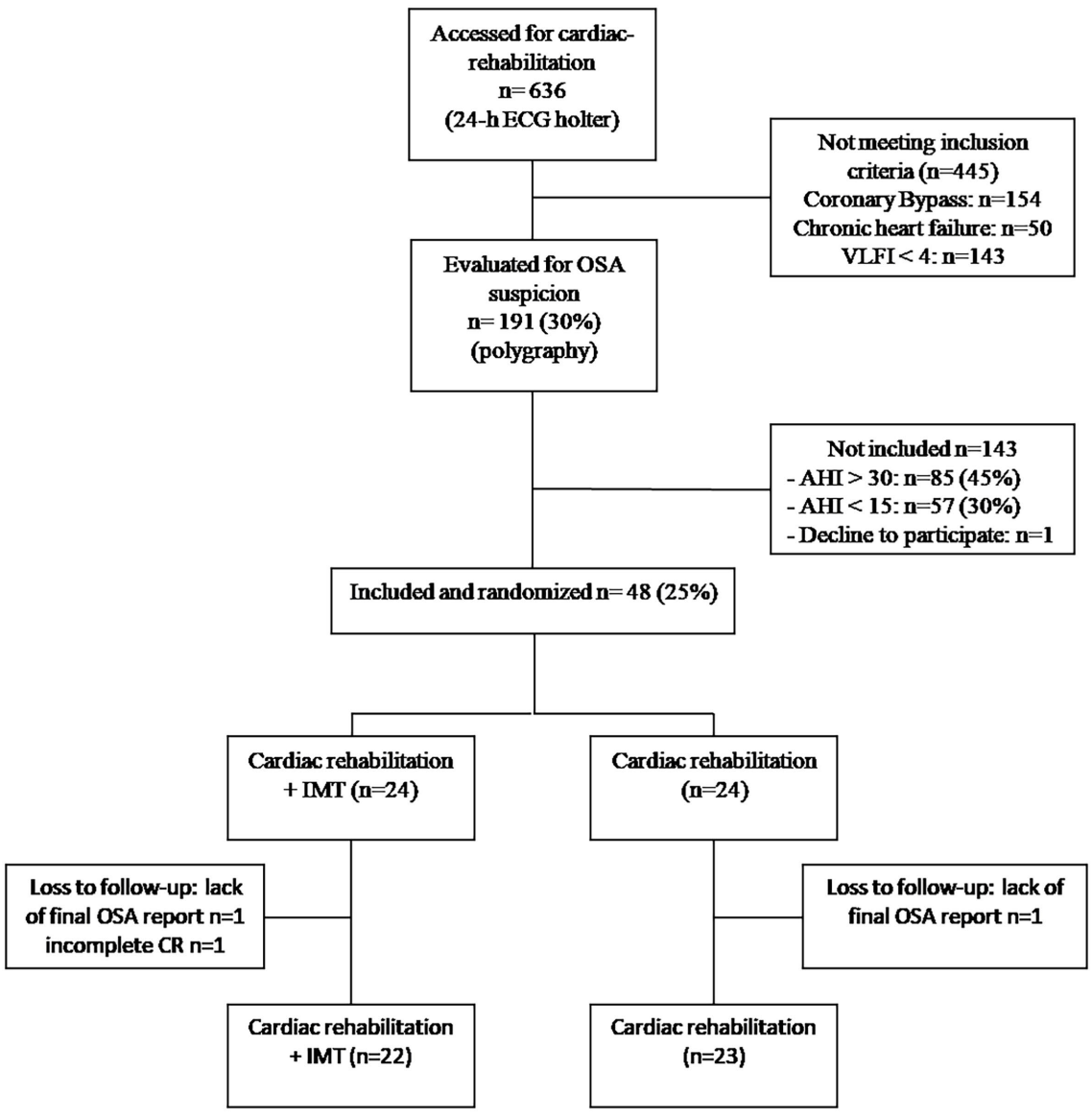

FIGURE 1 | Flowchartof the study. OSA, obstructive sleep apnea; AHI, apnea-hypopnea index; IMT, inspiratory muscle training; CR, cardiac rehabilitation; and VLFI, very low frequency power spectral density of heart rate increment.

difference between groups was also significant after intervention ($p = 0.01$). MIP significantly increased with the IMT intervention compared to the control ($p = 0.003$; **Table 2**).

Cardiopulmonary Exercise Test

At the end of the cardiac rehabilitation, both groups showed a significant improvement in maximal oxygen uptake from 20.7 ± 4.9 before CR to 24.7 ± 5.6 ($ml^{-1}min^{-1}kg^{-1}$; $p < 0.0001$) after CR for the IMT group and from 20.6 ± 4.9 before CR to 23.7 ± 5.3 ($ml^{-1}min^{-1}kg^{-1}$; $p = 0.0003$) after CR for the control group. Maximum power output during CPET after CR increased by 23.6 ± 23.7 W ($p < 0.0001$) for the IMT group and by 27.5 ± 22.9 W ($p < 0.0001$) for the control group **Table 2**.

TABLE 1 | Baseline characteristics.

	CTL Group *n* = 23	IMT Group *n* = 22	Value of *p*
Age (y)	59.3 ± 10.3	61.0 ± 8.4	0.55
Females	4 (18)	1 (4)	
Height (cm)	173.0 ± 8.4	169.5 ±	0.21
Weight (kg)	83.9 ± 18.9	81.0 ±14.1	0.58
BMI	28.1 ± 5.8	27.9 ±4.1	0.91
Neck size (cm)	40.1 ± 2.6	38.8 ±2.8	0.15
High blood pressure	8 (35)	10 (42)	0.64
Dyslipedemia	9 (39)	7 (29)	0.55
Smokers	13 (57)	14 (58)	0.65
Diabetes	6 (26)	7 (29)	0.82
Obesity	6 (26)	9 (38)	0.41
LVEF (%)	53.9 ± 9.6	57±9.4	0.28
Coronary intervention			
Thrombolysis	3 (13)	6 (25)	0.31
Angioplasty without stent	3 (13)	3 (13)	0.96
Single-stem stent	13 (57)	10 (42)	0.25
Multi-stem stents	8 (34)	10 (42)	0.53
Treatment			
Beta blocker	20 (87)	18 (75)	0.31
Aspirin	21 (91)	21 (88)	0.68
Double APT	23 (100)	23 (96)	0.33
Statin	21 (91)	17 (71)	0.08
ACEI/ARB	19 (83)	18 (75)	0.53
FEV-1%	99.0 ± 13.7	97.2±19.1	0.78

Values are mean ± standard deviation or n (%). BMI, body mass index; LVEF, left ventricular ejection fraction; APT, anti-platelet; ACEI/ARB, angiotensin-converting enzyme inhibitor/angiotensin receptor blocker; and FEV-1, Forced expiratory volume in 1 s.

Polygraphic Data

At baseline, polygraphy data between groups were similar (**Table 3**). Post-intervention, the mean reduction in AHI score was only significant for the IMT group, which showed a decrease of 6.5 ± 9.5 $events.h^{-1}$ (95% CI: 2.015 to 10.08, $p = 0.005$). By contrast, the control group which only carried out the CR showed a nonsignificant decrease of 0.7 ± 13.4 $events.h^{-1}$ (**Table 3**; **Figure 2**). The difference in AHI of 5.8 ± 3.2 points between the two groups was close to significance ($p = 0.09$). ODI was significantly decreased in the IMT group by 6.4 ± 8.02 $events.h^{-1}$ (95% CI: −9.910 to −2.977, $p = 0.0009$); for the CTL group, no significant change in ODI was observed ($p = 0.14$). If we only consider the obstructive part of sleep apnea (AHI minus central apnea index), we observed a significant decrease of 6.7 ± 8.5 $events.h^{-1}$ ($p = 0.004$).

Questionnaires

ESS only decreased significantly for the IMT group (−2.2 ± 3.2 points, $p = 0.0176$), and PSQI was close to significant with a mean score below 5 (3.9 ± 1.6, $p = 0.053$). For the mental health score of SF-12, the IMT group showed a significant increase of 5.58 ± 7.4 points ($p = 0.008$), while it did not change in the CTL group ($p = 0.7$). As a result, we found for this mental health score a significant difference between groups of 6.5 ± 2.6 points ($p = 0.01$). We found no other significant difference in the questionnaires, either intragroup or intergroup (**Table 3**).

DISCUSSION

Our study showed that 6 weeks of resistance IMT in patients with moderate OSA admitted in cardiac rehabilitation for

TABLE 2 | Average values of characteristics and cardiopulmonary exercise test obtained before and after cardiac rehabilitation in the control (CTL) and inspiratory muscle training (IMT) groups.

	CTL group			IMT group			Value of *p* intergroup
	Before	After	Value of *p*	Before	After	Value of p	
Characteristics							
Weight (kg)	81.0 ± 14.1	83.9 ± 18.9	0.92	83.9 ± 18.9	80.2 ± 14.3	0.09	0.16
BMI	27.9 ± 4.1	28.1 ± 5.9	0.83	28.1 ± 5.8	27.4 ± 3.8	0.07	0.11
Neck circumference (cm)	40.1 ± 2.6	40.5 ± 2.2	0.18	38.8 ± 2.8	38.3 ± 3.1	**0.02**	**0.01**
MIP (cmH_2O)	89.6 ± 17.7	92.4 ± 22.3	0.65	97.6 ± 29.7	115.4 ± 30.2	**0.0004**	**0.003**
Cardiopulmonaryexercise test							
VO_2 peak ($ml^{-1}.min^{-1}.kg^{-1}$)	20.6 ± 4.9	23.7 ± 5.3	**0.0003**	20.7 ± 4.9	24.7 ± 5.6	**<0.0001**	0.19
Pmax (W)	133.6 ± 39.3	164.4 ± 39.2	**<0.0001**	136.9 ± 32.3	159.4 ± 48.9	**<0.0001**	0.59
Heart rate at peak (% predicted)	75.3 ± 11.9	81.9 ± 13.5	**0.02**	79.8 ± 15.9	80.7 ± 14.1	0.57	0.15

Values are mean ± standard deviation. Significant values ($p < 0.05$) are indicated in bold.
BMI: body mass index; MIP: maximum inspiratory pressure; VO2peak: maximal oxygen uptake; Pmax: maximal power output. .

TABLE 3 | Average values of polygraphy and questionnaires before and after cardiac rehabilitation in the control (CTL) and inspiratory muscle training (IMT) groups.

	CTL group			IMT group			
	Before	**After**	**Value of *p***	**Before**	**After**	**Value of *p***	**Value of *p* intergroup**
Polygraphic recording							
AHI (events.h^{-1})	22.0 ± 7.1	21.4 ± 13.9	0.29	24.9 ± 7.8	18.8 ± 10.1	**0.003**	0.09
ODI (events.h^{-1})	22.5 ± 5.2	21.4 ± 14.1	0.14	23.1 ± 8.0	16.6 ± 9.6	**0.0004**	0.12
Hypopnea Index (events.h^{-1})	14.1 ± 5.1	14.8 ± 13.1	0.45	15.4 ± 4.9	11.8 ± 6.1	**0.03**	0.15
Obstructive apnea Index (events.h^{-1})	6.9 ± 3.5	6.4 ± 4.6	0.33	6.3 ± 5.6	4.0 ± 3.2	**0.02**	0.29
Obstructive part of OSA (events.h^{-1})	21.2 ± 4.9	21.8 ± 16.0	0.29	21.8 ± 7.1	15.8 ± 7.7	**0.004**	0.09
Central apnea Index (events.h^{-1})	1.7 ± 2.3	1.1 ± 1.1	0.37	2.4 ± 2.9	1.4 ± 1.8	0.32	0.78
Lowest SpO_2 (%)	83.8 ± 2.7	82.9 ± 5.2	0.57	83.3 ± 4.1	85.7 ± 2.2	0.08	0.12
Mean SpO_2 (%)	92.5 ± 1.6	92.1 ± 1.7	0.52	93.0 ± 1.4	93.4 ± 1.4	0.30	0.14
SpO2 < 90% (min)	13.7 ± 17.5	24.8 ± 57.5	0.33	12.3 ± 26.6	4.6 ± 6.3	0.25	0.13
Questionnaires							
ESS	7.4 ± 3.6	6.2 ± 4.0	0.24	7.3 ± 5.2	4.3 ± 2.9	**0.02**	0.26
PSQI	6.0 ± 3.7	5.4 ± 3.2	0.28	6.1 ± 3.7	3.9 ± 1.6	0.053	0.38
SF-12 mental score	46.4 ± 9.6	48.8 ± 8.8	0.68	44.6 ± 11.2	52.3 ± 9.6	**0.008**	**0.013**
SF-12 physical score	44.4 ± 9.6	44.9 ± 10.5	0.80	41.2 ± 10.0	42.6 ± 12.5	0.30	0.70

Values are mean ± standard deviation. AHI, apnea-hypopnea index; ODI, oxygen desaturation index, OSA, obstructive sleep apnea; SpO_2, oxygen saturation value; ESS, Epworth Sleepness Scale; PSQI, Pittsburgh Sleep Quality Index; and SF-12, 12-itemShort Form Survey. Significant values (p < 0.05) have been indicated in bold.

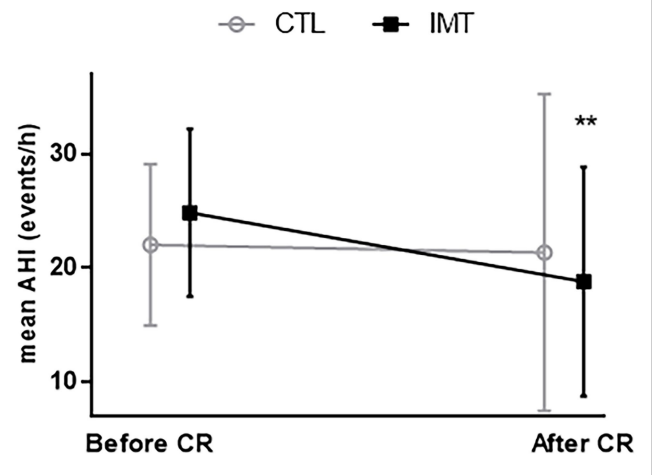

FIGURE 2 | Evolution of apnea-hypopnea index (AHI) before and after cardiac rehabilitation (CR). $^{**}p = 0.003$; significant decrease of AHI after cardiac rehabilitation only for IMT group (black square).

coronary artery disease allowed a decrease in AHI and ODI. This is in line with the effect of IMT on pharyngeal tone. In this sense, How et al. (2007) showed in awake subjects with calm breathing that an acute bout of inspiratory muscle exercise allowed to increase the activation of specific upper airway dilator muscles and that an IMT could, by increasing the passive tone of the upper airway dilatators, be more favorable in OSA patients with greater compliance of their pharyngeal wall (Isono et al., 1997).

In our study, patients undergoing IMT showed an improvement of their MIP (+ 18%). Although not measured, we hypothesize that IMT, initially focused on improving diaphragmatic strength and probably endurance, also allowed an improvement of the upper airway dilatator muscle tone during sleep. The decrease in the obstructive part of the sleep disorders recorded (AHI minus central apnea index) suggests that IMT could reduce collapsibility of the upper airway during sleep, even in the absence of neural drive. According to a study by Guimarães et al. (2009), in patients who performed oropharyngeal exercises for 12 weeks, the authors suggested an upper airway remodeling linked to a significant decrease in neck circumference. They also showed that these changes in neck circumference were negatively correlated with improvement in AHI. Finally, in our study, the significant decrease in neck circumference might be related to the improvement in the tone of the upper airway, which would require further investigational studies.

Noteworthy, IMT allowed a significant reduction in hypoxemic load in the present study. In fact, in the IMT group, the patients presented a significantly lower ODI and a decrease in the time spent below 90% of SpO2 after intervention. The improvement of these factors is of strong interest in the management of coronary patients with OSA considering the major role of intermittent hypoxemia in terms of morbidity and mortality as well as in the development of cardiovascular risk factors (Garvey et al., 2009; Dewan et al., 2015).

Our results suggest that, at baseline, coronary patients with moderate OSA present few OSA-related symptoms. Indeed, the subjective daytime sleepiness scores assessed by the Epworth questionnaire before CR were <10 (which would indicate excessive daytime sleepiness) in the whole population, while the patients reported poor sleep quality. Of note, cardiac rehabilitation had no significant effect on these two scores in the control group. On the other hand, in the IMT group, the significant improvement in AHI is in line with a significant improvement in daytime sleepiness as well as an improvement of sleep quality. Due to the high prevalence of OSA in the population with coronary artery disease (69% of our patients

were assessed for suspicion of OSA; Hupin et al., 2018), it seems important to systematically look for sleep disorders in patients who have had a heart attack.

STRENGTHS AND LIMITATIONS

The strength of our study was its randomized controlled design with an easy access IMT device, an IMT simple to perform and requiring little daily training time (60 repetitions per day, i.e.,20 min). Patient feedbacks were very positive, whereby most found a benefit and continued IMT after the end of their cardiac rehabilitation. Another strength of our study is the centralized blind reading of the polygraphic signals avoiding any analysis bias.

Our study also had limitations. First, we did not find any significant difference between the group performing IMT in addition to CR compared to the group performing CR alone. This lack of difference could be explained by the fact that physical training *per se*, *as* provided during CR, could improve the AHI in some patients included in the control group (Berger et al., 2018). Indeed, physical exercise could decrease fat mass (Kline et al., 2011; Desplan et al., 2014), greater overall muscle tone, and improved respiratory function (Sengul et al., 2009). This set of elements may have reduced the impact of IMT in our study. Indeed, physical training induces an increase in voluntary ventilation which, in the long term, allows better ventilatory control, an increased sensitivity to respiratory drive, and an increase in the strength of the inspiratory muscles (a median increase of +7 cmH_2O was observed for the control group and a maximum gain of 22 cmH_2O for a patient). This slight increase in MIP may have resulted in a lower rate of complete obstructive apnea in favor of a higher rate of hypopnea in the control group.

Another limitation to note is the lack of objective evaluation of adherence to IMT training since the device used is mechanical and does not have a repetition counter. For the sake of more precise evaluation, the use of an electronic device would have allowed rigorous monitoring of the training. Finally, our study did not allow to establish a causality link between improved MIP and increased resistance to upper airway collapse during sleep. Future physiological studies evaluating the effect of IMT on upper airway closure pressures during sleep are needed to confirm our data.

CLINICAL IMPLICATION AND CONCLUSION

In case of CPAP treatment failure, it appears necessary to offer various alternatives to OSA patients to reduce the severity of sleep disorders. In that view, our study brings a new piece to the puzzle by showing that a simple IMT exercise, performed daily, could reduce OSA severity in nontreated patients. In the most severe patients, this treatment could also be offered in addition to CPAP to reduce the pressures insufflated into the airways and thus improve patient comfort. Further investigational studies are needed to determine the best IMT modality (strength training or endurance training) and the potential combined effect with expiratory muscle training, as previously shown by Puhan et al. (2006) and Ward et al. (2012).

In conclusion, IMT for 6 week decreases AHI in coronary patients with moderate asymptomatic OSA. IMT could be an adjuvant therapy for patients who are unwilling or unable to tolerate nightly CPAP.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Saint-Etienne University Hospital ethics committee: CPP Sud Est I 1408189-2015-A00030-49. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PL, MB, and DH: writing of original draft. All authors had a substantial contribution to the conception and design of the study. PL, MB, AZ, FR, and DH: responsible for data collection and formal analysis. MB, J-CB, FR, DH, and AG: supervision. All authors contributed to the article and approved the submitted version.

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Effects of High-Volume Versus High-Load Resistance Training on Skeletal Muscle Growth and Molecular Adaptations

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We evaluated the effects of higher-load (HL) versus (lower-load) higher-volume (HV) resistance training on skeletal muscle hypertrophy, strength, and muscle-level molecular adaptations. Trained men ($n = 15$, age: 23 ± 3 years; training experience: 7 ± 3 years) performed unilateral lower-body training for 6 weeks (3× weekly), where single legs were randomly assigned to HV and HL paradigms. Vastus lateralis (VL) biopsies were obtained prior to study initiation (PRE) as well as 3 days (POST) and 10 days following the last training bout (POSTPR). Body composition and strength tests were performed at each testing session, and biochemical assays were performed on muscle tissue after study completion. Two-way within-subject repeated measures ANOVAs were performed on most dependent variables, and tracer data were compared using dependent samples t-tests. A significant interaction existed for VL muscle cross-sectional area (assessed *via* magnetic resonance imaging; interaction $p = 0.046$), where HV increased this metric from PRE to POST (+3.2%, $p = 0.018$) whereas HL training did not (–0.1%, $p = 0.475$). Additionally, HL increased leg extensor strength more so than HV training (interaction $p = 0.032$; HV < HL at POST and POSTPR, $p < 0.025$ for each). Six-week integrated non-myofibrillar protein synthesis (iNon-MyoPS) rates were also higher in the HV versus HL condition, while no difference between conditions existed for iMyoPS rates. No interactions existed for other strength, VL morphology variables, or the relative abundances of major muscle proteins. Compared to HL training, 6 weeks of HV training in previously trained men optimizes VL hypertrophy in lieu of enhanced iNon-MyoPS rates, and this warrants future research.

Keywords: higher-load resistance training, higher-volume resistance training, muscle hypertrophy, non-myofibrillar protein, myofibrillar protein

INTRODUCTION

Skeletal muscle hypertrophy has been defined as an increase in the weight or cross-sectional area of muscle (Tesch and Larsson, 1982; Folland and Williams, 2007), with the increased volume of muscle coming from an enlargement muscle fibers (Morpurgo, 1897; Gollnick et al., 1972; Goldberg et al., 1975). It is generally recognized that resistance training results in skeletal muscle growth through proportional increases in myofibrillar and non-myofibrillar protein content (Helander, 1961; Goldspink, 1964; Gordon et al., 1967; Seiden, 1976). Myofibril proteins are defined herein as the proteins that make up the rigid structure of muscle (e.g., dystrophin, actinin, titin, and nebulin) as well as contractile proteins (e.g., actin and myosin isoforms). In contrast, non-myofibrillar proteins are enzymes involved with signal transduction, energy synthesis and breakdown (e.g., sarcoplasmic and mitochondrial enzymes), and other metabolic processes (Haun et al., 2019b).

Recently, there has been interest regarding whether higher-load (HL) versus higher-volume (HV) resistance training elicits differential training adaptations at the macroscopic, molecular, and functional levels. HL training involves lifting heavier weights per set with fewer repetitions (e.g., 5 sets of 5 repetitions @ 85% of a 1-repetition maximum for a given exercise). HV training involves lifting lighter weights per set with more repetitions (e.g., 5 sets of 10–12 repetitions @ 60–65% of a 1-repetition maximum for a given exercise). HV training bouts yield a higher total volume load (the product of weight × total number of repetitions) where the total weight lifted is generally higher relative to HL training bouts. Research has typically suggested that HL training elicits superior increases in strength and muscle fiber hypertrophy compared to lower-load HV training (Fry, 2004). However, Mitchell and colleagues reported that 10 weeks of HL or HV resistance training led to similar increases in muscle hypertrophy as assessed through MRI and fiber histology (Mitchell et al., 2012). Subsequent literature indicates that both HL and HV training can: (i) elicit similar changes in skeletal muscle hypertrophy (assessed through either ultrasound or MRI; Schoenfeld et al., 2015; Jenkins et al., 2016; Morton et al., 2016; Ikezoe et al., 2017; Jenkins et al., 2017) and (ii) elicit similar strength adaptations (Ikezoe et al., 2017; Dinyer et al., 2019), although equivocal evidence exists suggesting HL training elicits superior strength adaptations (Schoenfeld et al., 2015; Jenkins et al., 2016, 2017). Reasons for similar outcomes between HL and HV training could be due to total volume lifted being comparable between paradigms. However, few HV versus HL studies have sought to modulate training loads with the intent of accumulating more training volume during HV conditions.

Our laboratory recently reported that 6 weeks of extremely HV resistance training decreased the relative abundances of myosin heavy chain and actin protein content per milligram of dry tissue (Haun et al., 2019a). Our findings, as well as those of others who have reported moderate-to-higher volume resistance training elicits similar molecular adaptations (MacDougall et al., 1982; Roth et al., 1999; Meijer et al., 2015), led us to postulate that a disproportionate increase in the sarcoplasmic space relative to myofibril protein accretion (i.e., sarcoplasmic hypertrophy) may be a training adaptation to HV resistance training (Roberts et al., 2020a). More recently, our laboratory demonstrated that lower volume, higher-load resistance training (3–5 sets of 2–6 repetitions at 65–90% 1RM) resulted in a maintenance of type I muscle fiber cross-sectional area (fCSA) while increasing type II fCSA. Additionally, no changes in non-myofibrillar protein concentrations were observed despite a modest but significant decrease in actin protein concentrations (Vann et al., 2020). While preliminary, these two studies from our laboratory suggest that HV resistance training may facilitate a more robust expansion of non-contractile proteins in myofibers, whereas HL training may promote a proportional increase in myofibril protein accretion with muscle growth. However, no single study to date has examined whether HV versus HL training differentially alter the molecular milieu in skeletal muscle; in particular the relative abundances of prominent myofibrillar proteins as well as the long-term synthesis rates of myofibrillar versus non-myofibrillar proteins.

The purpose of this study was to elucidate whether 6 weeks of unilateral HV versus HL lower-body resistance training differentially affected metrics of skeletal muscle hypertrophy, strength, and/or molecular variables assessed from skeletal muscle biopsies sampled from the vastus lateralis (VL). We sought to ensure HV training achieved more training volume relative to HL training. We hypothesized no differences would exist between HV and HL training when examining changes in VL muscle area assessed *via* magnetic resonance imaging (MRI), or VL thickness assessed *via* ultrasound. Additionally, we hypothesized that HL training would elicit superior increases in various indices of strength. However, we posited HV training would result in increased non-myofibrillar protein concentrations and a concomitant decrease in the relative abundances of contractile proteins, whereas HL training would result in no changes in these markers. Additionally, we hypothesized that the integrated non-myofibrillar (iNon-MyoPS) rates would be greater in HV versus HL training, whereas integrated myofibrillar protein synthesis (iMyoPS) rates would be greater in HL versus HV training. Finally, we aimed to determine if HV versus HL training adaptations persisted 10 days following the cessation training given that our laboratory and others have demonstrated features sarcoplasmic hypertrophy occur during ~8–10 days following 6–12 weeks of resistance training (Kadi et al., 2004; Haun et al., 2019a). Thus, all measures (except for protein synthesis assessments) were obtained prior to training as well as 3 days and 10 days following training.

MATERIALS AND METHODS

Ethical Approval and Pre-screening

Prior to study initiation, this protocol was reviewed and approved by the Auburn University Institutional Review Board and was conducted in accordance to the standards set by the latest revision of the Declaration of Helsinki (IRB approval #: 19-245 MR 1907), except this study was not registered in a database.

College-aged resistance-trained men from the local community were solicited to participate in this study. Participants were provided informed consent documents, which clearly outlined all procedures of the study including the collection of muscle biopsies. In addition, participants were instructed that they were free to withdraw from the study at any time without jeopardy. Eligible participants that provided verbal and written informed consent and were screened 4–7 days prior to the start of the study. Participants had to be free of cardio-metabolic diseases (e.g., morbid obesity, type II diabetes, and severe hypertension), or any conditions that preclude the collection of a skeletal muscle biopsy. Participants were queried for the use of medications or performance-enhancing drugs, and none of the participants reporting using drugs for medical or recreational purposes. Additionally, training status for participants was determined by two criteria: (i) self-reported resistance training >1 year at least three times weekly, and (ii) a tested barbell back squat of ≥1.5× bodyweight [estimated from a 3 repetition maximum (3RM) test] in accordance to standards designated by the National Strength and Conditioning Association (NSCA; Haff et al., 2016). At the conclusion of the screening visit, participants were asked to maintain their current nutritional practices and to cease all training outside of the study.

Study Design

A schematic of the study design is provided in **Figure 1**. Briefly, participants performed a testing battery prior to the start of training (PRE), 72 h following the last bout of training after 6 weeks of unilateral lower-body resistance training (POST), and 10 days following the last bout of training (POSTPR). The testing batteries are detailed below, following a description of the training intervention and tracer methodologies.

Resistance Training

Participants performed progressive unilateral lower-body resistance training (i.e., single-leg leg press and single-leg leg extension) 3 days per week in conjunction with compound upper body exercises (i.e., barbell bench press, pronated grip barbell row, barbell stiff-leg deadlift). Notably, participants were randomly assigned to lower-body training conditions prior to the start of the study, where some participants performed HV training on the left leg and HL training on the right leg or vice versa. All upper body exercises were performed for 3 sets of 10 repetitions at 70% of tested 1RM. Progression for the lower-body training can be found in **Figure 1**.

The HV training scheme was programed *a priori* considering that an individual engaged in a higher-volume training block would perform sets of 10 repetitions ~60% 1RM, and incrementally increase volume on a weekly basis. The HL training scheme was programed *a priori* considering that an individual engaged in strength training block would perform sets of 5 repetitions where the initial loads were ~ 80% 1RM, and incrementally increase training intensity on a weekly basis.

Auburn University staff supervised training, and weight lifted for each participant was logged in real time. Throughout training, we elected a systematic approach to adjust load within

A

Event	Wk 0			Wk 1 (Sat-Fri)							Wk 2 (Sat-Fri)							Wk 3 (Sat-Fri)							Wk 4 (Sat-Fri)							Wk 5 (Sat-Fri)							Wk 6 (Sat-Fri)							Wk 7 (Sat-Fri)							
Testing†				X																																																		
Training	*					X		X		X			X		X		X			X		X		X																														
D_2O#	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Salivette	X	X	X	X	X	X	X	X	X	X	X	X	X		X		X			X		X		X																															

D_2O was dosed at 1ml·kg^{-1} of lean body mass. * 72 hours prior to PRE participants orally consumed 6 servings of D_2O over 8 hours spaced ~90 minutes apart.
† Participants arrived in an overnight fasted state for testing consisting of: USG, height, weight, DXA, US, MRI, blood draw, VL muscle biopsy, isokinetic dynamometry, strength testing.

B

Training Protocol*

		High-volume leg					High-load leg				
Wk	Leg Extensor Exercise	M	W	F	Total/wk	% 1RM	M	W	F	Total/wk	% 1RM
1	Unilateral Leg Press & Unilateral Leg Extension	2x10	1x10	2x10	5x10	60%	3x5	3x5	3x5	9x5	82.5%
2	Unilateral Leg Press & Unilateral Leg Extension	2x10	2x10	2x10	6x10	60%	3x5	3x5	3x5	9x5	85.0%
3	Unilateral Leg Press & Unilateral Leg Extension	2x10	3x10	2x10	7X10	60%	3x5	3x5	3x5	9x5	87.5%
4	Unilateral Leg Press & Unilateral Leg Extension	3x10	2x10	3x10	8x10	60%	3x5	3x5	3x5	9x5	90.0%
5	Unilateral Leg Press & Unilateral Leg Extension	3x10	3x10	3x10	9X10	60%	3x5	3x5	3x5	9x5	92.5%
6	Unilateral Leg Press & Unilateral Leg Extension	3x10	4x10	3x10	10X10	60%	3x5	3x5	3x5	9x5	95.0%
7	Cessation of training										

*Participants also completed BB bench press, pronated grip BB row, and BB SLDL for 9x10 @ 70% of estimated 1RM. All sets and reps are shown as weekly prescriptions.

FIGURE 1 | Study design. Panel **(A)** provides an overview of testing, training, D_2O administration, and saliva collection times. Panel **(B)** provides a schematic of training by day and total training for each week. wk., week; D_2O, deuterium oxide.

each training session if target repetitions per set were not met (e.g., a 5–10% reduction in load for the next set if 9/10 repetitions completed). However, this was only necessary on a few occasions, and most of the training was executed according to the planned study design.

Tracer Protocol

Deuterium oxide (D_2O; Cambridge Isotope Laboratories, Inc.; Andover, MA, United States) was provided to the participants 3 days prior to and over the first 6 weeks of the study at 1 ml•kg^{-1} of lean body mass. For rapid enrichment of deuterium (^2H) participants were instructed to orally consume 6 doses of D_2O over an eight-hour period, 3 days prior to the first data collection (PRE), and were instructed to consume a top-up dose daily thereafter consisting of one dose of D_2O until data collection was performed at the conclusion of week 6 of the study (POST). Saliva samples were taken utilizing sterile salivettes (SARSTEDT AG &Co, Nümbrecht, Germany). Briefly, participants were instructed to chew on the cotton swab for 1 min and place the swab back into the top compartment of the salivette. This process was completed daily for the first 10 days of the study and every Monday, Wednesday, and Friday thereafter. Participants were instructed to place salivettes in their home freezers on days when saliva was donated outside of the laboratory. Samples were stored at −20°C until further processing as described below.

Testing Sessions

Urine-Specific Gravity Testing for Adequate Hydration

Upon arrival to each testing session, participants submitted a urine sample (~5 ml) for urine-specific gravity (USG) assessment. Measurements were performed using a handheld refractometer (ATAGO; Bellevue, WA, United States). USG levels in all participants were ≤1.020 indicative of a euhydrated state and, thus, were considered adequately hydrated for further testing.

Body Composition Testing

Following hydration testing, participants underwent height and body mass assessments utilizing a digital scale (Seca 769; Hanover, MD, United States) with body mass collected to the nearest 0.1 kg and height to the nearest 0.5 cm. Participants were then subjected to a full body dual-energy X-ray absorptiometry (DXA) scan (Lunar Prodigy; GE Corporation, Fairfield CT, United States). Our laboratory (Kephart et al., 2016) has previously shown same day reliability of the DXA during test–calibrate–retest on 10 participants yields an intra-class correlation coefficient (ICC) of 0.998 for total body lean mass and an absolute standard error of the measurement (SEM) of 0.47 kg. Associated software was used to derive whole-body lean soft tissue mass (bone-free; abbreviated as LSTM) and fat mass. In addition, regions of interest were drawn around the upper left and right legs from the inguinal crease to the top of the knee to obtain upper leg LSTM, upper leg fat mass, and upper leg total mass (i.e., LSTM + fat mass + bone mass).

Measurements of Muscle Morphology

Following body composition testing, participants were tested for VL muscle thickness and muscle pennation angle *via* ultrasound. VL thickness of both legs was assessed by placing a 3–12 MHz multi-frequency linear phase array transducer (Logiq S7 R2 Expert; General Electric, Fairfield, CT, United States) midway between the iliac crest and lateral epicondyle of the femur. Measurements were taken from a standing position and participants were instructed to bear most of their weight on the leg contralateral to the leg being measured. VL pennation angles were taken immediately following thickness measures by placing the transducer longitudinally at the same site mentioned above. VL thickness was measured as the distance between the superficial and deep aponeurosis while VL pennation angle was measured as the angle of the deep aponeurosis as it relates to the individual fascicles. Estimated fiber length was calculated using methods similar to those described by Fukunaga et al. (1997) as seen in the equation below.

$$est.\,fiber\ length = \frac{a}{\cos\left(90° - \theta\right)}$$

In the equation, a is equal to the distance between the superficial fascia and the deep aponeurosis and θ is equal to the angle of pennation. Importantly, to minimize variability in measurements as suggested in previous studies (Lohman et al., 2009; Lockwood et al., 2017), all measures were taken by the same investigator (S.C.O.), and this person in a test–retest validation on 10 participants had an ICC of 0.991 and an SEM of 0.06 cm. Critically, this investigator was not privy to the training condition for each participant's leg. Moreover, the location of measurements was marked, using anatomical landmarks, by the investigator so that the subsequent MRI scans and muscle biopsies could be obtained from the same plane of measurement.

MRI for Muscle Cross-Sectional Area

Following ultrasound assessments, participants were shuttled to the Auburn University MRI Research Center to perform dual-leg mid-thigh MRI scans. All measurements were performed on a 3 T VARIO system (Siemens, Erlangen, Germany). Briefly, participants were placed in a supine position for 10 min to allow for body fluid stabilization to occur. A volume coil was used for RF transmit and body and spine array coils placed around the legs were used for signal reception. 3D gradient echo sequence (3D fast low angle shot) was used to acquire fat-suppressed images with the following parameters: TR/TE = 10/4.92 ms; flip angle = 10°; bandwidth = 510 Hz/pixel, in-plane resolution 1 mm×1 mm and slice thickness = 2.2 mm. An axial 3D 35.2 mm thick slab (a6 partitions) was placed to image both thighs with the thickness dimension carefully centered on the participant biopsy marking. Following the conclusion of the study, MRI scans were digitized using Osirix MD software (Pixmeo, Geneva, CHE), and software tools were used to manually trace the border of the VL yielding mCSA values. Measures were taken by the same investigator (R.J.B.)

who did not possess knowledge of the training condition for each participant's legs, and this person in a test–retest validation on 10 participants had an ICC of 0.999 and an SEM of 0.31 cm^2.

Collection of Muscle Tissue

Following MRI scans, right and left leg VL muscle biopsies were collected using a 5-gauge needle under local anesthesia as previously described (Roberts et al., 2018, 2019). Immediately following tissue procurement, tissue was teased of blood and connective tissue, wrapped in pre-labeled foils, flash-frozen in liquid nitrogen, and subsequently stored at −80°C for processing described below.

Strength Testing

Following muscle skeletal muscle biopsies, participants underwent isokinetic dynamometry (Biodex System 4; Biodex Medical Systems, Inc., Shirley, NY, United States) for leg extensor peak torque and 3RM testing. For right and left leg extensor peak torque testing, participants were fastened to the isokinetic dynamometer. Each participant's lateral epicondyle was aligned with the axis of the dynamometer, and seat height was adjusted to ensure the hip angle was approximately 90°. Prior to torque assessment, each participant performed a warm-up consisting of submaximal to maximal isokinetic knee extensions. Participants then completed five maximal voluntary isokinetic knee extension actions at 1.05 rad/s (60°/s) and 2.09 rad/s (120°/s). Participants were provided verbal encouragement during each contraction. The isokinetic contraction resulting in the greatest value was used for analyses. Peak torque measurements were not gravity-corrected. Following isokinetic dynamometry participants performed maximum strength testing for the exercises utilized over the duration of the study (single-leg leg press, single-leg leg extension, barbell bench press, pronated grip barbell row, and barbell stiff-leg deadlift). Briefly, participants performed 3 warm-up sets starting at ~50% of their self-selected opening weight for 10 repetitions, then 75% of their self-selected opening weight for 5 repetitions, and 90% of their self-selected opening weight for 3 repetitions. Following warm-ups, participants executed their opening attempt for 3 repetitions with 5–10% increases being made from there on until a 3RM was achieved. Given the advanced training status of participants, most had performed and were familiar with unilateral leg exercises, so this likely mitigated learning effects. All strength testing was performed by investigators holding the NSCA certified strength and conditioning specialist credential (C.G.V. and C.L.S.). Strength testing for single-leg leg press, single-leg leg extension, barbell bench press, pronated grip barbell row, and barbell stiff-leg deadlift occurred at PRE in order to properly program exercises throughout the study. However, given single-leg exercises were outcome variables of interest, these were the only two exercises that were strength tested at POST and POSTPR.

Biochemical Assays

Non-myofibrillar and myofibrillar protein isolation. Isolation of protein fractions was performed using the proteomic validated "MIST" or "myofibrillar isolation and solubilization technique" (Roberts et al., 2020b). This method was validated through proteomic analysis showing that the myofibrillar fraction was exclusively enriched with myofibril proteins (i.e., MYH2, MHY1, MYH7, MYH4, ACTC1, and TTN), and none of these proteins were detected in the non-myofibril fraction. Additionally, several metabolic enzymes were enriched in the non-myofibrillar fraction (i.e., CKM, MB, ENO3, and PYGM), and these proteins were either not detectable or marginally present in the myofibrillar fraction. 1.7 ml polypropylene tubes were pre-filled with ice-cold buffer (300 µl; Buffer 1: 25 mm Tris, pH 7.2, 0.5% Triton X-100, protease inhibitors) and placed on ice. Skeletal muscle foils were removed from −80°C, placed on a liquid nitrogen-cooled ceramic mortar and pestle, and tissue was pulverized into 2–4 mm^3 chunks. Chunks (~20 mg) were weighed using a scale with a sensitivity of 0.0001 g (Mettler-Toledo; Columbus, OH, United States) and placed into 1.7 ml polypropylene tubes with buffer and placed on ice. Samples were homogenized using tight-fitting pestles and centrifuged at 1,500 g for 10 min at 4°C. Supernatants (non-myofibrillar fraction) were collected and placed in new 1.7 ml polypropylene tubes on ice. As a wash step, the resultant myofibrillar pellet was resuspended in 300 µl of Buffer 1 and centrifuged at 1,500 g for 10 min at 4°C. The supernatant was discarded and the myofibrillar pellet was solubilized in 300 µl of ice-cold resuspension buffer (20 mm Tris–HCl, pH 7.2, 100 mm KCl, 20% glycerol, 1 mm DTT, 50 mm spermidine, protease inhibitors). After this step, tubes were visually inspected for insoluble connective tissue that may not have been teased out following tissue collection. Protein concentrations for the non-myofibrillar fraction were determined the same day as protein isolations to minimize freeze–thaw artifact. The myofibrillar fraction was prepared to analyze the relative abundances of major myofibril proteins and stored at −80°C until analysis occurred.

Determination of Non-myofibrillar Protein Concentrations

Non-myofibrillar protein resuspensions were batch-assayed for determination of protein concentration using a commercially available bicinchoninic acid (BCA) kit (Thermo Fisher Scientific; Waltham, MA, United States). Samples were assayed in duplicate using a microplate assay protocol where a small volume of sample was assayed (20 µl of 5× diluted sample + 200 µl Reagent A + B). The average duplicate coefficient of variation for non-myofibrillar protein concentration was 2.27%.

SDS-PAGE and Coomassie Staining for Relative Contractile Protein Abundance

Determination of the relative abundances of major myofibril proteins per mg wet tissue was performed as previously described by our laboratory and others (Cohen et al., 2009; Roberts et al., 2018; Dowling et al., 2019; Haun et al., 2019a). Briefly, SDS-PAGE sample preps were made using 10 µl resuspended myofibrils, 65 µl distilled water (diH2O), and 25 µl 4× Laemmli buffer. Samples (5 µl) were then loaded on precast gradients (4–15%) SDS-polyacrylamide gels in duplicate (Bio-Rad Laboratories) and subjected to electrophoresis at 180 V for

40 min using pre-made 1× SDS-PAGE running buffer. Following electrophoresis, gels were rinsed in diH2O for 15 min and immersed in Coomassie stain (LabSafe GEL Blue; G-Biosciences; St. Louis, MO, United States) for 2 h. Gels were then de-stained in diH_2O for 60 min, and band densitometry was performed with a gel documentation system and associated software (ChemiDoc; Bio-Rad Laboratories, Hercules, CA, United States). Given that a standardized volume from all samples was loaded onto gels, band densities of different myofibril proteins were normalized to input muscle weights to derive arbitrary density units (ADU) per mg wet muscle. All values were then divided by the mean of the PRE time point to depict relative protein abundances of myosin heavy chain (MyHC). Our laboratory has reported that this method yields exceptional sensitivity in detecting 5–25% increases in relative actin and MyHC abundances (Roberts et al., 2018). Average duplicate coefficients of variation for relative protein abundances of actin, MyHC, tropomyosin, and troponin herein were 1.95, 1.90, 2.22, and 3.54%, respectively.

Six-Week Integrated Myofibrillar and Non-myofibrillar Protein Synthesis Rates

Protein isolations were performed using ~30 mg of tissue utilizing the MIST method as described above. Prior to preparation for tracer analysis, the non-myofibrillar protein fraction was lyophilized and precipitated in 1 ml of 1 M perchloric acid to form a pellet. The myofibrillar pellet was purified by adding 500 µl of DDH_2O followed by vortexing for 5 s and centrifugation at 1,500 rpm at 4°C for 10 min. Following centrifugation, 1 ml of 0.3 M NaOH was added to the sample and then vortexed for 5 s followed by being placed in a heat block at 50°C for 30 min of which samples were vortexed for 5 s every 10 min. Samples then underwent centrifugation at 10,000 rpm at 4°C for 10 min. The supernatant (non-myofibrillar or myofibrillar fraction) was transferred into a 4 ml glass screw-top tube. 1 M perchloric acid was then added to tubes, and tubes were centrifuged at 2,500 rpm at 4°C for 10 min. The supernatant was removed, and the remaining pellet was washed in 70% ethanol and centrifuged at 2,500 rpm at 4°C for 10 min twice. Amino acids were extracted through the addition of 1 ml of 1 Dowex resin (50WX8-200 resin; Sigma-Aldrich) and 1 ml of 1 M HCl prior to heating at 110°C for 72 h. Cation exchange columns were used to isolate the free amino acids after which the amino acids were analyzed for deuterated-alanine content (^{2}H-alanine) using a gas chromatography pyrolysis isotope ratio mass spectrometer. The amino acids were derivatized as their *n*-methoxycarbonyl methyl esters. Dried samples were suspended in 60 µl distilled water and 32 µl methanol, and following vortex, 10 µl of pyridine and 8 µl of methyl chloroformate were added. Samples were vortexed for 30 s and left to react at room temperature for 5 min. The newly formed *n*-methoxycarbonyl methyl esters of amino acids were then extracted into 100 µl of chloroform. A molecular sieve was added to each sample for ~20 s before being transferred to a clean glass gas chromatography insert. Incorporation of deuterium into protein bound alanine was determined by gas chromatography–pyrolysis–isotope ratio mass spectrometry (Delta V Advantage) alongside a standard curve of known l-alanine-2,3,3,3-d4 enrichment to validate measurement accuracy of the instrument (Wilkinson et al., 2014).

Saliva deuterium analysis was performed to assess whole-body isotope enrichment. Briefly, the water phase of saliva was injected 6 times with the average of the last 3 injections being used for data analysis. The ^{2}H isotope enrichments for both muscle and saliva were initially expressed as δ^2H% and then converted to atom percent excess using standard equations as reported by Wilkinson et al. (2014). Fractional synthetic rates for the myofibrillar and non-myofibrillar protein fractions were calculated using the standard precursor-product method as described by other laboratories (Chinkes et al., 1993; Bell et al., 2019; McKendry et al., 2019).

$$FSR\left(\%day^{-1}\right) = \left[\frac{\left(E_{Ala2} - E_{Ala1}\right)}{E_{BW} \times t}\right] \times 3.7 \times 100$$

In the equation above, E_{Ala1} and E_{Ala2} represent ^{2}H enrichment at PRE and POST, respectively, (in atom percent excess) from skeletal muscle biopsies. E_{BW} is the average ^{2}H enrichment (in atom percent excess) of total body water between time points and *t* is time in the number of days D_2O was ingested. Multiplying by 3.7 adjusts for average ^{2}H atoms that can be bound to alanine and multiplying by 100 converts this to a percentage per day (MacDonald et al., 2013; Wilkinson et al., 2014).

Statistical Analyses

Statistical analyses were performed using SPSS (Version 26; IBM SPSS Statistics Software, Chicago, IL, United States), open-source software JASP (Version 0.11.0; JASP Team; 2019), and RStudio (version 1.1.463, R Foundation for Statistical Computing, Vienna, AT). Prior to analysis, assumptions testing for normality was performed using Shapiro–Wilk's test for all dependent variables. If the assumption of heteroscedasticity was violated for repeated measures, a Greenhouse–Geisser correction factor was applied. Most dependent variables were analyzed using multi-factorial repeated measures ANOVAs, and if an interaction or main effect of time were observed ($p < 0.05$), manual Bonferroni adjustments were used to assess differences in dependent variables for leg or time. In this regard, significance for *post hoc* tests was established as $p < 0.025$ given that: (i) in the case of main time effects or interactions, two comparisons were made over time (POST versus PRE and POSTPR versus PRE), and (ii) in the case of interactions, two comparisons were made between legs at the POST and POSTPR time points. Tracer data were analyzed using dependent samples *t*-tests given that there was no time component to these data. Data are presented throughout as mean ± standard deviation (bar graphs) or box and whiskers plots including median (central horizontal line), 25th and 75th percentile (box), minimum and maximum values (vertical lines), and mean values (cross). Notably, a sample size of 15 participants was chosen *a priori* given the feasibility and logistics of performing individualized training sessions along with performing various techniques and assays that were resource intensive.

RESULTS

Participant Characteristics

Baseline participant characteristics are presented in **Table 1**. Briefly, 15 college-age males (23 ± 3 years) with an average training age of 7 ± 3 years volunteered for this study. At PRE, participants weighed 89.5 ± 11.6 kg, with 69.1 ± 7.4 kg being LSTM and 17.3 ± 7.5 kg being fat mass, on average. Additionally, participants had an average relative squat to body mass ratio of 1.9× body mass (167 ± 34 kg).

Training Volume and Strength Metrics

Training volumes and strength metrics are presented in **Figure 2**. Data for 14 of 15 participants are presented for unilateral leg press and unilateral leg extension one repetition maximums (1RM) due to one participant feeling lower extremity discomfort at POST with these exercises.

There was a condition×time interaction observed for lower-body training volume ($p < 0.001$, $\eta_p^2 = 0.914$; **Figure 2A**). Additionally, there was a main effect of condition ($p = 0.003$, $\eta_p^2 = 0.467$) where the HV condition completed more volume than the HL condition (8,100 ± 480 kg versus 7,296 ± 421 kg, respectively). Lower-body training volume changed over time ($p < 0.001$, $\eta_p^2 = 0.955$, **Figure 2A**) and within each condition over time (HV: $p < 0.001$, $\eta_p^2 = 0.952$; HL: $p < 0.001$, $\eta_p^2 = 0.954$, **Figure 2A**). *Post hoc* analysis revealed lower training volumes

TABLE 1 | Participant characteristics.

Variable	Mean ± SD
Age (years)	23 ± 3
Height (cm)	182 ± 8
Weight (kg)	89.5 ± 11.6
Lean soft tissue mass (kg)	69.1 ± 7.4
Fat tissue mass (kg)	17.3 ± 7.5
Fat-free mass index	20.9 ± 2.2
Est. 1RM Squat (kg)	167 ± 34
Squat relative to body weight	1.9 ± 0.4

N = 15 participants. Est. 1RM, estimated 1 repetition maximum. All measures taken prior to onset of training intervention.

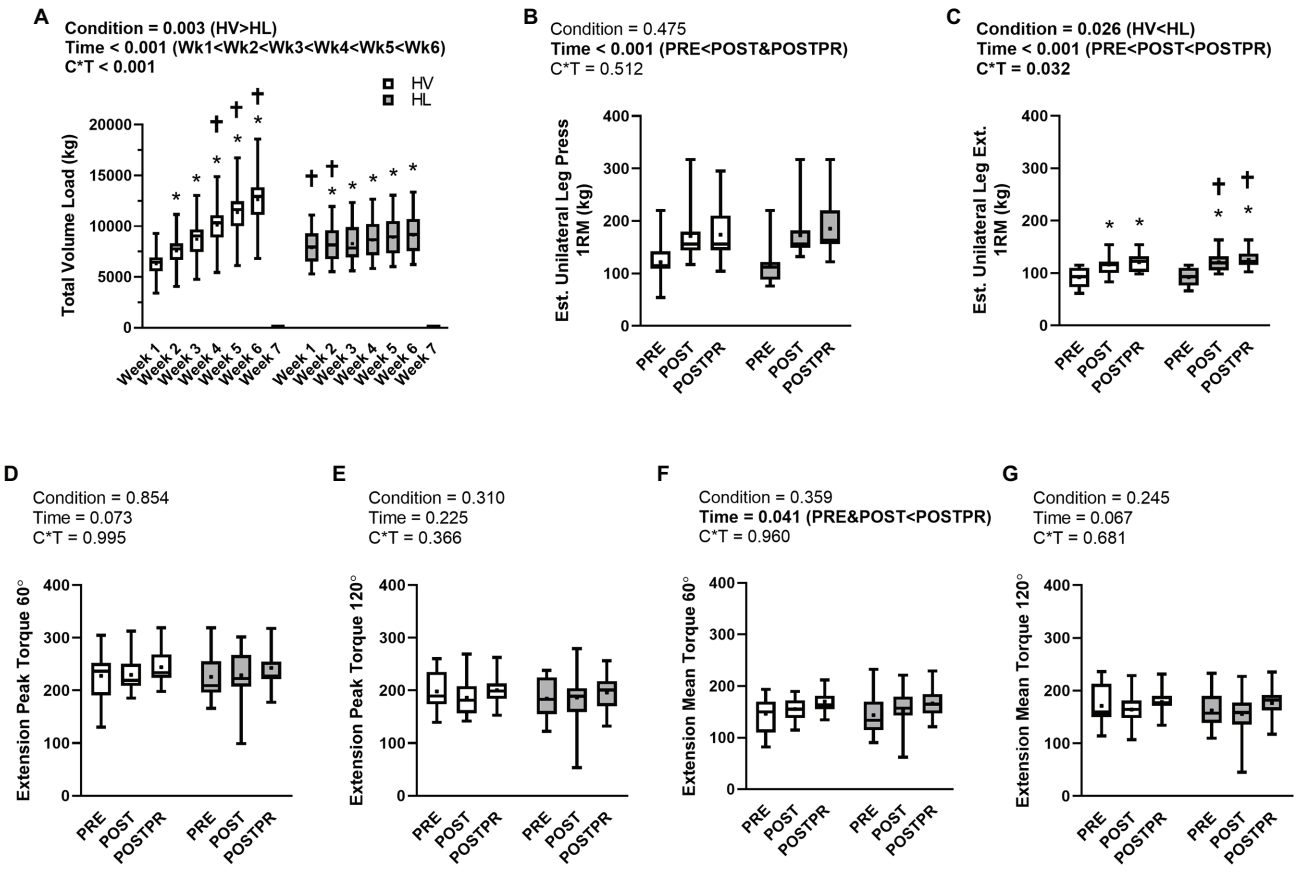

FIGURE 2 | Training Volume and Strength Metrics. Legend: Data are presented as box and whiskers plots including median (central horizontal line), 25th and 75th percentile (box), minimum and maximum values (vertical lines), and mean values (cross) for training volume load (panel **A**), unilateral leg press (panel **B**), unilateral leg extension (panel **C**), knee extension peak torque at 60°/s (panel **D**), knee extension peak torque at 120°/s (panel **E**), knee extension mean torque at 60°/s (panel **F**), and knee extension mean torque at 120°/s (panel **G**). Abbreviations: HV, high-volume; HL, high-load. Symbols: *indicates increase from PRE within condition; †indicates HV > HL or HL > HV at a given time point.

at weeks 1 and 2 in the HV leg compared to the HL leg ($p < 0.001$ at each time point), no differences between conditions at week 3, and higher training volumes in the HV leg at weeks 4–6 as compared to the HL leg ($p < 0.001$ at each time point).

A condition×time interaction ($p = 0.512$, $\eta_p^2 = 0.050$, **Figure 2B**) was not observed for estimated unilateral leg press 1RM. Additionally, no main effect of condition ($p = 0.475$, $\eta_p^2 = 0.040$, **Figure 2B**) was observed. There was a main effect of time ($p < 0.001$, $\eta_p^2 = 0.818$, **Figure 1B**) where estimated unilateral leg press 1RM at POST ($p < 0.001$) and POSTPR ($p < 0.001$) were greater than PRE.

A condition×time interaction was observed for estimated unilateral leg extension 1RM ($p = 0.032$, $\eta_p^2 = 0.265$, **Figure 2C**). A main effect of condition ($p = 0.026$, $\eta_p^2 = 0.328$, **Figure 2C**) was also observed where the HL condition (grand mean = 113 ± 5 kg) estimated unilateral leg extension 1RM was higher than the HV condition (grand mean = 109 ± 5 kg). Estimated unilateral leg extension 1RM also changed over time ($p < 0.001$, $\eta_p^2 = 0.885$, **Figure 2C**) and within each condition over time (HV: $p < 0.001$, $\eta_p^2 = 0.858$; HL: $p < 0.001$, $\eta_p^2 = 0.884$). *Post hoc* analysis revealed no differences in estimated unilateral leg extension 1RM at PRE; however, the HL condition had higher values at POST and POSTPR compared to the HV condition ($p < 0.025$ at each time point). Given the significant interaction, we also calculated POST-PRE and POSTPR-PRE change scores for the HV and HL conditions and compared these scores using dependent samples t-tests as an additional *post hoc* analysis. Comparison of POST-PRE change scores indicated HL was greater than HV (30 ± 10 kg versus 25 ± 11 kg, respectively, $p = 0.029$). Similarly, comparison of POSTPR-PRE change scores indicated HL was greater than HV (34 ± 14 kg versus 29 ± 11 kg, respectively, $p = 0.039$). These results collectively indicate that HL training increased leg extensor strength more so than HV training.

There was no condition×time interaction observed for knee-extensor peak torque at 60°/sec ($p = 0.995$, $\eta_p^2 < 0.001$, **Figure 1D**) and 120° ($p = 0.366$, $\eta_p^2 = 0.069$, **Figure 2E**), or knee-extensor mean torque at 120°/sec ($p = 0.681$, $\eta_p^2 = 0.027$, **Figure 2G**). Additionally, there were no main effects of condition or time observed for the aforementioned variables. Knee-extensor mean torque at 60°/sec showed a significant time effect ($p = 0.041$, $\eta_p^2 = 0.204$, **Figure 2F**) where knee-extensor mean torque at 60°/sec trended higher at POSTPR than at PRE ($p = 0.029$) and POST ($p = 0.043$). However, these value of ps did not achieve a level of significance according to manual Bonferroni corrections (i.e., $p < 0.025$). There were no differences observed between PRE and POST ($p = 0.805$).

Body Composition

PRE, POST, and POSTPR whole-body composition changes for all participants are presented in **Table 2**; notably, these data were derived from dual-energy X-ray absorptiometry (DXA) scans. Total body mass increased over time ($p < 0.001$, $\eta_p^2 = 0.435$), where POST ($p = 0.001$) and POSTPR ($p = 0.012$) body masses were greater than PRE. However, no differences were observed between POST and POSTPR body masses ($p = 0.119$). Whole-body LSTM increased over time ($p = 0.003$, $\eta_p^2 = 0.338$) where POST was greater than PRE ($p = 0.002$) and POSTPR ($p = 0.014$). No significant differences in LSTM were observed between PRE and POSTPR ($p = 0.286$). No differences were observed for DXA measured whole-body fat mass ($p = 0.097$).

TABLE 2 | Body composition changes during training.

Variable	PRE	POST	POSTPR	ANOVA *p*-Value
	Mean ± SD	Mean ± SD	Mean ± SD	
Total Body Mass (kg)	89.3 ± 11.5	90.8 ± 11.9	90.4 ± 12.2	<0.001†
DXA Whole-body LSTM (kg)	69.1 ± 7.4	70.2 ± 7.5	69.5 ± 7.5	0.003*
DXA Whole-body Fat Mass (kg)	17.3 ± 7.5	17.4 ± 7.8	17.7 ± 7.8	0.097

*N = 15 participants. DXA, dual-energy X-ray absorptiometry; LSTM, lean soft tissue mass. *indicates measurement was higher at POST than at PRE and POSTPR ($p < 0.05$); †indicates measurement was higher at POST and POSTPR than PRE ($p < 0.05$).*

Segmental Upper Leg Composition

There were no condition×time interactions observed for DXA-derived upper leg mass ($p = 0.069$, $\eta_p^2 = 0.173$, **Figure 3A**), upper leg LSTM ($p = 0.174$, $\eta_p^2 = 0.117$, **Figure 3B**), or upper leg fat mass ($p = 0.959$, $\eta_p^2 = 0.003$, **Figure 3C**). A main effect of time was observed for upper leg mass ($p = 0.001$, $\eta_p^2 = 0.392$, **Figure 3A**) where POST ($p = 0.002$) and POSTPR ($p = 0.013$) were higher than PRE. No differences were observed between POST and POSTPR ($p = 0.240$). A main effect of time was observed for DXA upper leg LSTM ($p = 0.001$, $\eta_p^2 = 0.418$, **Figure 3B**) where POST ($p < 0.001$) and POSTPR ($p = 0.002$) were higher than PRE. No differences were observed between POST and POSTPR ($p = 0.148$). No main effects of condition ($p = 0.102$) or time ($p = 0.595$) were observed for DXA upper leg fat mass.

Vastus Lateralis Muscle Morphology

A condition×time interaction was observed for magnetic resonance image (MRI)-derived VL cross-sectional area ($p = 0.046$, $\eta_p^2 = 0.211$, **Figure 4A**); however, no main effects of condition ($p = 0.490$, $\eta_p^2 = 0.037$) or time ($p = 0.351$, $\eta_p^2 = 0.077$) were observed. *Post hoc* analysis revealed no differences between conditions at PRE ($p = 0.246$), POST ($p = 0.673$), or POSTPR ($p = 0.247$). However, POST was greater than PRE in the HV condition ($p = 0.018$), whereas this was not the case in the HL condition (POST versus PRE $p = 0.475$). Given the significant interaction, we also calculated POST-PRE and POSTPR-PRE change scores for the HV and HL conditions and compared these scores using dependent samples t-tests as an additional *post hoc* analysis. Comparison of POST-PRE change scores indicated HV was greater than HL (1.3 ± 2.1 cm^2 versus 0.0 ± 2.1 cm^2, respectively, $p = 0.004$). However, between-condition differences were not evident when comparing POSTPR-PRE change scores (HV = 0.7 ± 2.2 cm^2, HL = 0.7 ± 1.8 cm^2; $p = 0.991$). These results collectively indicate that VL hypertrophy occurred from PRE to POST in the HV versus HL condition.

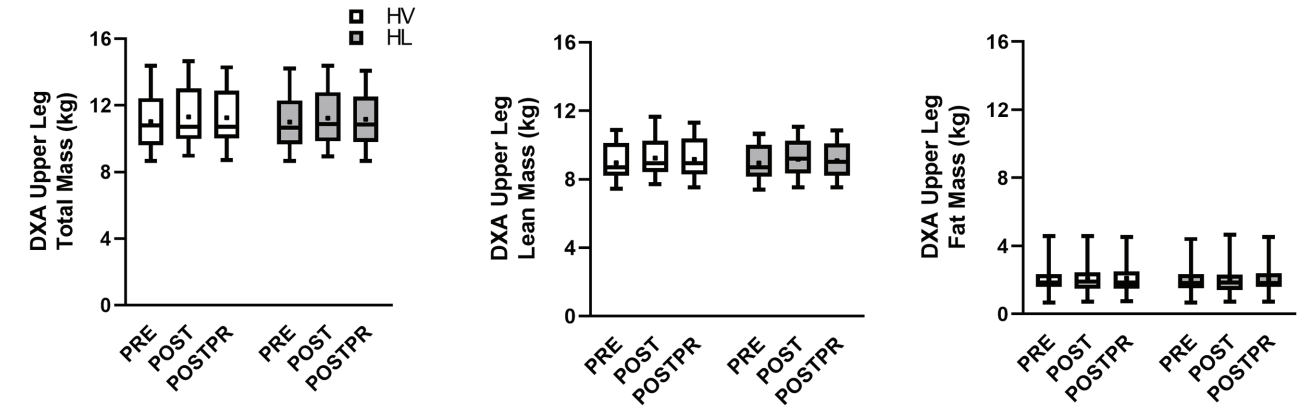

FIGURE 3 | Segmental Upper Leg Composition. Legend: Data are presented as box and whiskers plots including median (central horizontal line), 25th and 75th percentile (box), minimum and maximum values (vertical lines), and mean values (cross) for DXA upper leg total mass (panel **A**), DXA upper lean mass (panel **B**), and DXA upper leg fat mass (panel **C**). Abbreviations: HV, high-volume; HL, high-load; DXA, dual-energy X-ray absorptiometry.

There was no condition×time interaction ($p=0.338$, $\eta_p^2=0.075$, **Figure 4D**) or main effect of condition ($p=0.457$, $\eta_p^2=0.040$) observed for ultrasound measured VL thickness. VL thickness changed over time ($p=0.035$, $\eta_p^2=0.241$) where POST values trended greater than PRE ($p=0.026$) and were greater than POSTPR ($p=0.003$). No differences were observed between PRE and POSTPR ($p=0.614$). There were no interactions observed for muscle pennation angle of the VL ($p=0.393$, $\eta_p^2=0.064$, **Figure 4F**) or estimated VL muscle fiber length ($p=0.602$, $\eta_p^2=0.036$, **Figure 4C**). Additionally, there were no main effects of condition or time for the aforementioned variables ($p>0.05$). Representative images from the MRI and ultrasound are provided in **Figures 4B,E,G**.

Muscle Protein Adaptations

There was no condition×time interaction observed for non-myofibrillar protein concentrations per mg of wet tissue weight ($p=0.112$, $\eta_p^2=0.159$, **Figure 5A**). There was a main effect of condition ($p=0.002$, $\eta_p^2=0.497$, **Figure 5A**) where non-myofibrillar protein concentrations in the HV group were higher than the HL group (44.8 ± 1.6 versus 42.6 ± 1.3 respectively). Additionally, there was a main effect of time ($p=0.022$, $\eta_p^2=0.239$, **Figure 5A**) where PRE non-myofibrillar protein concentrations trended higher than POST ($p=0.038$) and POSTPR ($p=0.032$). However, these values of p did not achieve a level of significance according to manual Bonferroni corrections (i.e., $p<0.025$). No differences in non-myofibrillar protein concentrations were observed between POST and POSTPR ($p=0.524$).

There were no condition×time interactions observed for relative MyHC protein abundance per mg of wet tissue weight ($p=0.668$, $\eta_p^2=0.028$, **Figure 5B**) or relative actin protein abundance per mg of wet tissue weight ($p=0.254$, $\eta_p^2=0.093$, **Figure 5C**). Additionally, no main effects of condition or time were observed for these variables ($p>0.05$). There was no condition×time interaction ($p=0.180$, $\eta_p^2=0.115$, **Figure 5D**) or main effect of condition ($p=0.762$, $\eta_p^2=0.007$, **Figure 5D**) observed for relative tropomyosin protein abundance per mg wet tissue weight. However, a main effect of time was observed for this variable ($p=0.008$, $\eta_p^2=0.294$, **Figure 5D**) where PRE was greater than POST ($p=0.009$) and POSTPR ($p=0.010$). No differences were observed between POST and POSTPR ($p=0.704$). There was no condition×time interaction ($p=0.112$, $\eta_p^2=0.145$, **Figure 5E**) or a main effect of condition ($p=0.912$, $\eta_p^2=0.001$, **Figure 5E**) observed for relative troponin protein abundance per mg wet tissue weight. A main effect of time was observed for this variable ($p<0.001$, $\eta_p^2=0.431$, **Figure 5E**) where PRE was greater than POST ($p<0.001$) and POSTPR ($p=0.005$). No differences were observed between POST and POSTPR ($p=0.865$). **Figure 5F** is a representative Coomassie gel.

Six-Week Integrated Myofibrillar and Non-myofibrillar Protein Synthesis

Figure 6A shows whole-body deuterium enrichment assessed *via* saliva samples for $n=12$ participants. Following the loading phase (6 doses of D_2O over an eight-hour period at $1\,ml\bullet kg^{-1}$ of lean body mass), deuterium enrichment increased significantly above baseline values ($APE=0.572\pm0.087$; $p<0.001$). No difference was observed in iMyoPS rates between the HV and HL conditions ($p=0.687$; $d=-0.106$; **Figure 6B**). A significant difference was observed for iNon-MyoPS rates where the HV condition exhibited a higher value than the HL condition ($p=0.018$; $d=0.693$; **Figure 6C**).

DISCUSSION

Chief findings from the current study include: (i) VL hypertrophy with HV training, but not HL training, from PRE to POST,

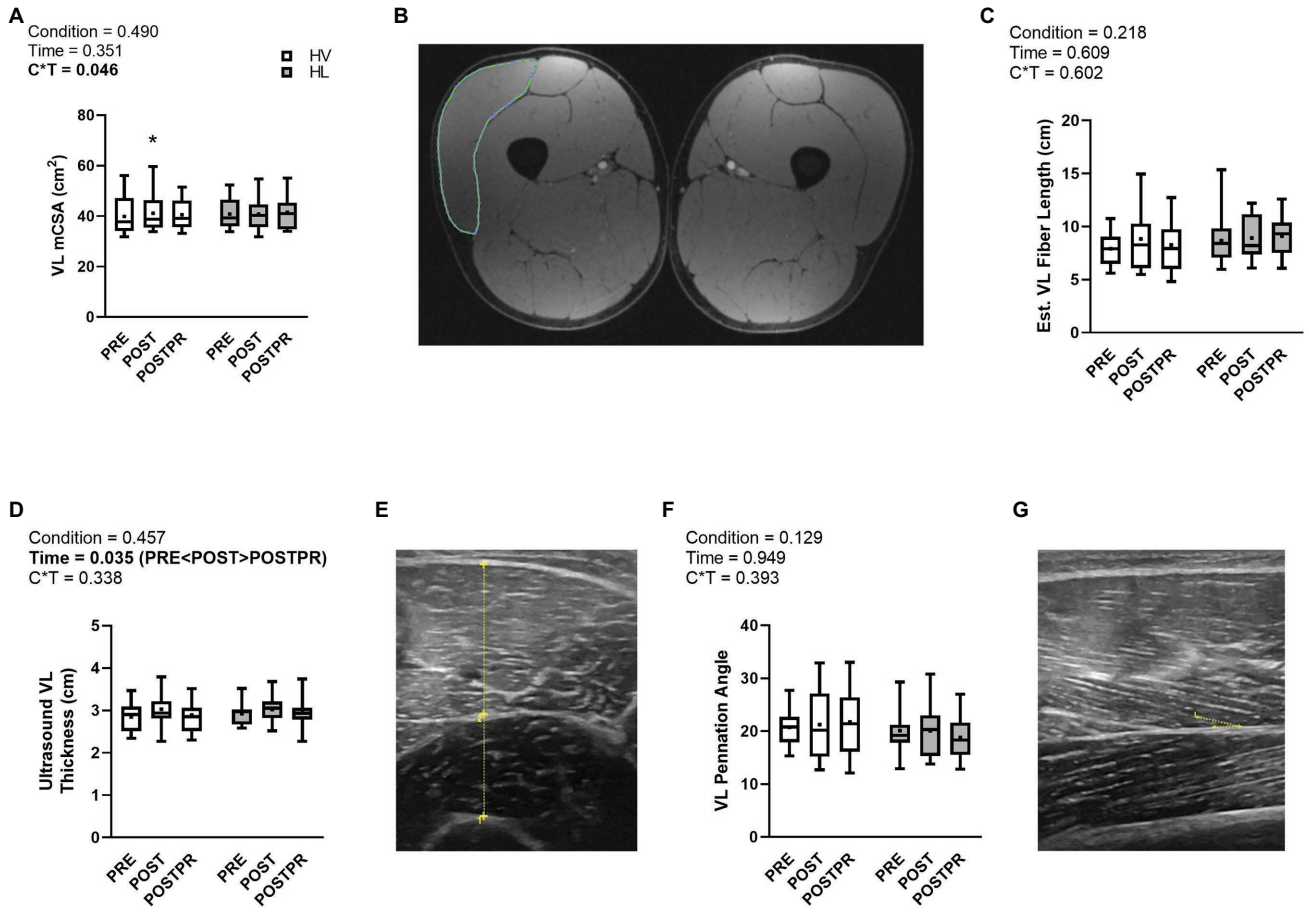

FIGURE 4 | Vastus Lateralis Muscle Morphology. Data are presented as box and whiskers plots including median (central horizontal line), 25th and 75th percentile (box), minimum and maximum values (vertical lines), and mean values (cross) for VL mCSA (panel **A**), Est. VL fiber length (panel **C**), VL thickness (panel **D**), and VL muscle pennation angle (panel **F**). Representative images: Dual-leg MRI for VL mCSA (panel **B**), ultrasound cross section for VL thickness (panel **E**), ultrasound cross section for pennation angle (panel **G**). No significance was observed following decomposition of condition × time interaction for VL mCSA. Abbreviations: HV, high-volume; HL, high-load; VL, vastus lateralis; mCSA, muscle cross-sectional area; Est., estimated; Symbol: *indicates increase from PRE within condition.

(ii) greater increases in leg extensor strength with HL training, and (iii) iNon-MyoPS being greater in the HV versus HL condition. Notably, these results are for previously trained male participants, thus these findings may not hold true in other populations. The relevance of these as well as other findings are discussed below. A significant limitation is a lack of histology data detailing type I and II fiber type adaptations, and this is discussed later.

There is prior literature that has interrogated differences between HV and HL training paradigms. Holm and colleagues (Holm et al., 2008) reported that high-load (~70% 1RM) versus very low-load (~15.5% 1RM) leg extensor training increased quadriceps CSA; however, the change in the high-load condition was greater than the change in the low-load condition. Chestnut and Docherty (1999) reported similar increases in muscle CSA of the upper arm following 10 weeks of upper body resistance training using ~85% of 1RM for 6 sets of 4 repetitions versus ~70% for 3 sets of 10 repetitions. Mitchell and colleagues reported that performing three sets of knee-extensor training to fatigue at 30% or 80% of 1RM resulted in similar increases in quadriceps volume measured by MRI (Mitchell et al., 2012). Both modalities yielded greater quadriceps hypertrophy than performing one set at 80% 1RM to voluntary failure. Furthermore, a systematic review and meta-analysis conducted by Schoenfeld and colleagues concluded that similar skeletal muscle growth can be realized across a variety of loading ranges (Schoenfeld et al., 2017). While it is challenging to form a cohesive model based on our data and the literature cited above, our finding that VL mCSA increased from PRE to POST with HV training further implies that higher-volume training at loads corresponding to ~60% 1RM can be used to optimize hypertrophy in previously trained men. However, in accordance with the current data and some of these previously mentioned studies, the training loads utilized with HV training paradigms likely need to be between 30 and 85% 1RM to optimize hypertrophy.

As mentioned above, HL training increased leg extensor 1RM values more so relative to HV training. Several studies have examined changes in strength between different loading

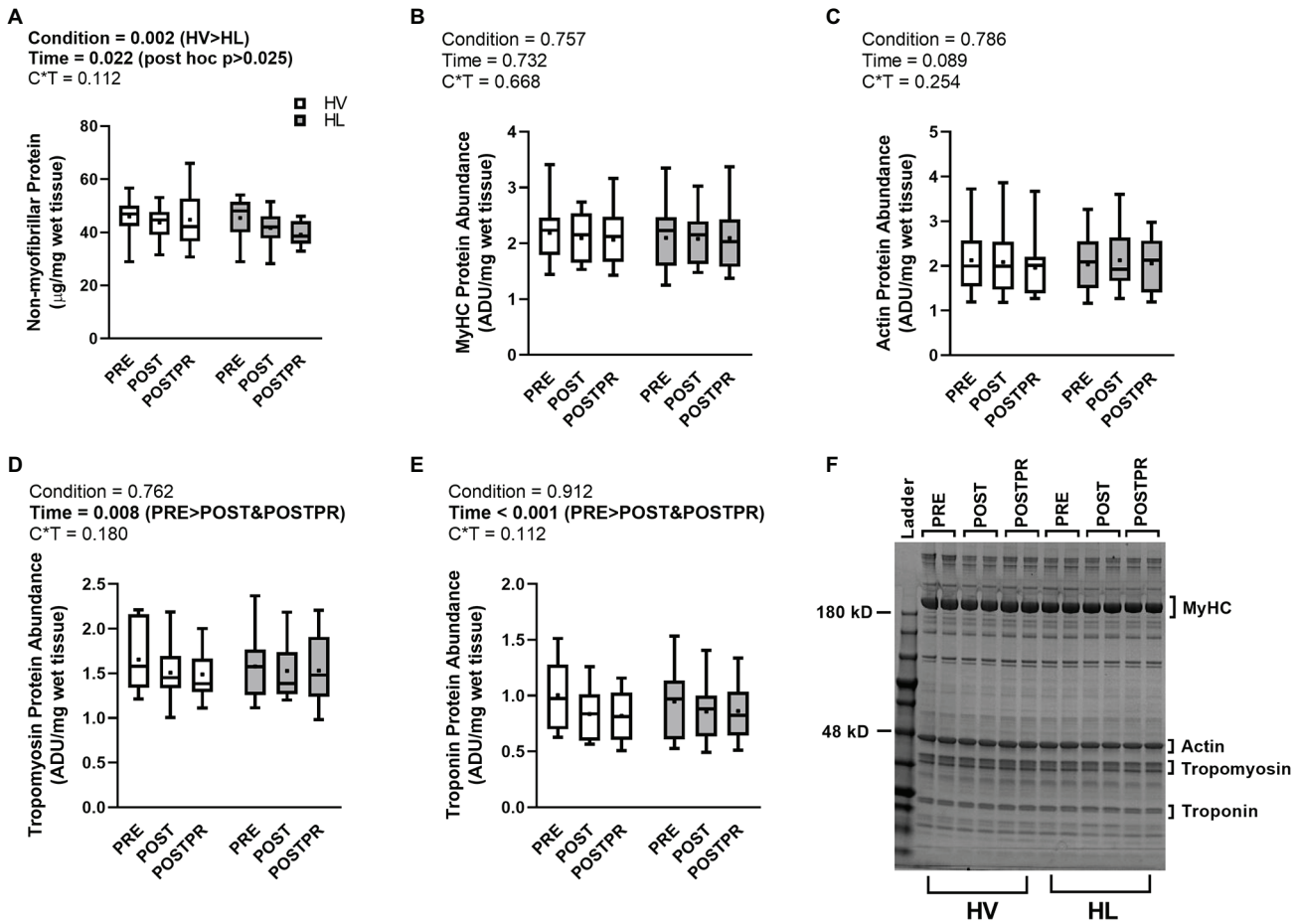

FIGURE 5 | Muscle Protein Adaptations. Data are presented as box and whiskers plots including median (central horizontal line), 25th and 75th percentile (box), minimum and maximum values (vertical lines), and mean values (cross) for non-myofibrillar protein concentrations (panel **A**), MyHC protein abundance (panel **B**), actin protein abundance (panel **C**), tropomyosin protein abundance (panel **D**), and troponin protein abundance (panel **E**). Representative image: Coomassie blue stained poly-acrylamide gel for protein abundance (panel **F**). Abbreviations: HV, high-volume; HL, high-load; VL; MyHC, myosin heavy chain; ADU, arbitrary density units; kD, kilodalton.

paradigms. Campos and colleagues reported high-load resistance training (3-5RM) over an 8-week period yielded greater leg extension strength increases compared to high-volume resistance training (20-28RM; Campos et al., 2002); however, no differences in strength adaptations were reported between the 3-5RM group and a third group which performed training using 9-11RM loads. Additionally, Jenkins et al. published two studies comparing 30% 1RM versus 80% 1RM leg extensor training (Jenkins et al., 2016, 2017). Results from both studies suggest that higher-load training elicited greater strength increases due to neural factors. Jessee et al. (2018) reported that unilateral training (4 sets to volitional failure) over an 8-week period resulted in greater strength adaptations for HL training (70% 1RM/no blood flow restriction) than low-load conditions with or without blood flow restriction. Furthermore, Schoenfeld and colleagues reported increased barbell back squat strength with lower (30–50% 1RM) and higher-load (70–80% 1RM) training, with higher-load training resulting in greater strength adaptations (Schoenfeld et al., 2015). When considering our findings in the context of these studies, it seems plausible that training at 60–90% 1RM over shorter-term periods may elicit similar strength alterations with certain strength tests; in this case the leg press and isokinetic dynamometer. However, given that leg extensor 1RM values increased more so with HL versus HV training and as implicated in prior research (Spitz et al., 2020), it also remains possible that strength adaptations for certain exercise tests that are practiced through higher-load training may also be optimized.

A novel aspect of the current study was to compare how HL versus HV training affected molecular markers from muscle biopsies. This interrogation was prompted by select literature suggesting that a disproportionate increase in non-contractile proteins in myofibers may occur following high-volume resistance training. However, as reviewed by Jorgenson and colleagues, several studies have shown that mechanical-load induced skeletal muscle hypertrophy is largely attributed to proportional increases in the contractile and non-contractile elements of the myofiber (Jorgenson et al., 2020). In the current study, no significant

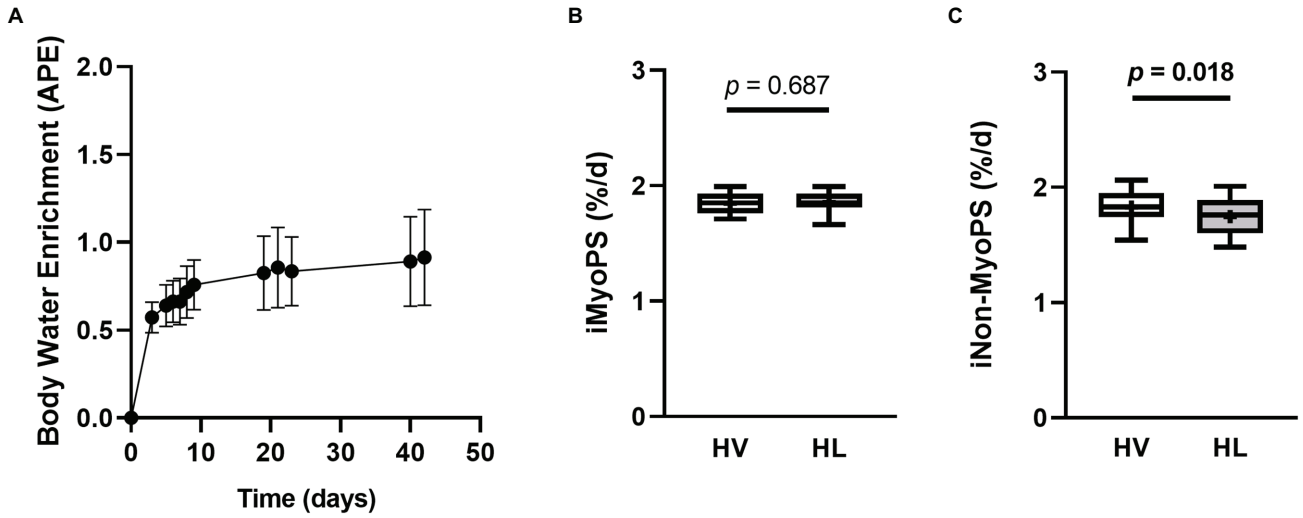

FIGURE 6 | Six-week Integrated Myofibrillar and Non-Myofibrillar Protein Synthetic Rates. (Panel **A**) shows D_2O enrichment from saliva analysis for 12 participants (means ± SD values). Data are presented as box and whiskers plots including median (central horizontal line), 25th and 75th percentile (box), minimum and maximum values (vertical lines), and mean values (cross) for iMyoPS (panel **B**) and iNon-MyoPS (panel **C**). No significant differences were observed for iMyoPS between conditions. iNon-MyoPS was significantly lower in the HL condition as compared to the HV condition. Abbreviations: HV, high-volume; HL, high-load; iMyoPS, integrated myofibrillar protein synthesis; iNon-MyoPS, integrated non-myofibrillar protein synthesis.

changes in the relative protein abundances of actin and MyHC were observed in either condition. Prior to discussing the implications of these data, it is important to understand the logistics of the contractile protein assay used herein, and readers are referred to a methods paper as well as a recent review on the topic from our laboratory for further details (Roberts et al., 2020a,b). In the presence of muscle hypertrophy (e.g., increases in VL mCSA), if sarcoplasmic protein concentrations and the relative abundances of contractile proteins remain unaltered from pre-to-post training then this likely indicates a proportional expansion of myofibril and non-myofibril components during growth; this being a phenomenon we have termed as "conventional hypertrophy" (Roberts et al., 2020a). On the other hand, if values, and in particular the relative abundances of myofibril proteins decrease from pre-to-post training then this indicates a "dilution" effect wherein hypertrophy occurs in the midst of sarcoplasmic (or fluid) expansion. Although our data largely imply conventional hypertrophy occurred with HV training, a handful of studies exist showing that a disproportionate increase in non-contractile proteins and cellular spacing may occur following months to years of resistance training (Penman, 1969; MacDougall et al., 1982; Toth et al., 2012; Meijer et al., 2015). Recently, our laboratory has reported decreases in the relative abundances of MyHC and actin protein abundances per mg of dry tissue weight following 6 weeks of extremely high-volume resistance training in previously trained college-aged men (Haun et al., 2019a). We previously posited that this was reflective of sarcoplasmic hypertrophy. Our laboratory subsequently reported that small (but significant) decrements occurred in actin protein abundance in previously trained college-aged males that partook in a 10-week low-volume, high-load training paradigm (Vann et al., 2020), and again we interpreted this as being reflective of sarcoplasmic hypertrophy. When considering the findings from both studies, we hypothesized that HV training in those with prior training experience might facilitate sarcoplasmic hypertrophy, whereas HL training may facilitate proportional accretion of contractile and non-myofibrillar proteins with whole-muscle hypertrophy (i.e., conventional hypertrophy). Although our current data disagree with prior findings from Haun et al., it is important to note the key differences that exist between the high-volume components of each study. In particular, Haun et al. used a 6-week intervention starting at 10 sets of 10 repetitions per week (for each exercise) and finishing with 32 sets of 10 repetitions per week where loads were standardized at 60% 1RM (Haun et al., 2018). The current study started the HV leg at 10 sets of 10 repetitions per week (split between two exercises) at week 1 and finished with 20 sets of 10 repetitions per week at week 6 where loads were standardized at 60% 1RM. Thus, although the HV leg was exposed to more training volume compared to the HL leg herein, the HV leg did not experience nearly the amount of volume as both legs incurred in the study by Haun et al. Moreover, the total training volume data in **Figure 1** indicates that the HV leg was only exposed to ~11% more volume compared to the HL leg. We speculate that similar molecular adaptations between legs may have been due a relatively small difference in total training volume between legs throughout the duration of the study.

Despite the null findings discussed above, it is intriguing that HV training increased iNon-MyoPS rates versus HL training. This partially supports the notion that HV training may affect the non-myofibril protein pool more so than HL training. It is difficult to determine mechanisms associated with these observations given that time course biopsies were not procured to examine molecular signaling pathway differences between legs acutely following a single HV versus HL bout. We and others

have shown that mTORC1 signaling markers, as well as the expression of mRNAs associated with skeletal muscle hypertrophy, are largely similar acutely following a HV versus LV training bout (Burd et al., 2010; Haun et al., 2017). Burd and colleagues also demonstrated that sarcoplasmic protein synthesis rates were elevated 24 h following a single HV versus HL exercise bout, and MAPK signaling was transiently elevated 4 h following the HV versus HL bout. While speculative, it may be possible that HV training herein stimulated MAPK signaling following each exercise bout more so than HL training, and this led to greater increases in iNon-MyoPS rates in the former condition. This hypothesis is supported by limited *in vitro* work demonstrating MAPK inhibition reduces protein synthesis rates (Servant et al., 1996; Kelleher et al., 2004). HV training may also increase intracellular calcium levels in a transient fashion more so than HL training, and heightened intracellular calcium levels have been shown to increase MAPK signaling (White and Sacks, 2010). These indirect lines of evidence lead to a hypothetical model where HV training, through elevated intracellular calcium concentrations and MAPK signaling, lead to greater increases in sarcoplasmic protein synthesis (i.e., iNon-MyoPS) rates relative to HL training. However, it has not been determined if elevated MAPK signaling in skeletal muscle leads to preferential increase in sarcoplasmic, versus myofibrillar, protein synthesis rates. Thus, this potential mechanism requires further investigation.

As with many studies examining the effects of training interventions, the present study is limited due to a small sample size. The procurement of skeletal muscle tissue *via* percutaneous muscle biopsy inherently has a finite tissue yield. We lacked an adequate amount of tissue to perform histology as we have done in the past with HV and HL training paradigms. Moreover, we recently developed a method to discriminate cell area occupied by myofibrils in type I and II fibers (Ruple et al., 2021), and this (along with tracking changes in type I and II fiber cross-sectional areas) would have added extraordinary insight to the current dataset. Data related to leg fluid shifts (e.g., leg segmental bioelectrical impedance spectroscopy or BIS) or tissue fluid content (e.g., lyophilization and comparing wet and dry tissue masses) were also not performed herein due to logistical constraints. Again, this remains an unresolved limitation. While protein synthesis rates were measured herein, it is notable that muscle protein breakdown rates were not assessed. The former are commonly measured, whereas the latter are rarely measured given the technical challenges that are often cited [reviewed in (Tipton et al., 2018)]. We speculate that protein breakdown rates are likely volume-dependent, and over longer time courses (i.e., > 6 weeks), this may affect phenotype outcomes given that net protein balance would be higher in HL versus HV training. However, no data exist supporting this contention, and this needs to be formally assessed. In spite of collecting training volume throughout the course of the study, we lack time under tension data and this would have been insightful to include in the current dataset. In spite of this limitation, Jenkins et al. (2017) have shown that individuals performing a similar type of unilateral HV training accumulated approximately three times the amount of time under tension relative to the HL-trained leg over a 6-week period. Thus, while we lack these data, we suspect that our participants experienced time under tension stimuli between legs. A final limitation of the current study is the length of training as well as our programming. With regard to the former, previous literature has shown 3–6 weeks of resistance training increases measures of hypertrophy in untrained to recreationally trained men (Seynnes et al., 2007; DeFreitas et al., 2011; Haun et al., 2018). In this regard, we posit that the training status of the cohort in the current study may have precluded our ability to detect any meaningful training adaptations over the 6-week training period. With regard to programming, we contend that a strength includes the real-world applicability; namely, HV and HL load progressions would likely follow similar patterns in recreational gym-goers. However, limitations to our approach include *a priori* programming being a bit arbitrary as well as weekly volume loads being more accelerated in the HV versus HL condition.

In conclusion, HV training elicited VL hypertrophy, whereas HL training resulted in a greater increase in leg extension strength. The current data challenge our prior muscle-molecular findings given that no alterations were observed in myosin heavy chain and actin protein abundances following either training protocol. However, the current iNon-MyoPS findings suggest some muscle-molecular differences exist between HV and HL training and warrant further research.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Auburn University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CV, SP, and MR devised the project aims and outcomes, and DB, JMcD, and KY provided critical insight. MR, SP, KS, and PA devoted significant resources to project outcomes. CV, CS, SO, MS, CH, MR, NM, BR, JMcl, and JMcK were involved with multiple aspects of data collection and analyses. AB, RB, and KY developed methods for MRI analyses. CV, SP, and MR primarily drafted the manuscript, and all authors edited the final manuscript for submission.

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Effects of Cardiac Telerehabilitation During COVID-19 on Cardiorespiratory Capacities in Patients With Coronary Artery Disease

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Background: The COVID-19 pandemic led to the closure of most cardiac therapy centers. One of the solutions was to adapt the existing cardiac rehabilitation (CR) program in an institute to a remote approach offered by home-based telerehabilitation. The aim of this study was to measure the cardiorespiratory effects of telerehabilitation compared to conventional center-based CR.

Methods: Patients were assigned to two 3-week CR programs: telerehabilitation and conventional center-based CR. The telerehabilitation group wore a connected watch to monitor heart rate (HR) and gave their perception of effort according to a modified Borg scale. The exercise training (four sessions/week) consisted of 1-h aerobic endurance and strength training session at the target HR zone determined by results based on cardiopulmonary exercise test (CPET) and perception of effort, respectively. The exercise protocol was the same for conventional CR participants except the duration of session that lasted 2 h instead of one. The week before and after the training program, peak oxygen uptake (VO_2 peak), oxygen uptake at first ventilatory threshold (VO_2 at VT_1), peak workload, percent of predicted maximum HR, and the absolute differences in HR and systolic blood pressure between maximum and recovery at 1 and 3 min were measured using a CPET. A two-way ANOVA with one repeated measure and one independent factor was performed.

Results: Fifty-four patients (mean age: 61.5 ± 8.6 years, 10 women) equally split in the two groups were included in this experiment. A significant increase was observed in both groups on VO_2 peak (telerehabilitation: 8.1 ± 7.8% vs. conventional: 10.1 ± 9.7%, $p < 0.001$), VO_2 at VT_1 (telerehabilitation: 8.8 ± 4.4% vs. conventional: 7.3 ± 19.0%, $p = 0.02$) and peak workload (telerehabilitation: 16.6 ± 18.9% vs. conventional: 17.2 ± 7.0%, $p < 0.001$) after the 3-week telerehabilitation and conventional CR, respectively. No significant difference was noticed between both groups.

Conclusion: A 3-week exercise program improved patients' cardiorespiratory fitness. Telerehabilitation was as effective and represents a safe alternative CR program during the COVID-19 period. In the future, this approach could facilitate the continuity of care for patients unable to participate in center-based CR.

Keywords: telerehabilitation, physical activity, coronary artery disease, COVID-19, cardiorespiratory fitness, exercise training, cardiac rehabilitation

INTRODUCTION

Coronary artery disease, one of the most common cardiovascular (CV) diseases, accounts for a large proportion of deaths worldwide (Roth et al., 2017). Secondary prevention consists of decreasing as much as possible all CV risk factors in order to avoid the recurrence of cardiac events (Ades, 2001). Although preventive drug therapy is a priority after myocardial infarction, patients suffer from neuromuscular deconditioning, dyspnea, and poor quality of life (Cavalheiro et al., 2021). To restore or increase physical abilities and reduce CV risk, a cardiac rehabilitation (CR) program is required after myocardial infarction (Iliou et al., 2015). A predominant part of CR is physical exercise (Balady et al., 2007; Price et al., 2016; Ambrosetti et al., 2021). Nevertheless, a holistic management strategy is recommended (Balady et al., 2007). In addition to training, programs provide behavioral changes and lifestyle therapeutic education on coronary artery disease risk factor management and psychological assistance (Ambrosetti et al., 2021). The objective for active subjects is to regain their place in society and for older persons to maintain their independence (Pavy et al., 2012; Iliou et al., 2015). The benefits of CR are actually well described in the literature (Wisløff et al., 2007; Scrutinio et al., 2009; Piepoli et al., 2016). These include increased functional, muscular and cardiopulmonary capacities and also greater control of CV risk factors by adopting a better lifestyle, such as smoking cessation, a heart-healthy diet, and stress management (Scrutinio et al., 2009).

In December 2019, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) was discovered in China (Pericàs et al., 2020). Three months later, the World Health Organization declared the SARS-CoV-2 disease (COVID-19) a pandemic (Cucinotta and Vanelli, 2020). This virus spread very rapidly throughout the world and caused many economic, social, and health consequences (Pericàs et al., 2020). The latest repercussion led to the saturation of hospital services which were forced to close rehabilitation centers (Taylor et al., 2021). Therefore, in many institutes or specialized CR clinics, the programs were partially interrupted or suspended according recommendations of scientific and public health authorities (Haute Autorité de Santé, 2020; Ministère des Solidarités et de la Santé, 2020). An alternative CR delivery strategies should be used to remedy these barriers.

One of the solutions was to adapt the existing center-based CR program to a remote approach offered by telerehabilitation (Chan et al., 2016). Telerehabilitation is medical technology-assisted delivery model to provide healthcare services between healthcare professionals and home-based patients (Silva-Cardoso et al., 2021). This therapy includes remotely supervised exercise training and collective or individual cardiac prevention and management heart disease meetings by videoconference (Scherrenberg et al., 2021). To demonstrate the effectiveness of cardiac telerehabilitation, some studies have focused on quality of life, mainly assessed by questionnaires (Gooley et al., 2021; Taylor et al., 2021). Patients also suffer from physical limitations such as shortness of breath, lack of fitness, and fatigue during exercise. Hence, one of the priorities is to improve physical capacity.

The aim of this study was to investigate and measure the effects of home-based CR compared to conventional center-based CR on cardiorespiratory functions in coronary artery disease patients. We hypothesized that telerehabilitation would be as effective as traditional CR realized in a conventional hospital setting.

MATERIALS AND METHODS

Inclusion Criteria

Firstly, we controlled the low risk of patients experiencing an adverse event during CR. We based this on their postsurgical or medical intervention complications (no complications), asymptomatic, no ventricular arrhythmias, no heart failure, no left ventricular dysfunction, and test results as CV response during the cardiopulmonary exercise test (CPET) before the CR.

All participants had to be over 18 years old, had acute coronary syndrome treated within the last 6 months, had received coronary revascularization by percutaneous coronary intervention (angioplasty with stent implantation) or surgical operation (coronary artery bypass grafting). The exclusion criteria were uncontrolled ventricular rhythm disorders and articular or respiratory diseases.

In addition to meeting the above inclusion criteria, patients who followed the home-based CR program were requested to have internet access and an indoor exercise bike at home but were excluded if they had significant deconditioning that required on-site supervision.

All volunteers provided written informed consent before beginning the experimentation. The study was in accordance with the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the university hospital of Saint-Etienne, France (IRBN1022021/CHUSTE).

Experimental Design

The non-randomized investigation was conducted from September 2020 to March 2021 in a single rehabilitation center

(Saint-Etienne University Hospital, Saint-Etienne, France). CR was offered as soon as possible after 8 d of coronary intervention (Corbett et al., 2021). Eligible patients were assigned to two groups: home-based CR (telerehabilitation) and traditional center-based CR (conventional CR). Each group was composed of 4 patients per CR cycle (**Figure 1**). This multidisciplinary medical and paramedical care was offered to patients for 3 weeks. Exercise training represented an essential part of this CR since participants practiced four consecutive sessions of physical activity per week. The last day of the week was devoted to a group therapeutic education workshop. Before starting the intervention, patients were interviewed by a specialist nurse about their CV risk factors and personal objectives. According to their needs and in addition to exercise program and collective meetings, a medical, diet, psychology and/or tobacco expert could be individually proposed by videoconference or face-to-face according to the method of rehabilitation (McDonagh et al., 2021). At the end of CR, recommendations and guidance were given by physical activity specialist to maintain exercise training independently.

Physiological Assessments

Elementary anthropometric measurements such as body mass and body mass index (BMI) were assessed before and after the 3-week CR. BMI was calculated by dividing body mass (in kg) by the square of body height (in m).

An initial transthoracic echocardiography was carried out to define the left ventricular ejection fraction. Patients repeated this test only if an abnormality was detected the first time.

The week before and after the intervention program, the patients performed in hospital a maximum CPET on an electronically braked ergocycle (Vyntus CPX, CareFusion, San Diego, CA, United States). Two experienced medical doctors specialists in exercise physiology (DH, FR) used a ramp-type protocol, consisting of 2 min warming up to 10 W, followed by a 10-W progressive increment every minute until exhaustion (Writing Committee et al., 2012). The automated metabolic

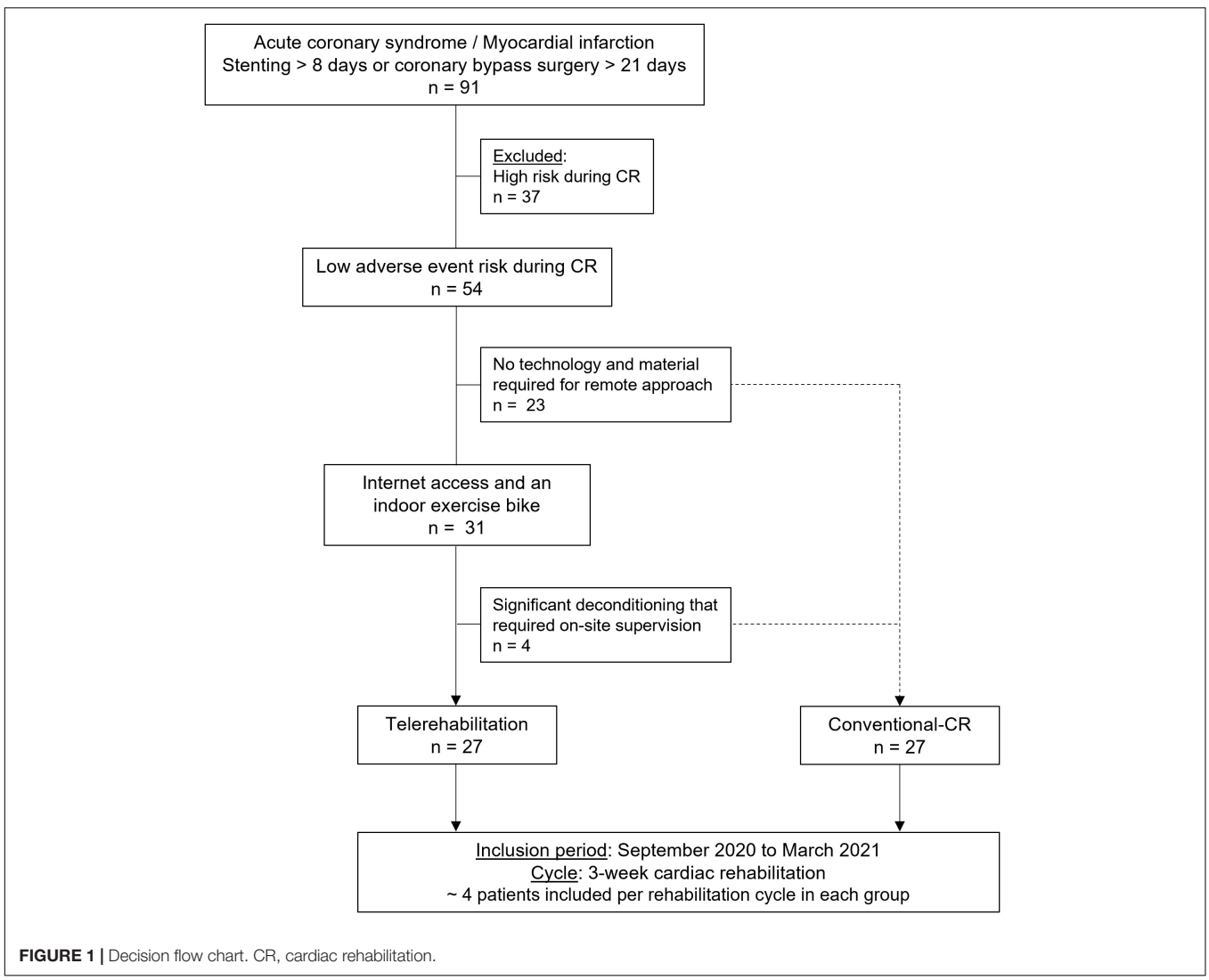

FIGURE 1 | Decision flow chart. CR, cardiac rehabilitation.

system analyzed respiratory gas exchange including oxygen uptake (VO_2). The average temperature and relative humidity in testing room were 21°C and 24%, respectively. Peak oxygen uptake (VO_2 peak) was determined as the mean value of the last 30 s of exercise. VO_2 at the first ventilatory threshold (VO_2 at VT_1) and the peak workload (PWL) were evaluated to illustrate the achievable efforts without dyspnea such as carrying out tasks of daily life without difficulty and the duration of the exercise, respectively (Writing Committee et al., 2012).

Participants were monitored continuously with electrocardiography (ECG). Hence, the heart rate (HR) was recorded. Systolic blood pressure (SBP) was measured manually by an experienced nurse using a random-zero sphygmomanometer when the participant was sitting on the cycle ergometer every 2 min during exercise, and at 1 and 3 min recovery from exercise.

More specifically, we focused on the percentage of the predicted maximum HR (%HRpeak = the ratio of peak measured HR and peak predicted HR). HR recovery (HRR) was measured at 1 and 3 min following peak HR during exercise. Peak HR was identified as the maximum HR during the exercise protocol. HRR 1 min (ΔHRR 1 min) was defined as the absolute change from peak HR to HR 1 min post peak HR (HRR1 = peak HR – HR at 1 min post peak HR) (Shetler et al., 2001; Arduini et al., 2011). Similarly, HRs of recovery 3 min (ΔHRR 3 min) was calculated as the absolute change from peak HR to HRs 3 min post peak HR (Peçanha et al., 2017). Maximal SBP was the highest value achieved during the exercise ECG. SBP recovery deltas between maximal and at 1-min (ΔSBP 1 min) and 3-min (ΔSBP 3 min) recovery from exercise were also measured.

Cardiac Telerehabilitation

The telerehabilitation process is described in **Figure 2**. Medical and paramedical teleconsultations were carried out by videoconference. Before the start of exercise training, the personal coach checked the correct functioning of network connection and presented the exercise training program. Patients performed 1-h physical activity sessions at home using digital technology available to them (computer or tablet). The training session consisted of 30 min of cycling and 20 min of strength training. The physical exercise program, the frequency and the intensity were the same for the conventional CR group, except the duration of training session lasted twice as long. To ensure safety and adapt the intervention for each participant, they wore

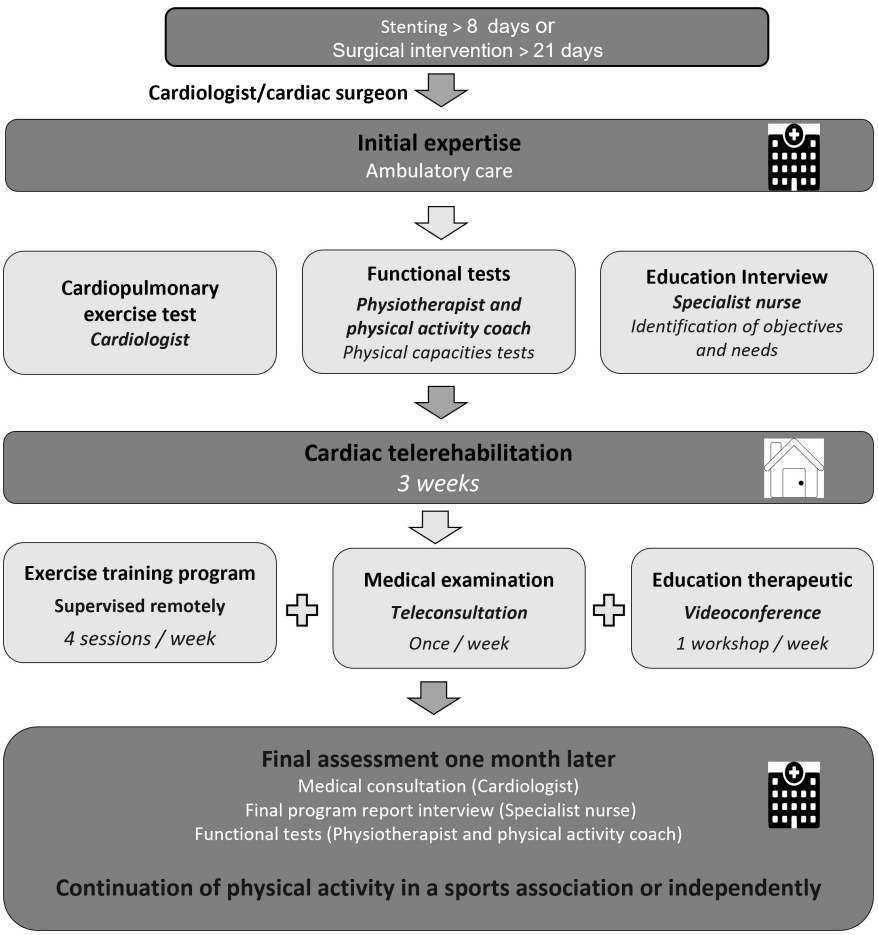

FIGURE 2 | Experimental design of telerehabilitation intervention.

a connected watch (Dona Care, Life Plus, Versailles, France) to monitor the HR and assess the number of steps per day during the program. They gave their perceived exertion using a modified Borg scale from 0 (no sweating, no shortness of breath, no exertion at all) to 10 (extremely hard exertion). In addition, before each training session, the adapted physical activity coach asked the patients about their fatigue or if they had pains.

Therapeutic Education Meetings

Once a week, a therapeutic education meeting was organized. These educational and recreational thematic workshops allowed to deal with the difficulties linked to a patient's pathology and to discuss the main issues related to their cardiac event (Janssen et al., 2013; Pavy et al., 2013). Thanks to these meetings, participants developed their knowledge of their CV disease and the options to reduce their CV risk factors. A first workshop was led by a cardiologist on cardiac medical intervention and pharmacological treatment. A dietician coordinated the second seminar relative to heart-healthy nutrition. The last topic by a physiotherapist was on CPET and physical activity.

Physical Exercise Program

Figure 3 illustrates the physical training program. The first half of the training session on a cycle ergometer was devoted to aerobic endurance. The intensity of this prolonged submaximal exercise was constant and adapted accordingly to the CPET results of each patient. More specifically, the HR at VT_1 defined the intensity of endurance training session (Ambrosetti et al., 2021). The physical exercise session included a warm-up period, a CV training period, and a cool down phase. In addition, an aerobic interval training was gradually offered as an alternative to continuous endurance exercise. The dynamic resistance training consisted of overall muscle strength training or focused more on the lower or upper limbs.

Statistical Analysis

Statistical analyses were performed using JASP (version 0.15). All data were reported as mean ± standard deviation (SD). We checked distribution of normality and the homogeneity of variances with the Shapiro-Wilk and Levene tests, respectively. The effect of the training program on cardiorespiratory parameters according to CR approach was evaluated using two-way repeated measures ANOVA, i.e., CR groups (telerehabilitation vs. conventional center-based) x time (pre-post). Where a significant interaction difference occurred, Tukey's *post hoc* analyses were performed. For all statistical comparisons, the level of significance was set at $p < 0.05$.

RESULTS

Fifty-four patients were recruited in this study (mean age: 61.5 ± 8.6 years, 11% women). Half of the patients completed conventional CR in hospital ($n = 27$). The second half followed the telerehabilitation program ($n = 27$). CV risk factors, cardiac

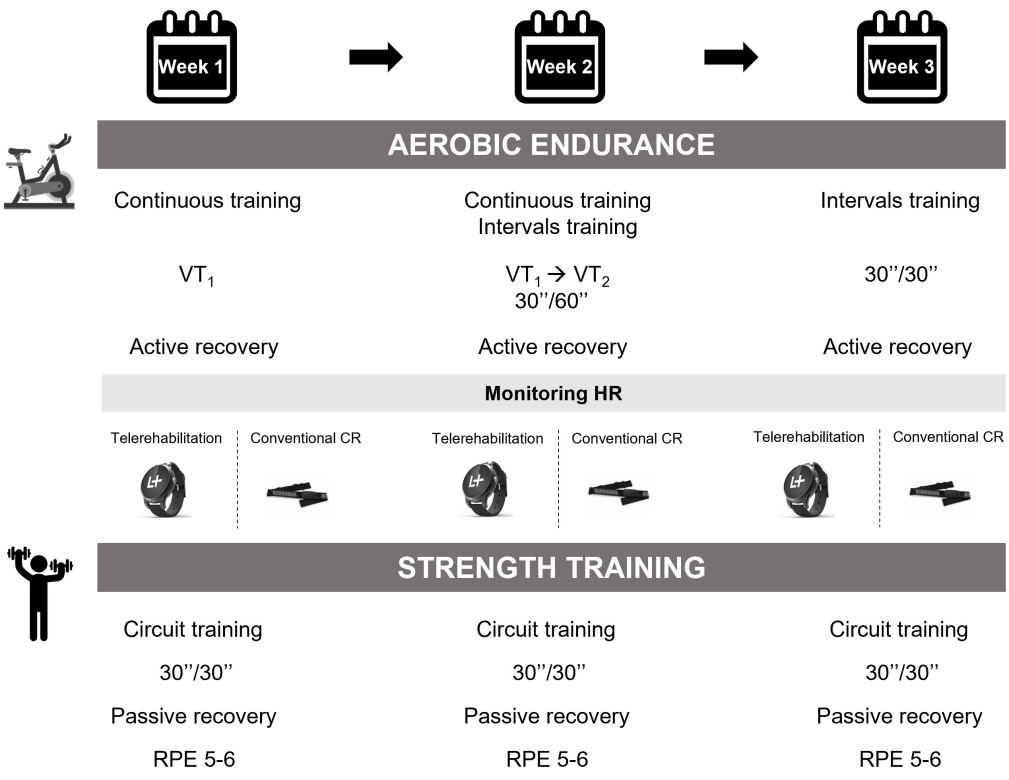

FIGURE 3 | Physical exercise intervention. VT, ventilatory threshold; HR, heart rate; CR, cardiac rehabilitation; RPE, rating of perceived exertion.

TABLE 1 | Baseline characteristics of the participants.

	Telerehabilitation (n = 27)	Conventional CR (n = 27)	p-value
Age (years)	63.3 ± 8.1	59.7 ± 9.1	0.13
Females	4 (15)	6 (22)	0.50
Distance from hospital (km)	41.2 ± 30.9	31.8 ± 22.4	0.36
Body mass (kg)	80.3 ± 17.3	72.1 ± 16.9	0.08
BMI (kg m^{-2})	27.0 ± 4.5	25.2 ± 4.6	0.16
LVEF (%)	54.9 ± 7.5	54.7 ± 9.5	0.53
LVEF < 50%	4 (15)	7 (26)	0.32
Beta blocker	23 (85)	25 (93)	0.40
Aspirin	25 (93)	27 (100)	0.16
Double APT	26 (96)	26 (96)	1.00
Statin	26 (96)	27 (100)	0.34
ACEI/ARB	23 (85)	27 (100)	0.33
High blood pressure	8 (30)	7 (26)	0.77
Dyslipidemia	8 (30)	8 (30)	1.00
Diabetes	2 (7)	0 (0)	0.16
Sleep apnea			
Total	6 (22)	9 (33)	0.37
Whose detect during CR	2 (33)	5 (56)	
Smoking status			0.14
Never	10 (37)	5 (19)	
Former	17 (63)	22 (81)	
Current	2 (7)	4 (15)	
Coronary artery intervention			
Medical	3 (11)	0 (0)	0.24
Angioplasty (stenting)	21 (78)	23 (85)	0.22
CABG	3 (11)	4 (15)	0.70

Values are mean ± SD or n (%). CR, cardiac rehabilitation; BMI, body mass index; CABG, coronary artery bypass grafting; APT, Anti-platelet; ACEI/ARB, angiotensin-converting enzyme inhibitor/angiotensin receptor blocker; LVEF, left ventricular ejection fraction.

TABLE 2 | Effect of 3-week exercise training on cardiorespiratory parameters between telerehabilitation and conventional cardiac rehabilitation program.

	Telerehabilitation (n = 27)		Conventional CR (n = 27)		Main time effect	Main group effect	Interaction effect
	Pre	Post	Pre	Post			
VO_2 peak (L min^{-1})	1.6 ± 0.4	1.7 ± 0.5***	1.4 ± 0.4	1.5 ± 0.4***	<0.001	0.124	0.984
VO_2 peak (ml min^{-1} kg^{-1})	20.0 ± 4.7	21.6 ± 5.0***	19.5 ± 4.3	21.5 ± 4.7***	<0.001	0.844	0.678
VO_2 at VT_1 (ml min^{-1} kg^{-1})	13.7 ± 3.3	14.9 ± 3.5*	12.9 ± 3.0	13.8 ± 3.6*	0.023	0.222	0.771
PWL (W)	123.9 ± 36.8	144.4 ± 43.7***	111.0 ± 34.1	128.1 ± 35.8***	<0.001	0.156	0.419
HR peak (bpm)	123.7 ± 22.6	127.9 ± 23.0***	118.5 ± 18.9	130.9 ± 27.0***†	<0.001	0.858	0.031
%HR peak (%)	79.7 ± 14.4	82.5 ± 14.8***	72.3 ± 18.3	82.8 ± 15.7***†	<0.001	0.379	0.038
SBP max (mmHg)	176.3 ± 26.4	179.9 ± 29.3	169.8 ± 22.8	173.0 ± 24.5	0.266	0.304	0.947
ΔHRR 1 min (bpm)	17.9 ± 7.4	19.9 ± 8.2*	13.0 ± 12.2	18.0 ± 10.5*	0.035	0.129	0.361
ΔSBP 1 min (mmHg)	−1.5 ± 15.2	5.8 ± 22.0	4.7 ± 21.7	2.7 ± 22.1	0.530	0.714	0.277
ΔHRR 3 min (bpm)	34.0 ± 12.7	39.7 ± 13.9***	27.3 ± 9.4	40.8 ± 16.4***†	<0.001	0.399	0.018
ΔSBP 3 min (mmHg)	18.6 ± 19.0	20.4 ± 23.7	15.2 ± 18.7	18.5 ± 22.1	0.518	0.846	0.552
Duration CPET (min)	9.1 ± 2.6	10.3 ± 2.5**	8.6 ± 3.0	9.5 ± 2.6**	0.007	0.300	0.691

CR, cardiac rehabilitation; VO_2 peak, peak oxygen uptake; VO_2 at VT_1, oxygen uptake at the first ventilatory threshold; PWL, peak workload; W, watts; HR, heart rate; HR peak, peak heart rate; %HR peak, ratio of peak measured HR out of peak predicted HR; bpm, beats per minute; SBP, systolic blood pressure; mmHg, millimeters of mercury; ΔHRR 1 min, difference between maximum heart rate and heart rate after 1 min of recovery; ΔHRR 3 min, difference between maximum heart rate and heart rate after 3 min of recovery; CPET, cardiopulmonary exercise test.

**Significantly difference between baseline ($p < 0.05$).*

***Significantly difference between baseline ($p < 0.01$).*

****Significantly difference between baseline ($p < 0.001$);*

† Time × method of cardiac rehabilitation interaction.

intervention, medical treatment and baseline characteristics of both groups are described in **Table 1**. No significant differences between telerehabilitation and conventional CR groups were observed at baseline. No adverse events were occurred during the CR period and all participants completed the exercise intervention.

The results are presented in **Table 2**. Except for SBP parameters, we noticed a significant increase of all variables within both groups, while there was no significant difference between groups. VO_2 peak and VO_2 at VT_1, are represented in **Figure 4**. The same conclusions were applicable for these physiological variables.

There were both time effect × CR method interaction for HR peak ($p = 0.031$), %HR peak ($p = 0.038$) and ΔHRR 3 min ($p = 0.018$) values. Tukey *post hoc* tests showed statistically significant time difference (pre vs. post) in conventional CR group for these two variables. In addition, we observed a significant difference between the conventional group at baseline and telerehabilitation group after 3-week training program only for ΔHRR 3 min parameter.

DISCUSSION

This study examined the cardiorespiratory benefits after a 3-week telerehabilitation compared to conventional CR in hospital. The main findings of this research were an improvement of VO_2 peak, VO_2 at VT_1 and PWL, irrespective of the CR strategy.

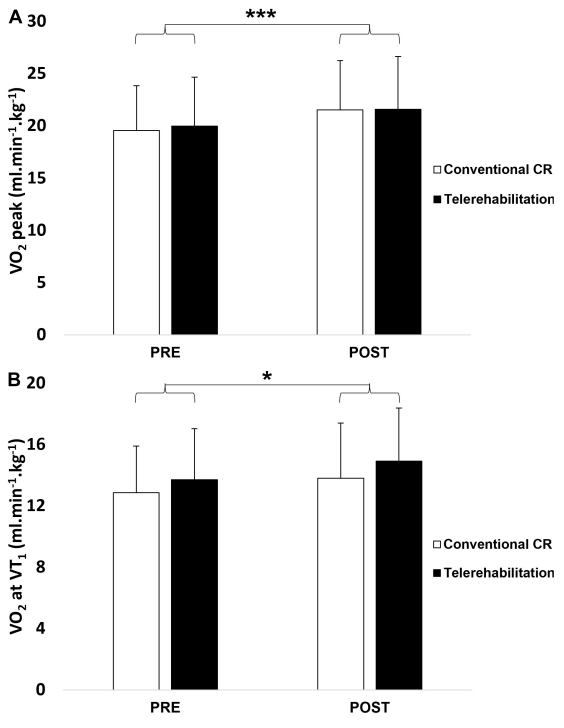

FIGURE 4 | Peak oxygen uptake **(A)** and oxygen uptake at the first ventilatory threshold **(B)** before and after a 3-week exercise program between telerehabilitation (black bars) and conventional CR (white bars) groups. CR, cardiac rehabilitation; VO_2 peak, peak oxygen uptake; VO_2 at VT_1, oxygen uptake at the first ventilatory threshold. *Significantly difference between baseline ($p < 0.05$); ***significantly difference between baseline ($p < 0.001$).

Enhanced cardiorespiratory fitness is one of the main objectives after acute coronary syndrome (Price et al., 2016; Pelliccia et al., 2021). In our experimentation, patients who followed a telerehabilitation program increased VO_2 peak, VO_2 at VT_1 and PWL, by 8.1, 8.8, and 16.6%, respectively. As expected, we achieved similar physiological improvements as the conventional center-based CR group since they increased by 10.1, 7.3, and 17.1%, respectively. The duration of the interventional CR strategy was shorter than other investigations (i.e., 3 weeks vs. 8–12 weeks) (Kraal et al., 2014; Batalik et al., 2020). This was due to a more intensive and frequent exercise training with four physical activity sessions per week. Therefore, the results were similar to several other studies (Karapolat et al., 2009; Kraal et al., 2014; Vysoký et al., 2015; Batalik et al., 2021).

Most studies which used telerehabilitation as an add-on to center-based CR or an alternative for conventional CR suggested that it was a safe and well tolerated approach for patients (Kraal et al., 2014; Taylor et al., 2015; Kikuchi et al., 2021). It was also the case in our study since all patients satisfactorily achieved this CR program and no adverse events were reported. Thanks to current technological advances (smartwatch, accelerometers, pedometers), it is easier to collect and record physiological constants in order to ensure the safety in home environment and adapt the physical training intervention (Frederix et al., 2015; Scherrenberg et al., 2021).

Moreover, one of the major issues of conventional center-based CR is the low adherence (Zhang et al., 2018). Telerehabilitation could increase the number of potential participations (Batalik et al., 2021). In France, despite reimbursed coverage and well organized post-infarction care, only 30% of patients benefit from a CR program. Indeed, remote technology assistance can overcome accessibility barriers such as socioeconomic and travel difficulties, professional constraints, geographical distance of CR center. The latter two obstacles represent the major limiting factors in France. Hence, telerehabilitation may be an additional feasible and effective solution due to better integration of CR into the daily life of patients.

In addition, Avila et al. (2020) assessed cardiorespiratory and strength variables after one-year follow-up and highlighted the preservation of exercise capacity for the telerehabilitation group.

The secondary outcome showed a time effect for all HR parameters as above cardiopulmonary values and an interaction (time × CR strategy) for the HR peak, %HR peak and ΔHRR 3 min. Traditional CR remains a reference and it appears to be more effective on the HR variables since a greater improvement of these findings was observed in conventional CR programs compared to telerehabilitation. The postexercise HR response recovery could serve to assess autonomic nervous system activity. The recovery HR reflects autonomic nervous system activity after the exercise phase and more specifically the reactivation of parasympathetic tone after cessation of effort (Peçanha et al., 2017). A slow decline in HR after exercise suggests non-optimal parasympathetic and orthosympathetic balance or cardiac autonomic impairment. It is also a strong independent

marker of CV mortality (Lipinski et al., 2004; Jouven and Courbon, 2005). Physical exercise training in CR optimizes the recovery kinetics postexercise. This is associated with improved findings (Jolly et al., 2011).

Although some HR variables which reflect autonomic nervous system components indicated lower improvements with the telerehabilitation program and suggested to favor a center-based CR, the various scientific evidence show that remote technology services can be proposed to meet the objectives of CR.

Strengths and Limitations

We emphasized that our investigation is one of the first studies conducted in France on the use of cardiac telerehabilitation during the COVID-19 pandemic with objective physiological measurements (few self-reported parameters). In addition, the patients followed a holistic cardiac telerehabilitation, i.e., lifestyle counseling, clinical examinations and exercise training.

We should add an autonomic nervous system measurement to illustrate an improvement in the parasympathetic part and a decrease in the orthosympathetic system. One limitation of the present research study was the non-measurement of telerehabilitation long-term effects. The duration of training session was not the same between the two groups. Telerehabilitation performed the exercise training program without interruption, while patients who followed a standard center-based CR had longer rest period. Furthermore, this time included the changing room, general health checking, physiological measurements like oxygen saturation and blood pressure. Our investigation was not a randomized study, was in a single rehabilitation hospital center and a control group was absent. To validate these findings, we need to propose a multicenter randomized controlled trial.

CONCLUSION

Following a 3-week exercise intervention effectively improved cardiorespiratory capacities in coronary artery patients. This investigation showed that telerehabilitation might become a relevant alternative to conventional center-based CR. This innovative healthcare delivery method appears to be a feasible, tolerable, safe and cost-effective solution. In the future, this approach could facilitate the continuity of care for people who encounter geographical or social accessibility difficulties.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité d'Ethique du CHU de Saint-Etienne IRBN1022021/CHUSTE. The patients provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MF and DH contributed to the conception or design of the study. MF, FR, and DH contributed to the analysis and/or interpretation of data. MF drafted the manuscript. MB, PL, FR, and DH critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of work ensuring integrity and accuracy.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ventricular-Vascular Uncoupling in Heart Failure: Effects of Arterial Baroreflex-Induced Sympathoexcitation at Rest and During Exercise

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Autonomic alterations in blood pressure are primarily a result of arterial baroreflex modulation of systemic vascular resistance and cardiac output on a beat-by-beat basis. The combined central and peripheral control by the baroreflex likely acts to maintain efficient energy transfer from the heart to the systemic vasculature; termed ventricular-vascular coupling. This level of control is maintained whether at rest or during exercise in healthy subjects. During heart failure, the ventricular-vascular relationship is uncoupled and baroreflex dysfunction is apparent. We investigated if baroreflex dysfunction in heart failure exacerbated ventricular-vascular uncoupling at rest, and during exercise in response to baroreceptor unloading by performing bilateral carotid occlusions in chronically instrumented conscious canines. We observed in healthy subjects that baroreceptor unloading caused significant increases in effective arterial elastance (Ea) at rest (1.2 ± 0.3 mmHg/ml) and during exercise (1.3 ± 0.2 mmHg/ml) that coincided with significant increases in stroke work (SW) (1.5 ± 0.2 mmHg/ml) and (1.6 ± 0.2 mmHg/ml) suggesting maintained ventricular-vascular coupling. Heart Failure significantly increased the effect of baroreceptor unloading on Ea at rest (3.1 ± 0.7 mmHg/ml) and during exercise (2.3 ± 0.5 mmHg/ml) whereas no significant increases in stroke work occurred, thus signifying further ventricular-vascular uncoupling. We believe that the enhanced ventricular-vascular uncoupling observed during baroreceptor unloading only worsens the already challenged orthostatic and exercise tolerance and thereby contributes to poor exercise performance and quality of life for heart failure patients.

Keywords: arterial baroreflex, ventricular-vascular coupling (VVC), baroreceptor unloading, orthostatic hypotension, orthostatic intolerance

INTRODUCTION

Beat by beat control of blood pressure is crucial to the maintenance of cardiovascular performance. The primary mechanism by which blood pressure control occurs is through arterial baroreflex mediated changes in sympathetic and parasympathetic activity to modify heart rate and vascular resistance which thereby maintains blood pressure within normal ranges in response to changes in posture, exercise, and activation of other cardiovascular reflexes or local vascular responses (Melcher and Donald, 1981; Chen et al., 1991; Olivier and Stephenson, 1993; Iellamo et al., 1997; DiCarlo and Bishop, 2001; Joyner, 2006; Sheriff, 2006; Drew et al., 2008; Chen et al., 2010; Davis, 2011; Fadel, 2015).

In heart failure arterial baroreflex regulation is significantly altered. Most notably the ability of the baroreflex to increase blood pressure in response to a hypotensive stimulus is attenuated and this is primarily an effect of an attenuated ability to increase cardiac output due to depressed ventricular function (Chen et al., 1991; Dall'Ago et al., 1997; DiCarlo and Bishop, 2001; Kim et al., 2004; Kim et al., 2005a; Olivier and Stephenson, 1993; Osterziel et al., 1995; Thames et al., 1993; Wang et al., 1996). Furthermore, the range and gain of heart rate responses to baroreceptor unloading is significantly depressed whereas the overall change in vasoconstriction remains largely unaffected (Olivier and Stephenson, 1993; Osterziel et al., 1995; Kim et al., 2004; Joyner, 2006). Sympatho-inhibitory responses and spontaneous baroreflex control are also significantly attenuated engendering sympatho-excitatory responses with attenuated negative feedback control (Ferguson et al., 1992; Drew et al., 2008; Ichinose et al., 2008; Chen et al., 2010). Thus, alterations in baroreflex function are variable under cardiovascular pathologies and require an assessment of the output effects of the respective increases or decreases in baroreflex function and how they relate to overall cardiovascular efficiency. One such measure is an assessment of ventricular-vascular coupling that can be assessed by changes in arterial elastance and stroke work (Sunagawa et al., 1985; Mannozzi et al., 2020).

Optimal ventricular-vascular coupling is paramount to the maintenance of efficient cardiovascular performance at rest and during exercise (Sunagawa et al., 1983; Sunagawa et al., 1985; Burkhoff and Sagawa, 1986; Nozawa et al., 1988; Asanoi et al., 1989; Kelly et al., 1992a; Kelly et al., 1992b; Eichhorn et al., 1992; Hayashida et al., 1992; Little and Cheng, 1993; Kass, 2005; Chantler et al., 2008; Otsuki et al., 2008; Cote et al., 2013; Ky et al., 2013; Mannozzi et al., 2020). This relationship is a ratio of Effective Arterial Elastance (E_a) vs. ventricular maximal elastance (E_{MAX}). E_a is a vascular property that was originally derived by relating total peripheral resistance with systolic, and diastolic time periods, as well as the diastolic time decay constant to assess the load imposed by the vasculature on the left ventricle (Sunagawa et al., 1983; Sunagawa et al., 1985; Kelly et al., 1992a). Later this series of variables was simplified by Kelly et al. (1992a) for assessments of vascular load in humans to the relationship of end systolic pressure divided by stroke volume. Within that series of derivations another method for measuring E_a focusing on vascular parameters utilizes total vascular resistance divided by cardiac cycle (reference). This latter method showed limited bias and statistical difference from the fully derived method (Kelly et al., 1992a; Mannozzi et al., 2020). Either method is valid for assessing E_a and they have been used to define the state of ventricular-vascular across a wide range of settings and pathologies (Sunagawa et al., 1985; Asanoi et al., 1989; Little and Cheng, 1991; Kelly et al., 1992a; Redfield et al., 2005; Otsuki et al., 2006; Chantler et al., 2008; Otsuki et al., 2008; Cote et al., 2013; Faconti et al., 2017; Reddy et al., 2017; Mannozzi et al., 2020). To date no study has quantified the effect of baroreflex activation on ventricular-vascular coupling nor on the impact of heart failure on baroreflex mediated changes in ventricular-vascular coupling at rest and exercise. In this study we hypothesized that in normal subjects arterial baroreflex unloading at rest and during exercise would cause improved ventricular-vascular coupling to produce optimal blood flow and thereby likely allow for timely, appropriate corrections in blood pressure. In heart failure the alterations in baroreflex control of arterial elastance will lead to further worsening of the already uncoupled ventricular-vascular relationship due to an inability to improve stroke work in parallel with increases in E_a, and this likely contributes to orthostatic and exercise intolerance.

METHODS

Five (1 male 4 females) adult mongrel canines 18–25 kg were selected based on their willingness to walk on a motor driven treadmill at 3.2 km/hr with no incline. We have shown previously that gender does not impact cardiovascular responses to activation of the muscle metaboreflex (Laprad et al., 1999). Furthermore, previous studies have shown gender in young adults does not impact sympathetically mediated baroreflex responses elicited via hypotension (Kim et al., 2011; Fu and Ogoh, 2019) as was done per this study. All animals used in this study underwent a minimum fourteen-day laboratory and personnel acclimation period prior to any volitional exercise. All experimental and surgical protocols outlined in this study were approved and comply with the Wayne State University Institutional Animal Care and Use Committee (IUCAC) and National Institutes of Health Guide to the Care and Use of Laboratory Animals respectively.

Surgical Instrumentation

All anesthetic, analgesic and surgical procedures used in this study have been documented in previous studies (O'Leary, 1993; O'Leary and Augustyniak, 1998; Augustyniak et al., 2000; Hammond et al., 2000; Hammond et al., 2001; O'Leary et al., 2004; Kim et al., 2005a; Kim et al., 2005b; Augustyniak et al., 2006; Sala-Mercado et al., 2007; Ichinose et al., 2008; Coutsos et al., 2010; Kaur et al., 2016; Kaur et al., 2018; Mannozzi et al., 2020; Mannozzi et al., 2021). Briefly, all animals in this study underwent three separate surgical anesthetic events using standardized aseptic surgical techniques with a minimum of a 14-day recovery period between each procedure and prior to any experiments. Approximately 30 min prior to induction of

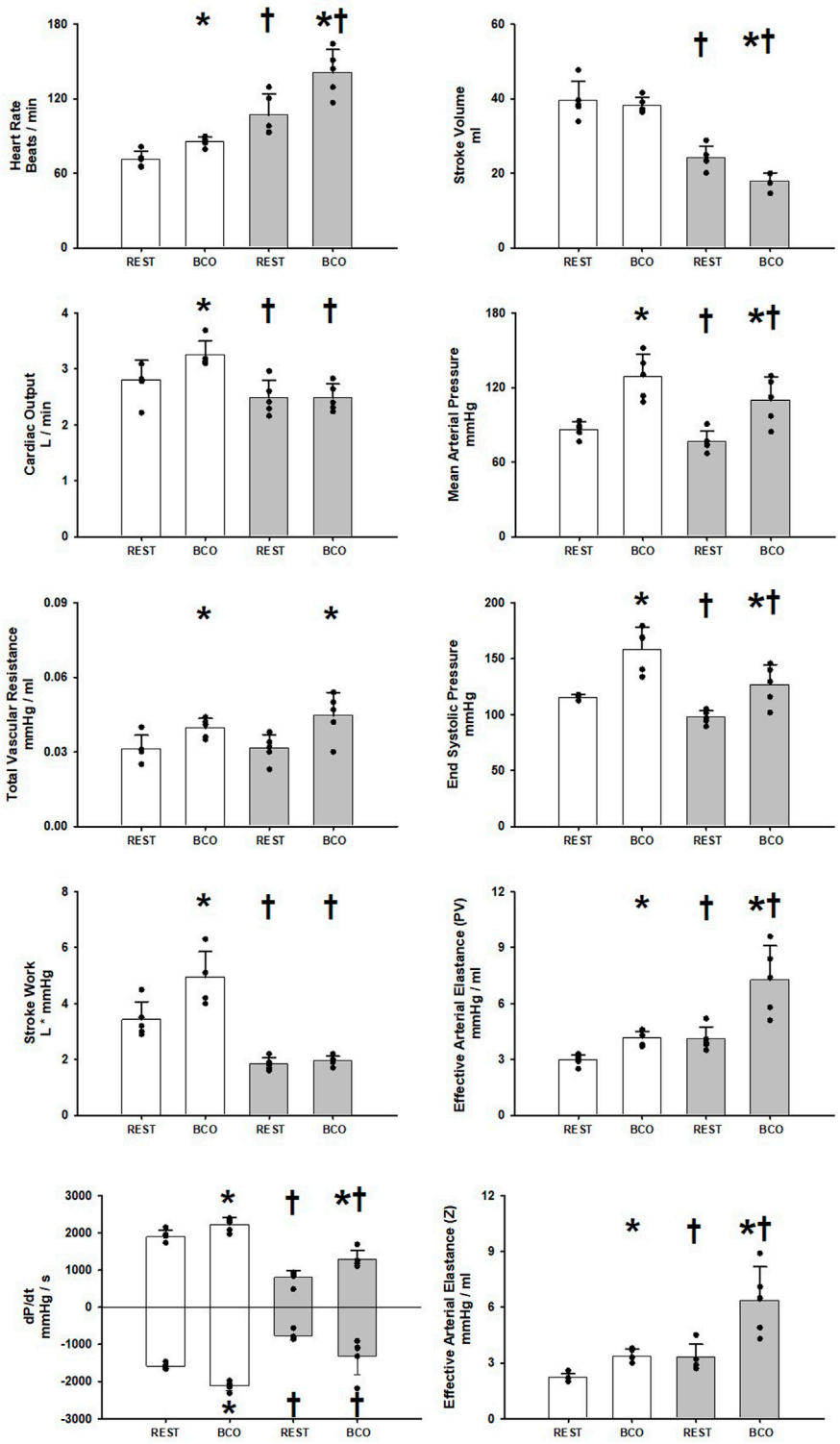

FIGURE 1 | One-minute averaged hemodynamics at rest and at rest with baroreceptor unloading via bilateral carotid occlusion (BCO) before (white bars) and after induction of heart failure (grey bars). Data are reported as mean with errors bars depicting the standard error of the mean. Actual observed data points are overlain on corresponding bar graphs. Statistical significance against the previous exercise workload depicted as $^{*}p < 0.05$ and significant against previous state at the same workload depicted as † where $p < 0.05$. ($N = 5$).

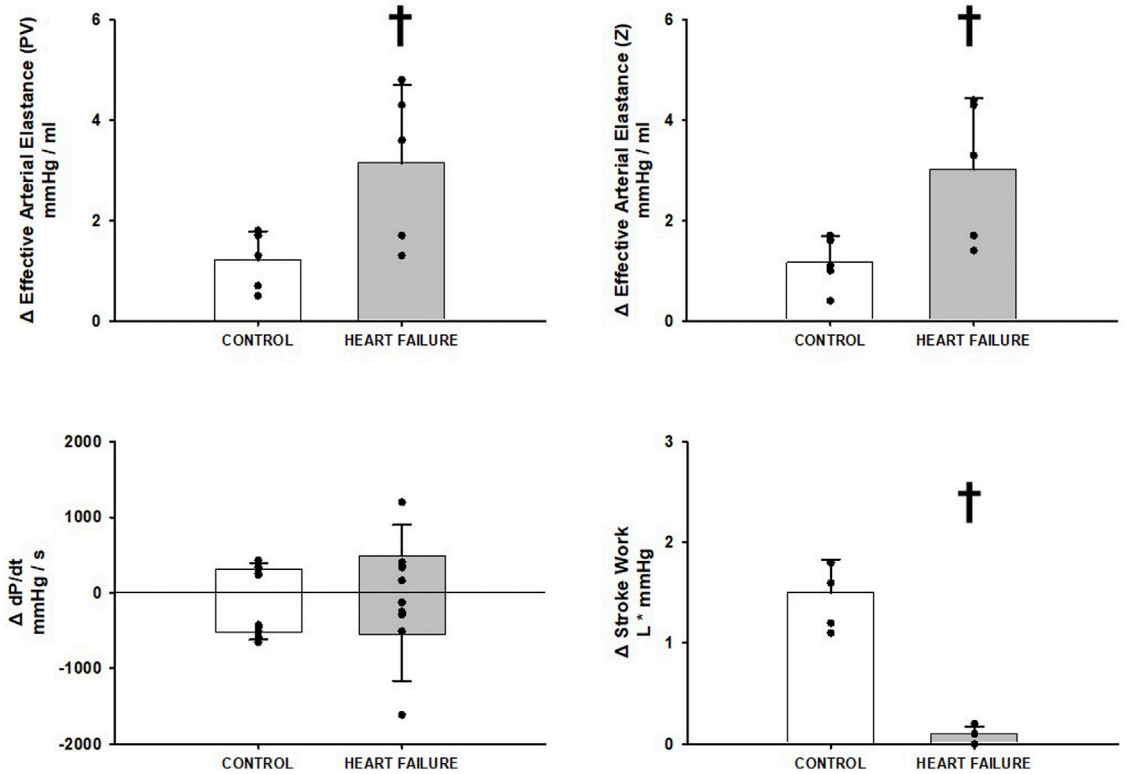

FIGURE 2 | Relative change in hemodynamic variables between rest and rest with baroreceptor unloading via bilateral carotid occlusion in control (white bars) and after induction of heart failure (grey bars). Data reported as means with errors bars depicting the standard error of the mean. Observed data points are overlain on corresponding bar graphs. Statistical significance depicted as † where $p < 0.05$. ($N = 5$).

anesthesia for each surgery, animals were sedated with an intramuscular injection of acepromazine (0.2–0.5 mg/kg). Anesthetic induction was accomplished through administration of ketamine (5 mg/kg) and diazepam (0.2–0.3 mg/kg), additionally analgesics carprofen (4.4 mg/kg IV) buprenorphine (0.03 mg/kg IM) and a fentanyl patch (50–125 μg/kg 72 h TD) were administered. Animals were anesthetically maintained pre and intraoperatively with (1%–3%) isoflurane gas. IV cefazolin (30 mg/kg IV) was administered pre and intraoperatively for prevention of acute infection. Post operatively animals were administered acepromazine (0.2–0.3 IV) and buprenorphine (0.01–0.03 IM) as needed. Prophylactic antibiotic cephalexin (30 mg/kg PO) was administered for the duration of the protocol to prevent infection of surgical sites.

In the first surgery a left thoracotomy was performed by entering the 3-4th intercostal space. The pericardium was incised to access the apex of the heart for insertion of a telemetric pressure sensor (Data Sciences International St. Paul, MN) and place 0-Flexon steel pacing leads (Ethicon Summerville, NJ) on the epicardium for measures of ventricular pressure and induction of heart failure respectively. Additionally, the ascending section of the aorta was dissected from its surrounding tissue for placement of a 20 PAU flow probe (Transonic Systems Ithaca, NY, United States) to measure cardiac output. The pericardium and ribs were reapproximated and the chest was closed in layers. All cables and wires were tunneled subcutaneously and exteriorized at the scapulae. Animals recovered for a minimum of 14 days prior to the next procedure.

The second surgery was a left retroperitoneal approach to access the terminal aorta and renal artery. A fluid 19-gauge polyvinyl catheter (Tygon, S54-HL, Norton Murdock Industrial Inc. Akron, OH) was placed into the most cranial lumbar artery branch of the terminal aorta for measures of systemic blood pressure. Additionally, a 10 PAU flow probe (Transonic Systems Ithaca, NY, United States) and two hydraulic vascular occluders (DocXS Biomedical Products Petaluma, CA, United States) were placed around the terminal aorta caudally to the catheter to measure and manipulate hindlimb blood flow for experiments unrelated to the current study. Lastly a 4 PSB flow probe (Transonic Systems Ithaca, NY, United States) was placed around the left renal artery for experiments unrelated to the current study. The retroperitoneal space was closed in layers and all cables, catheters and occluder lines were tunneled and exteriorized at the scapulae. Animals recovered a minimum of 14 days prior to the next surgery.

The final surgical procedure was performed using a single 4–5 cm vertical incision on the neck for placement of a vascular occluder (DocXS Biomedical Products Petaluma, CA,

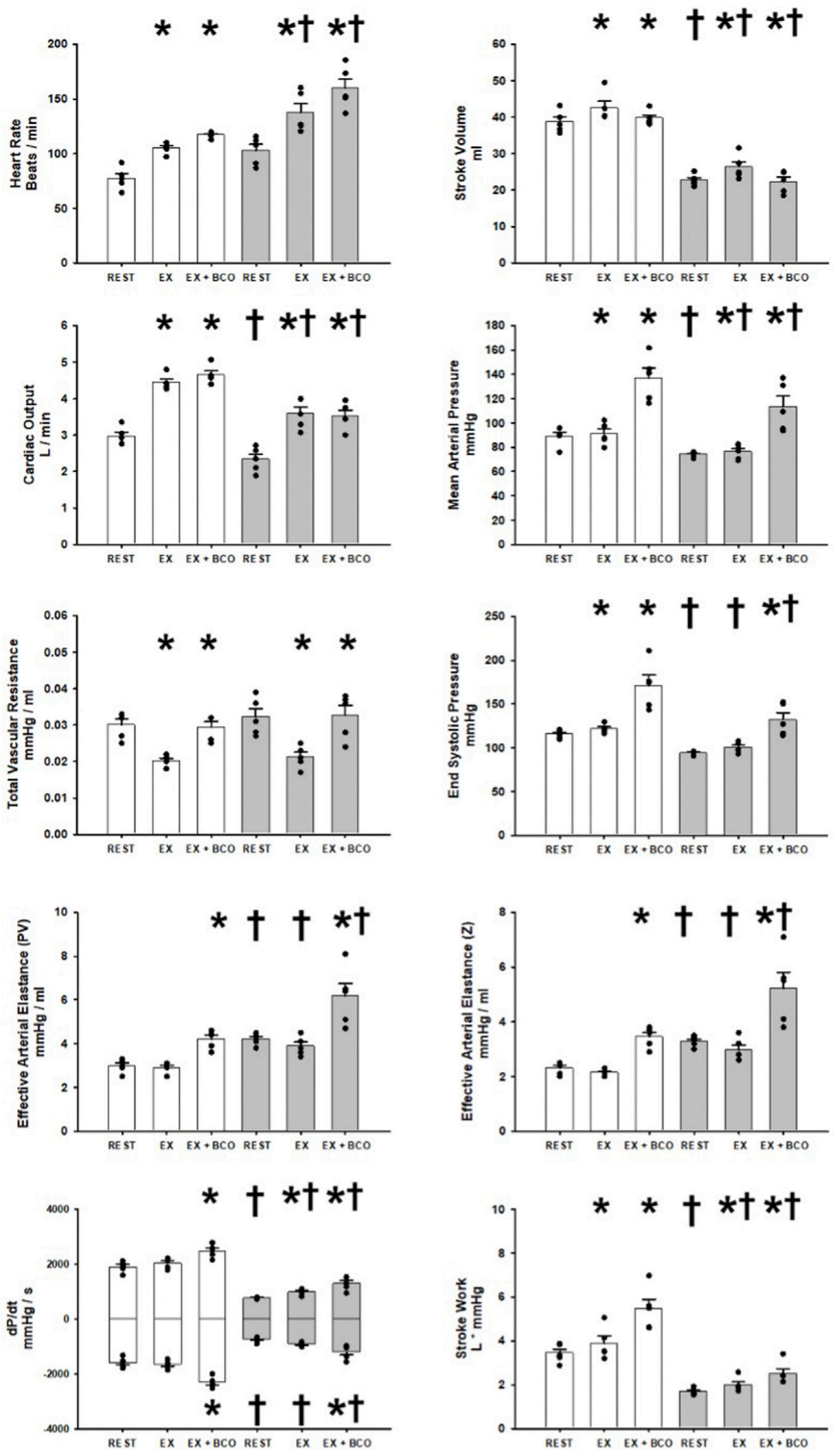

FIGURE 3 | One-minute averaged hemodynamics at rest, exercise, and exercise with baroreceptor unloading via bilateral carotid occlusion (BCO), before (white bars) and after induction of heart failure (grey bars). Data are reported as means with error bars depicting the standard error of the mean. Actual observed data points are overlain on corresponding bar graphs. Statistical significance against the previous exercise workload depicted as $^{*}p < 0.05$ and significant against previous state at the same workload depicted as † where $p < 0.05$. ($N = 5$).

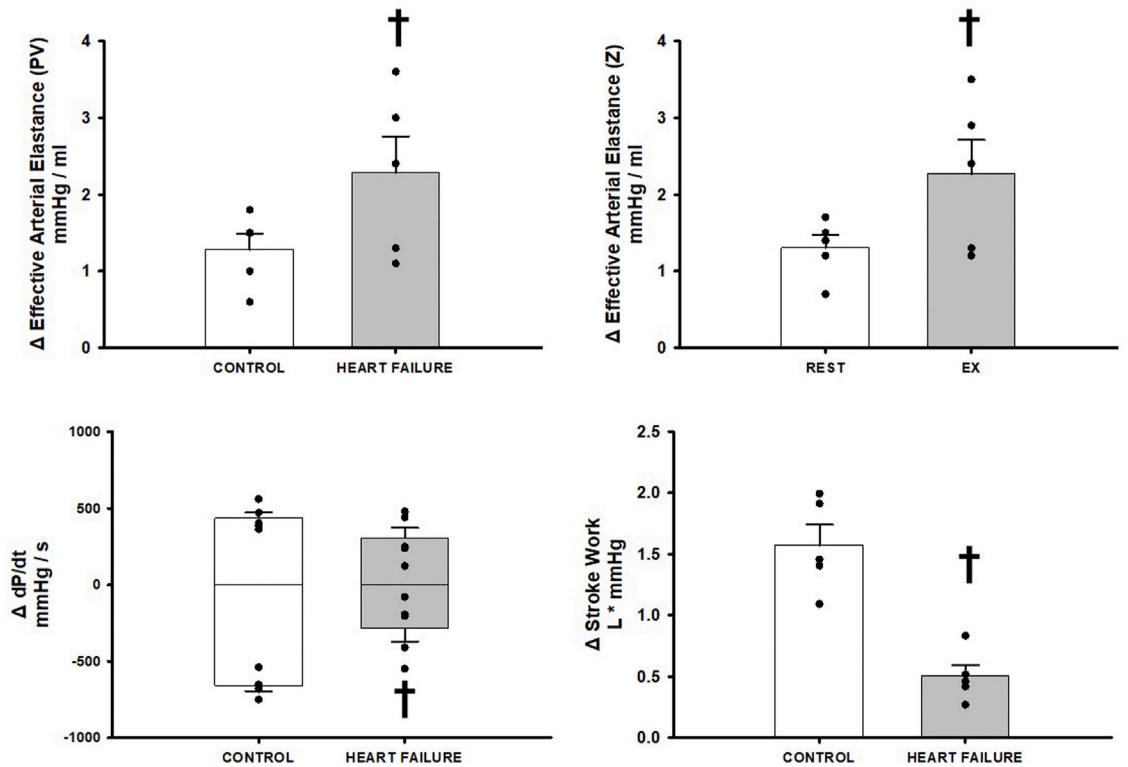

FIGURE 4 | Relative change in hemodynamic variables between exercise and exercise with baroreceptor unloading via bilateral carotid occlusion in control (white bars) and after induction of heart failure (grey bars). Data reported as means with errors bars depicting the standard error of the mean. Observed data points are overlain on corresponding bar graphs. Statistical significance depicted as † where $p < 0.05$. ($N = 5$).

United States) on each carotid artery caudally from the carotid body to be used for baroreceptor unloading. The neck incision was closed in layers and occluder lines were tunneled and exteriorized at the scapulae. Animals recovered for a minimum of 14 days prior to any experiments.

Data Acquisition and Experimental Procedures

Experiments were performed after a minimum of 14 days post-surgery. For each experiment animals were given 10–20 min to acclimatize to the laboratory environment. After acclimation all the fluid catheter was attached to a pressure transducer (Transpac IV, ICU Medical San Clamente, CA, United States) and the flow probes were connected to their respective flow channels on a TS420 flowmeter (Transonic Systems Ithaca, NY, United States). The telemetric DSI implant for the ventricular catheter was turned on to transmit data to the receiver (Data Sciences International St. Paul, MN, United States). All data was collected and analyzed using Labscribe acquisition software (iWorx Dover, NH). With the animals standing on the treadmill at rest the carotid vascular occluders were inflated for 2 min. The data were averaged over the last 1 minute of carotid occlusion. After a minimum of 20 min recovery at rest or on a separate day, the treadmill was started, and the speed raised to 3.2 km/h and maintained for at least 3–5 min to achieve steady state and the carotid occlusions were repeated. All experiments were repeated in the same animals after induction of heart failure induced via rapid ventricular pacing at 225 beats per minute for 31 ± 5 days. Thus, each animal was observed in every setting and condition: e.g., each animal served as its own control in this longitudinally designed study.

Data Analysis

Cardiac output, mean arterial pressure, and left ventricular pressure were measured and recorded continuously and all other variables shown in this study were derived from these waveforms or through calculations. Heart rate was derived from the left ventricular pressure wave form. Stroke Volume was determined by Cardiac Output/Heart Rate, Total Vascular Resistance was determined by Mean Arterial Pressure/Cardiac Output. End Systolic Pressure and rates of ventricular contractility were derived from the left ventricular pressure wave. Effective Arterial Elastance, the vascular component of the ventricular-vascular coupling relationship was calculated from two previously described methods. The first method is derived from the ventricle (Kelly et al., 1992a; Chantler et al., 2008; Amin et al., 2011; Chantler and Lakatta, 2012; Mannozzi et al., 2020) (End Systolic Pressure/Stroke Volume) and the second from the systemic vasculature (Sunagawa et al., 1985; Kelly et al., 1992a; Kelly et al., 1992b; Kass, 2005; Borlaug and Kass, 2008; Mannozzi et al., 2020) (Heart Rate x Total Vascular

Resistance). Finally Stoke work an index of optimal the ventricular-vascular coupling relationship, was calculated as [(Stroke Volume/1000) x Mean Arterial Pressure]. One-minute steady state averages were taken of each of the measured and derived variables at rest, rest with bilateral carotid occlusion, exercise, and exercise with bilateral carotid occlusion before and after induction of heart failure.

Statistical Analysis

All hemodynamic data in this study, observed and calculated are reported as means ± standard error. Statistical significance was determined using and α-level of $p < 0.05$. A two-way ANOVA with repeated measures was performed with Systat 13 statistical analysis software. When a significant interaction was observed a C matrix was used to test for Simple Effects to compare individual means. The impact of heart failure on the changes induced by bilateral carotid occlusion in each state rest and exercise was compared using Students Paired T-Tests.

RESULTS

Figure 1 shows the one-minute average observed and calculated hemodynamic variables at rest and during rest with baroreceptor unloading via bilateral carotid occlusion (BCO) before and after induction of heart failure. In control BCO caused a significant increase in Heart Rate, Cardiac Output, Mean Arterial Pressure, Total Vascular Conductance, End Systolic Pressure, Stroke Work, dP/dt MAX and MIN and both measures of Effective Arterial Elastance derived from ventricular components (PV) and peripheral vascular components (Z). After induction of heart failure resting Heart Rate, and both measures of Effective Arterial Elastance (PV, Z) were significantly increased. Stroke Volume, Cardiac Output, Mean Arterial Pressure, End Systolic Pressure, Stroke Work, dP/dt MAX and MIN were all significantly reduced at rest after induction of heart failure. In Heart Failure BCO significantly increased Heart Rate, Mean Arterial Pressure, End Systolic Pressure, both indexes of elastance, and dP/dt MAX. Stroke Work, and Cardiac Output did not significantly change in heart failure whereas Stroke Volume significantly fell. Total Vascular Resistance did not significantly change with the induction of heart failure; however, it did significantly increase during BCO at rest in heart failure.

Figure 2 shows the changes induced by BCO at rest before and after induction of heart failure in both measures of Effective Arterial Elastance (PV, Z), Stroke Work, and dP/dt MAX and MIN. Heart failure significantly increased both Effective Arterial Elastance (PV, Z) responses to BCO. The Stroke Work response to BCO was significantly attenuated in heart failure and no significant difference was observed in the relative change of dP/dt MAX or MIN during BCO, albeit the baseline levels of both were significantly reduced with heart failure (**Figure 1**).

Figure 3 shows the averaged observed and calculated hemodynamic responses comparing rest, exercise, and exercise with BCO before and after induction of heart failure. In healthy subjects the transition from rest to exercise induced significant increases in heart rate, stroke volume, cardiac output, end systolic pressure, mean arterial pressure, and stroke work. No significant change was observed in dP/dt MAX and MIN and a significant reduction in total vascular resistance was observed. Activation of the baroreflex during exercise via BCO induced statistically significant increases in heart rate, cardiac output, mean arterial pressure, total vascular resistance, end systolic pressure, both measures of Effective Arterial Elastance (PV, Z), dP/dt MAX and MIN, and stroke work. Whereas stroke volume was significantly reduced with BCO during exercise. After induction of heart failure significant reductions in stroke volume, cardiac output, mean arterial pressure, end systolic pressure, both measures of Effective Arterial Elastance (PV and Z), dP/dt MAX and MIN, and stroke work. Alternatively, no change was observed in total vascular resistance heart rate or dP/dt MIN. In the transition from rest to exercise in heart failure significant increases were observed in heart rate, stroke volume, cardiac output, mean arterial pressure, dP/dt MAX, and stroke work. Conversely significant reductions in total vascular resistance as well as no significant change in either measure of Effective Arterial Elastance or end systolic pressure were observed in the transition from rest to exercise. BCO activation during exercise in heart failure yielded significant increases in heart rate, mean arterial pressure, total vascular resistance, end systolic pressure, Both measures of Effective Arterial Elastance (PV, Z), dP/dt MAX and MIN, and stroke work. In contrast, stroke volume and cardiac output were significantly reduced during exercise with BCO Heart Failure.

Figure 4 illustrates the effect of BCO during exercise on indices of Effective Arterial Elastance, dP/dt MAX and MIN, and stroke work, in control and after induction of heart failure. Heart failure significantly increased the reflex increases in both measures of Effective Arterial Elastance and caused a significant reduction in stroke work relative to control. No significant changes were observed on dP/dt MAX however a significant reduction in dP/dt MIN was observed.

DISCUSSION

This is the first study to demonstrate the impact of baroreflex induced sympatho-activation on the ventricular-vascular coupling relationship in chronically instrumented conscious canines. We used 2 separate indices of Effective Arterial Elastance as one portion of the ventricular-vascular coupling relationship and we simultaneously measured Stroke Work as an index of how optimal the relationship is functioning. We observed that baroreflex unloading via bilateral carotid occlusion in healthy animals at rest and during exercise elicits profound increases in both ventricular and arterial assessments of Effective Arterial Elastance that coincide with significant increases in Stroke work. Thus, in normal animals arterial baroreflex unloading promotes improved ventricular-vascular coupling and thereby likely improves systemic perfusion and arterial blood pressure homeostasis. In heart failure the already uncoupled ventricular-vascular relationship is worsened by arterial baroreflex unloading due to substantial increases in Effective Arterial Elastance that likely contributes importantly to the inability to improve Stroke Work. The inability to improve Stroke Work is a direct index of further significant impairment in ventricular-vascular coupling and thereby a likely reflects an

attenuated ability to improve systemic perfusion and maintain blood pressure homoeostasis at rest and during exercise.

In healthy subjects the arterial baroreflex acts as a negative-feedback proportional control mechanism which maintains arterial blood pressure within a normal range on a beat-by-beat basis and can be assessed as spontaneous baroreflex activity or baroreflex sensitivity (gain) (Chen et al., 1991; Iellamo et al., 1997; DiCarlo and Bishop, 2001; Loimaala et al., 2003; Ogoh et al., 2003; Joyner, 2006; Hesse et al., 2007; Drew et al., 2008; Ichinose et al., 2008; Chen et al., 2010; Dutoit et al., 2010; Hart et al., 2010; Sala-Mercado et al., 2014; Hureau et al., 2018). The arterial baroreflex acts to modify blood pressure primarily via modulating autonomic outflow which then induce changes in cardiac output (mostly via changes in heart rate) as well as alterations in total vascular resistance. This dynamic control affords healthy subjects the ability to maintain blood pressure homoeostasis in response to hypotensive bouts engendered by postural changes and rest immediately after severe isometric exercise, through swift retraction of parasympathetic activity and activation of sympathetic outflow. Inasmuch as the arterial baroreflex can combat hypotensive stimuli it can also inhibit hypertensive stimuli such as those elicited by other cardio sympathetic reflexes such as the muscle metaboreflex during exercise (Ichinose et al., 2002; Kim et al., 2005a; Kim et al., 2005b; Iellamo et al., 2007; Drew et al., 2008; Ichinose et al., 2008; Fisher et al., 2010; Ichinose et al., 2012; Choi et al., 2013; Kaur et al., 2016; Ichinose et al., 2017; Kaur et al., 2018). This is a powerful blood pressure raising reflex that occurs during exercise and has previously been observed to maintain optimal ventricular-vascular coupling through large increases in both ventricular maximal elastance as well as Effective Arterial Elastance which together promote robust increases in stroke work (Sala-Mercado et al., 2006; Sala-Mercado et al., 2007; Mannozzi et al., 2020; Mannozzi et al., 2021). However, in healthy subjects the arterial baroreflex buffers the vascular component of the muscle metaboreflex and likely plays a significant role in the maintenance of the ventricular-vascular coupling relationship by modifying sympathetic outflow to the peripheral vasculature (Ansorge et al., 2005; Kim et al., 2005a; Kim et al., 2005b; Iellamo et al., 2007; Ichinose et al., 2008; Kaur et al., 2016; Kaur et al., 2018). In heart failure multiple characteristics of the arterial baroreflex are altered such that they likely contribute to orthostatic and exercise intolerance. However, the exact alterations in the reflex characteristics that likely cause these effects varies depending on the aspect of the baroreflex being assessed. For instance, in heart failure the arterial baroreflex exhibits an attenuated ability to buffer sympathetic outflow during exercise and a decreased response to baroreceptor unloading under pharmacological induced hypotension (White, 1981; Ferguson et al., 1992; Olivier and Stephenson, 1993; Thames et al., 1993; Osterziel et al., 1995; Kim et al., 2004; Kim et al., 2005a; Kim et al., 2005b; Ichinose et al., 2008). Conversely, although buffering capacity is reduced, control of sympathetic activity is maintained in regard to the functional range of baroreflex response (Thames et al., 1993; Wang et al., 1996).

In this study we observed a diminished BCO induced pressor response likely due to the inability to increase cardiac output which was caused by significant reductions in stroke volume. Significant increases in heart rate were observed like as a compensatory mechanism to offset the afterload induced reductions to stroke volume. Additionally, we observed the increase in ventricular performance in healthy animals was significantly diminished in heart failure as observed by reductions in dP/dt MAX and MIN and a reduction in peak increase in end systolic pressure. Thus, the ability to regulate the ventricular component of the ventricular-vascular coupling relationship is likely nearly abolished in heart failure. In addition to these deficits, we observed that in response to BCO both indexes of Effective Arterial Elastance were significantly increased in control and, after the induction of heart failure, the increases in E_a were significantly enhanced. By breaking these separate measures of elastance into their components we observe that the vascular component of the ventricular-vascular relationship is impacted by multiple factors. Effective Arterial Elastance PV was significantly increased because of significant albeit attenuated increase in End Systolic Pressure coupled with a significant reduction in Stroke Volume during bilateral carotid occlusion in both exercise and rest. Alternatively Effective Arterial Elastance Z was significantly increased during bilateral carotid occlusion at rest and during exercise because of the maintained increase in Total Vascular Resistance in heart failure coupled with the significantly enhanced heart rate. Together, the ventricular deficits coupled with the enhanced Effective Arterial Elastance significantly impairs energy transfer from the left ventricle and the systemic circulation and thus we observe the significant attenuation of Stroke Work and ultimately these effects are indicative of further ventricular-vascular uncoupling. Furthermore, the enhancements in Effective Arterial Elastance (PV, Z) likely further attenuate not just transfer but the overall propagation of energy throughout the arterial system thereby limiting systemic perfusion and contributing to orthostatic and exercise intolerance.

LIMITATIONS

We used the classic technique of BCO to unload the carotid arterial baroreceptors in order to elicit baroreflex mediated autonomic responses. Previous studies utilizing an isolated carotid sinus technique in conscious dogs have shown that both the arterial pressure and heart rate responses to BCO are similar to those observed in response to reducing pressure in the isolated carotid sinus to sub-threshold levels (Stephenson and Donald, 1980). However, with this approach only one side of the carotid sinus stimulus - response relationship was observed as the responses to maximal activation of carotid sinus baroreceptors could not be performed with the techniques we utilized. This could be a great future interest inasmuch as baseline autonomic activity is changed with heart failure: sympathetic activity is elevated, and parasympathetic activity is lower. In the current study, it is unknown whether further baroreflex mediated increases in sympathetic activity are limited due to the elevated baseline sympathetic tone. However, given that increases in Effective Arterial Elastance, in response to BCO, were significantly greater

in heart failure both at rest and during exercise indicates that despite elevated baseline levels, substantial baroreflex—induced increases in sympathetic activity still occurred. With the elevated levels of sympathetic activity in heart failure, it is possible that responses to arterial baroreceptor loading could be affected. These questions await further investigation.

We conclude that arterial baroreflex unloading at rest and during exercise acts to maintain ventricular-vascular coupling and thereby preserve blood pressure homeostasis by improving both ventricular and vascular aspects of the relationship. In heart failure significant ventricular-vascular uncoupling is apparent and drastically impairs any reflex increase in ventricular. Baroreceptor unloading in heart failure induces significant increases in Effective Arterial Elastance that in turn limit ventricular energy transfer as indexed by significant reductions in Stroke Work which likely inhibits propagation of ventricular energy throughout the arterial system. Thus, arterial baroreflex control not only is directly altered by the pathology of heart failure but also baroreflex-induced sympatho-activation actively worsens the ability of the reflex to improve perfusion pressure.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Wayne State University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

DO'L and JM conception and design of research and performed experiments; DO'L, JM, M-HA-H, JK, BL, AA, LM and TB, analyzed data and performed experiments. DO'L and JM, interpreted results of experiments; JM prepared figures; DO'L and JM drafted manuscript; DO'L, JK, and JM edited and revised manuscript; All authors approved final version of manuscript.

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Differences in Blood Flow Patterns and Endothelial Shear Stress at the Carotid Artery Using Different Exercise Modalities and Intensities

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Endothelial dysfunction is the first pathophysiological step of atherosclerosis, which is responsible for 90% of strokes. Exercise programs aim to reduce the risk of developing stroke; however, the majority of the beneficial factors of exercise are still unknown. Endothelial shear stress (ESS) is associated with endothelial homeostasis. Unfortunately, ESS has not been characterized during different exercise modalities and intensities in the carotid artery. Therefore, the purpose of this study was to determine exercise-induced blood flow patterns in the carotid artery. Fourteen apparently healthy young adults (males = 7, females = 7) were recruited for this repeated measures study design. Participants completed maximal oxygen consumption (VO2max) tests on a Treadmill, Cycle-ergometer, and Arm-ergometer, and 1-repetition maximum (1RM) tests of the Squat, Bench Press (Bench), and Biceps Curl (Biceps) on separate days. Thereafter, participants performed each exercise at 3 different exercise intensities (low, moderate, high) while a real-time ultrasound image and blood flow of the carotid artery was obtained. Blood flow patterns were assessed by estimating ESS *via* Womersley's estimation and turbulence *via* Reynold's number (Re). Data were analyzed using a linear mixed-effects model. Pairwise comparisons with Holm-Bonferroni correction were conducted with Hedge's g effect size to determine the magnitude of the difference. There was a main effect of intensity, exercise modality, and intensity * exercise modality interaction on both ESS ($p < 0.001$). Treadmill at a high intensity yielded the greatest ESS when compared to the other exercise modalities and intensities, while Bench Press and Biceps curls yielded the least ESS. All exercise intensities across all modalities resulted in turbulent blood flow. Clinicians must take into consideration how different exercise modalities and intensities affect ESS and Re of the carotid artery.

Keywords: atherosclerosis, blood lactate levels, aerobic exercise, resistance exercise, stroke

INTRODUCTION

Cardiovascular (CV) diseases, including coronary artery disease and stroke, are the leading cause of death worldwide. One in every 19 deaths are produced by a stroke, and there are more than 610,000 new cases of stroke per year. The total direct and indirect costs of CV for the USA are estimated to be around 43.6 billion dollars (Urbich et al., 2020). Atherosclerosis is responsible for 9 in every 10 cases of Stroke (Qaja and Bhimji, 2017); in addition, CV comorbidities are common features among stroke survivors (Tang et al., 2009).

Endothelial dysfunction is recognized as the first step for the development of 90% of all CV diseases (Benjamin et al., 2017), is a pathological condition characterized by an unbalance between vasodilatory and vasoconstrictory mechanisms (Flammer et al., 2012), and is generally defined as the decrease in nitric oxide (NO) bio-availability within the endothelium (Harris et al., 2010). The primary physiological mechanism that regulates endothelial function in endothelial shear stress (ESS), which is the frictional force produced between blood flow and endothelial cells (Sriram et al., 2016); where increments of ESS (e.g., during exercise) are known to improve endothelial nitric oxide synthase (eNOS) gene expression (Ishibazawa et al., 2011) and NO bioavailability (Rodríguez and González, 2014). Exercise programs are one of the best-suited approaches to prevent CV comorbidities and a subsequent stroke (Tang et al., 2009; Jurczak et al., 2014; Kirk et al., 2014; Marzolini et al., 2014; Prior et al., 2017), however, different exercise modalities and intensities, such as endurance *versus* resistance and low *versus* high intensities, could elicit different CV outcomes. Moreover, and to the best of our knowledge, there are no studies regarding carotid ESS during different modalities of exercises and intensities.

The purpose of this study was to determine exercise-induced blood flow patterns across different exercise modalities at three different intensities in the carotid artery. It was hypothesized that ESS and turbulent flow in the carotid artery would increase in an intensity-dependent manner and that exercises involving larger and more muscle groups would have larger ESS and more turbulent flow.

METHODS

Experimental Design

Twenty participants were recruited for a repeated-measures study design. Participation within the study involved 2 sessions for maximal testing and 2 sessions of submaximal testing with 24–48 h between sessions. A priori power analysis was conducted in Rstudio using R statistical programing language and the "pwr" library; a total of 14 subjects with stratification by sex (7 per group) at an alpha level (α) of 0.05 with a large effect size (f) of 0.4, was determined to be enough to obtain power (β) of 0.80. All study protocols were in accordance with the Declaration of Helsinki and were approved by the Institution Review Board at the University of Texas at El Paso (Reference number: 1250657). All participants signed an informed consent form before engaging in their first testing session. Females were tested within an 8-day period, spanned from 4 days before to 4 days after the start of menses, to reduce any hormonal influence on vascular response (Adkisson et al., 2010; Mattu et al., 2020).

Study Protocol

All testing was performed in a temperature-controlled room (24°C–26°C) and participants were asked to refrain of food, alcohol, and smoking for at least 8 h before any testing session. Participants completed demographic and screening questionnaires to determine eligibility. Height and mass were taken using a calibrated stadiometer and scale, respectively (Detecto PHR, Detecto, Webb, MO, United States). Then, resting blood pressure was obtained using an automated brachial blood pressure cuff (BP760, Omnron Healthcare, Inc., Lake Forest, IL, United States). In addition, and at the beginning of every visit, hematocrit (HemataStat II Hematocrit Analyzer, Separation Technology Inc., Sanford, FL, United States) and resting blood lactate (BLa) levels (Lactate Plus, Nova Inc., Boston, MA, United States) were obtained from the lower end of the earlobe as previously described (Rascon et al., 2020; Gurovich et al., 2021a). Then, for session 1, subjects completed 3 maximal strength tests (Squat, Bench Press, and Biceps curls), then subjects rested for at least 30 min (Tagesson and Kvist, 2007; Neto et al., 2015; García-Ramos et al., 2019) and performed a graded exercise test on the treadmill (Trackmaster TMX58, Newton, KS, United States) to determine maximal oxygen consumption (VO_2max) and lactate threshold. In addition, and to confirm recovery, BLa levels were obtained after the 30 min resting period and participants were not allowed to perform the next exercise testing if BLa leveles were not back to baseline levels. In session 2, participants performed two other graded exercise tests in the cycle-ergometer (Corival, Lode, Groningen, Netherlands) and arm-ergometer (Angio, Lode, Groningen, Netherlands) with at least 30 min between tests. All three VO_2max tests included blood draws from the earlobe to determine BLa at the end of each exercise stage. The 6 sub-maximal exercises (i.e., Squat, Bench Press, Biceps curls, treadmill, cycling, and arm-ergometer) were randomly assigned to sessions 3 and 4, and performed each exercise at three different exercise intensities. Participants performed three repetitions of Squat, Bench Press, and Biceps curls at low (45% 1-RM), moderate (65% 1-RM), and high intensity (85% 1-RM) each and 3-min steady-state exercise stages of treadmill, cycling, and arm-ergometer at low (BLa < 2 mmol/L), moderate (BLa 2–4 mmol/L), and high intensity (BLa > 4 mmol/L) (Rascon et al., 2020) (**Figure 1**). At least 30 min between sub-maximal exercise sets were provided to recovery.

All three graded exercise tests used a protocol with speed/workload increased every 2-min (Beltz et al., 2016). VO_2max was obtained using a metabolic cart (TrueOne 2400, Parvomedics Inc., Sandy, UT, United States). At 30 s before the end of each stage, BLa was drawn from the participant's earlobe, to determine BLa threshold, along with reported heart rate and rate of perceived exertion. A successful trial was considered if the following criteria were met: 1) BLa > 8.0 mmol/L, respiratory exchange ratio (RER) > 1.10, heart rate was within 10 bmp of

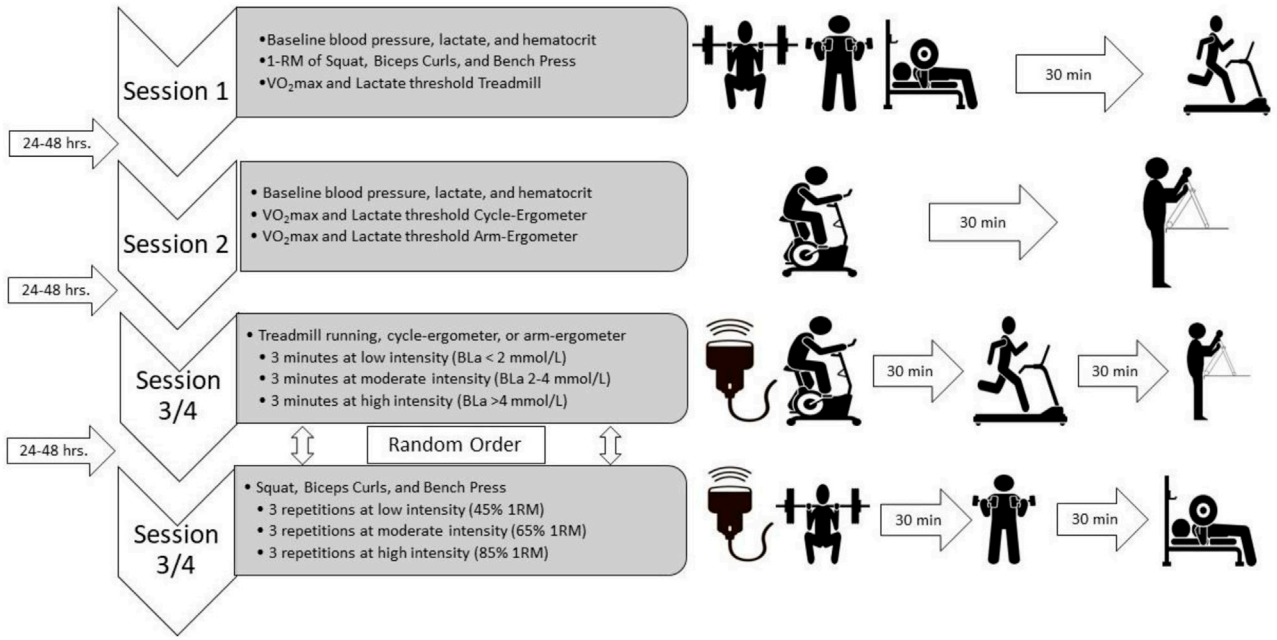

FIGURE 1 | Study design. Four exercise sessions, 2 for maximal tests and 2 for submaximal tests, with 24–48 h between sessions. Ultrasound assessment during both submaximal sessions and exercise modalities sets were randomly assigned to either session 3 or session 4. VO2max, maximal oxygen consumption; BLa, blood lactate levels.

estimated maximal heart rate (220—age), and RPE > 17 (Beltz et al., 2016).

The 1-RM testing consisted of a familiarization and technique inspection of the individual's exercise execution. Thereafter, participants were asked to predict the maximal load they could achieve. Then, participants performed 5–10 repetitions of the predicted load at a comfortable pace. The load was increased by 20% for the following set and performed for 2–3 repetitions. Then load was increased by 2.5–5 kg until participants reached failure (Seo et al., 2012; Montalvo et al., 2021). Technical execution analysis, as well as spotting, was performed by a Certified Strength and Conditioning Specialist (SM).

Blood Flow Pattern Testing

During sub-maximal exercise sets (sessions 3 and 4), real-time carotid artery longitudinal images and blood flow velocity were recorded with a 12 MHz ultrasound transducer and Doppler, (LA435, MyLab30 Gold, Esaote, Firenze, Italy), which has secure with a cervical probe holder placed on the participant's neck as previously described (Gurovich et al., 2021b; Morales-Acuna et al., 2020). Ultrasound images and Doppler signals were obtained on the common carotid artery 2 cm below the bifurcation of the anterior and posterior carotid arteries and then analyzed with edge detection technology (Vascular Analysis Integrative System, Medical Imaging Applications, Coralville, IA, United States) and a data acquisition system (MP150WSW, BIOPAC Systems Inc., Goleta, CA, United States) (**Figure 2**). ESS was obtained by Womersley's approximation and the presence of turbulent flow *via* Reynold's number (Re) as previously described (Gurovich and Braith, 2012; Morales-Acuna et al., 2019; Rascon et al., 2020; Gurovich et al., 2021a). The presence of laminar or turbulent flow was defined *via* Re, where undisturbed laminar flow values were < 200, disturbed blood flow values between 200–1800, and turbulent flow values > 2000 (Davies, 2009). Both ESS and Re were determined within a single cardiac cycle to minimize the effects that heart rate and cardiac output have in ESS, as previousle described (Gurovich and Braith, 2012; Morales-Acuna et al., 2019; Rascon et al., 2020; Gurovich et al., 2021b).

Statistical Analysis

Data were compiled into a master data spreadsheet (Excel, Microsoft 2021). Data were then exported into Rstudio Integrative Development Environment (Rstudio, 2020) and analyzed using a custom-built script in R statistical programming language (R 4.1.2). The "dplyr" package was used for grammar data manipulation, "forecats" for factor re-leveling, "ggplot2" and "ggpurb" for data visualization, "psych" for data descriptives, "cvcqv" for reliability analysis, "lm4" and "lmerTest" for linear mixed-effects models, "rstatix" for post-hoc pairwise comparisons, and effect sizes. Data distribution was assessed *via* Shapiro-Wilk test. Baseline demographic data were analyzed by a series of independent t-tests between males and females. Reliability of baseline ESS and Re were analyzed using a coefficient of variation (CV) and interpreted as < 10% as very good, 10%–20% as good, 20%–30% as acceptable, and >30% as poor (Campbell et al., 2010). Differences between exercise modalities and intensities were assessed using a general linear mixed-effects model for repeated measures with adjusting for individual differences as a random effect; the model was as follows: dependent variable ~ exercise modality + exercise

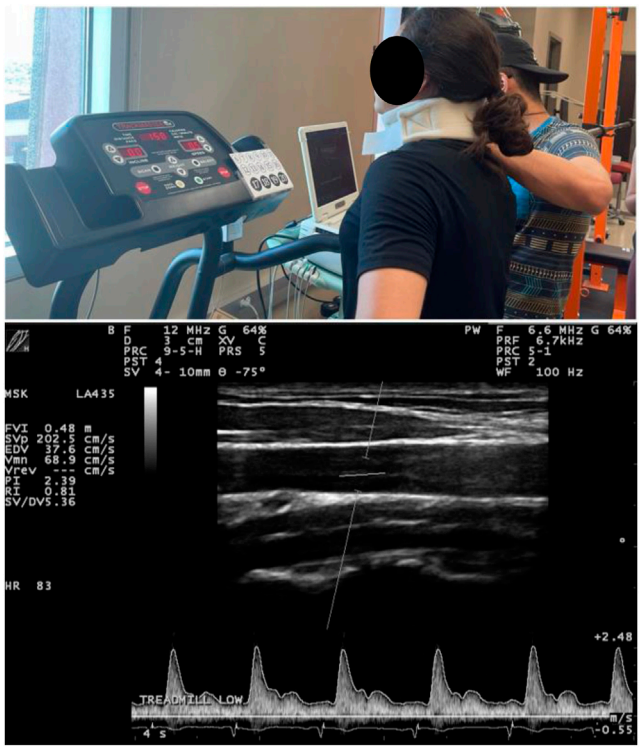

FIGURE 2 | Typical ultrasound testing setup with neck probe holder utilized during all exercise testing and representative ultrasound image.

intensity + Sex + exercise modality*exercise intensity + (1| Participant). Pairwise differences were analyzed post-hoc with a Holm-Bonferroni *p*-value correction (*p.adj*) when appropriate; the effect size was obtained through standardized mean differences using Cohen's D with a Hedge's g (ES_g) correction for a small sample size, and interpreted as follows: ES_g < 0.2 as very small, 0.2–0.49 as small, 0.5–0.79 as moderate, and > 0.8 as large (Hopkins, 2009). Statistical significance was set *priori* at an alpha level of 0.05. Re was analyzed by visual analysis using a 95% confidence interval (CI) as previously described (Gurovich et al., 2021a). Data and data analysis scripts are available in a repository for data analysis replication in https://github.com/Samuelmontalvo/Modalities.

RESULTS

Out of the 20 participants, 6 were unable to finish all 4 visits due to the COVID-19 lockdown. Hence, only 14 participants were able to complete the study. All data analyzed was normally distributed. Demographics and descriptive data for the final participants are provided in **Table 1**. Males were taller, had a higher VO_2max on treadmill ($t = 2$, $p = 0.002$), 1-RM Bench Press ($t = 6$, $p < 0.01$), and 1-RM Biceps curls ($t = 3$, $p = 0.02$) than females. Baseline reliability analysis showed a good inter-testing reliability on ESS [CV = 16.9 (95%CI = 12.3–21.5)] and acceptable inter-testing reliability on Re [CV = 22.7 (95%CI = 16.4–29.1)].

Overall, the model indicated a main effect of exercise modality ($F_{(2,247)} = 53.78$, $p < 0.001$) and intensity ($F_{(2,247)} = 63.16$, $p < 0.001$), and a significant intensity * modality interaction ($F_{(10,247)} = 2.99$, $p < 0.01$) on ESS (**Table 2**). However, there was no main effect of sex on ESS ($F_{(1,12)} = 2.01$, $p = 0.18$) or Re ($F_{(1,12)} = 0.12$, $p = 0.73$). Moreover, there was a significant random effect ($p < 0.001$), indicating significant individual variability in ESS. Due to the no effect of sex within our model, post-hoc pairwise comparisons were performed with all individuals as one group.

Post-hoc pairwise analysis within exercise modalities showed that almost all exercise modalities were influenced by intensity ($p < 0.01$) with large effect size between intensities (**Figure 2**; **Table 3**). Only Squat at low intensity vs. moderate intensity [t = -1.17, p.adj = 0.26, ES_g (*small*) = -0.29], Bench Press at low intensity vs. high intensity [t = -2.08, p.adj = 0.12, ES_g(*moderate*) = -0.52], and Bench Press at moderate intensity vs. high intensity [t = -0.81, p.adj = 0.43, ES_g(*small*) = -0.20] were not statistically different (**Figure 3**; **Table 3**).

Pairwise comparisons for ESS during low-intensity exercise showed significant differences and large effects between cycle-ergometer vs. Bench Press and Squat vs. Bench Press (**Figure 4**; **Table 4**). Similarly, there were significant differences and large effects at moderate exercise intensity between treadmill vs. arm-ergometer, treadmill vs. Squat, treadmill vs. Bench Press, treadmill vs. Biceps curls, and Squat vs. Biceps curls (**Figure 4**; **Table 4**). Finally, there were significant differences and large effects at high exercise intensity between cycle-ergometer vs. Squat, cycle-ergometer vs. Bench Press, cycle-ergometer vs. Biceps curls, treadmill vs. arm-ergometer, treadmill vs. Squat, treadmill vs. Bench Press, and treadmill vs. Biceps curls.

TABLE 1 | Demographic and descriptive data of the participants.

	All Mean ± SD	Males Mean ± SD	Females Mean ± SD	t	p
Age (yrs.)	23.00 ± 2.86	24.00 ± 3.56	22.00 ± 1.63	1.35	0.21
Height (m)	1.66 ± 0.09	1.73 ± 0.05	1.60 ± 0.08	3.48	<0.01
Weight (kg)	69.18 ± 11.03	73.94 ± 7.60	64.41 ± 12.37	1.73	0.11
BMI (kg/m2)	24.97 ± 3.45	24.73 ± 2.19	25.21 ± 4.57	0.25	0.80
SBP	113.29 ± 8.91	117.00 ± 9.07	109.57 ± 7.59	1.66	0.12
DBP	74.07 ± 6.83	75.14 ± 7.49	73.00 ± 6.51	0.57	0.57
Treadmill VO2 (ml/kg/min)	43.26 ± 9.99	50.6 ± 5.21	35.91 ± 7.95	4.08	<0.01
Cycle-ergometer VO2 (ml/kg/min)	32.00 ± 9.18	34.59 ± 10.16	29.41 ± 7.99	1.05	0.31
Arm-ergometer VO2 (ml/kg/min)	28.74 ± 9.47	32.34 ± 10.43	25.13 ± 7.43	1.49	0.16
1RM-Squat (kg)	83.34 ± 36.84	101.83 ± 43.04	64.86 ± 17.08	2.11	0.06
1RM-Bench (kg)	55.78 ± 24.97	76.86 ± 16.18	34.70 ± 7.26	6.28	<0.01
1RM-Biceps (kg)	33.73 ± 20.28	47.02 ± 21.36	20.43 ± 4.73	3.21	0.01

TABLE 2 | Endothelial Shear Stress (in dynes/cm^2) by exercise intensity and modality.

Rest		Low intensity		Moderate intensity		High intensity		Effect or interaction	
Modality	**Mean ± SD**	**Modality**	**Mean ± SD**	**Modality**	**Mean ± SD**	**Modality**	**Mean ± SD**	**F**	***p***
Baseline	23.8 ± 4.8	Arm-ergometer	39.4 ± 10.7	Arm-ergometer	47.8 ± 12.1	Arm-ergometer	57.8 ± 20.7	Intensity	
Baseline two	26.5 ± 3.3	Cycle-ergometer	48.0 ± 10.8	Cycle-ergometer	62.6 ± 19.4	Cycle-ergometer	77.5 ± 20.3	63.16	<0.01
—	—	Treadmill	47.5 ± 13.1	Treadmill	67.3 ± 17.9	Treadmill	84.7 ± 9.7	Modality	
—	—	Bench press	34.8 ± 13.0	Bench press	42.1 ± 12.0	Bench press	45.6 ± 13.7	53.79	<0.01
—	—	Biceps curls	37.3 ± 13.3	Biceps curls	41.3 ± 12.8	Biceps curls	50.7 ± 14.7	Intensity*Modality	
—	—	Squat	44.1 ± 14.4	Squat	48.8 ± 16.4	Squat	56.8 ± 13.57	2.99	<0.01

TABLE 3 | Pairwise comparisons between exercise modalities by exercise intensity for endothelial shear stress (ESS).

Intensity	Modality 1	Modality 2	t	p.adj	Hedges g	Effect
Low	Cycle-ergometer	Bench	3.639	0.042	0.915	Large
Low	Squat	Bench	3.950	0.025	0.994	Large
Moderate	Cycle-ergometer	Bench	3.739	0.022	0.940	Large
Moderate	Cycle-ergometer	Biceps	3.847	0.022	0.968	Large
Moderate	Treadmill	Arm-ergometer	4.081	0.017	−1.027	Large
Moderate	Treadmill	Squat	3.965	0.019	0.997	Large
Moderate	Treadmill	Bench	6.723	0.000	1.691	Large
Moderate	Treadmill	Biceps	5.742	0.001	1.444	Large
Moderate	Squat	Biceps	3.811	0.022	0.959	Large
High	Cycle-ergometer	Squat	3.382	0.044	0.851	Large
High	Cycle-ergometer	Bench	5.218	0.002	1.312	Large
High	Cycle-ergometer	Biceps	4.548	0.005	1.144	Large
High	Treadmill	Arm-ergometer	5.283	0.002	−1.329	Large
High	Treadmill	Squat	7.487	0.000	1.883	Large
High	Treadmill	Bench	7.289	0.000	1.834	Large
High	Treadmill	Biceps	7.725	0.000	1.943	Large

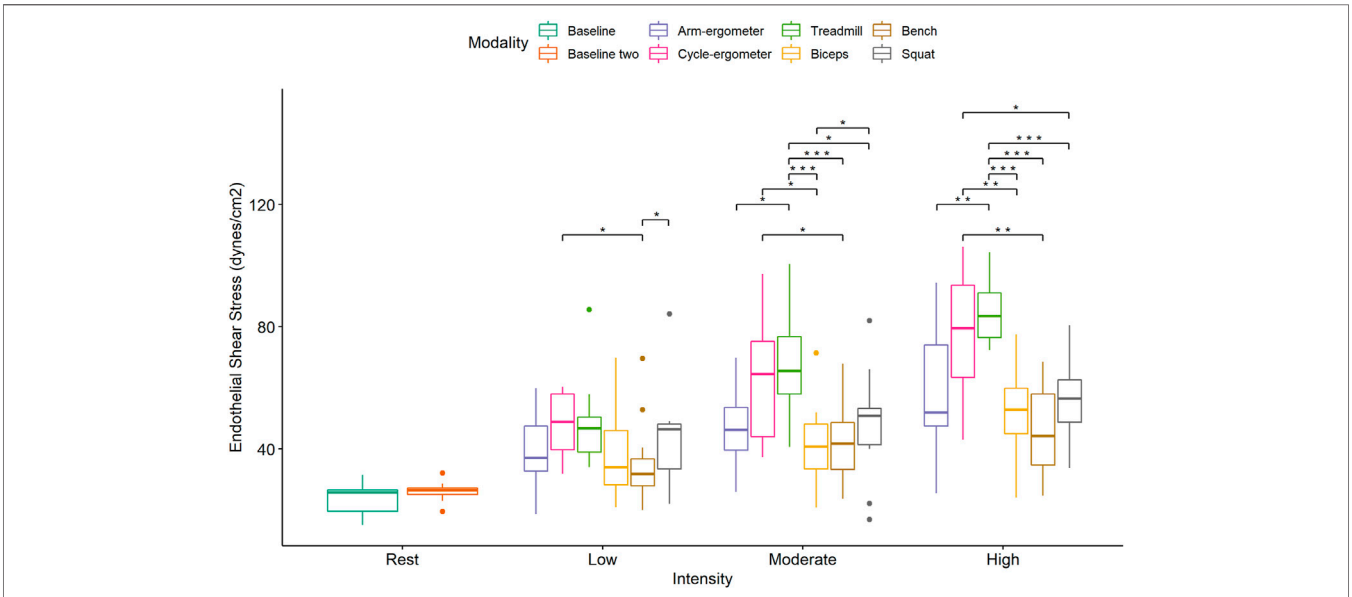

FIGURE 3 | Boxplot of Endothelial Shear Stress (ESS) by exercise modality and intensity with comparisons between modalities at each intensity. *$p < 0.05$; **$p < 0.01$; ***,$p < 0.001$.

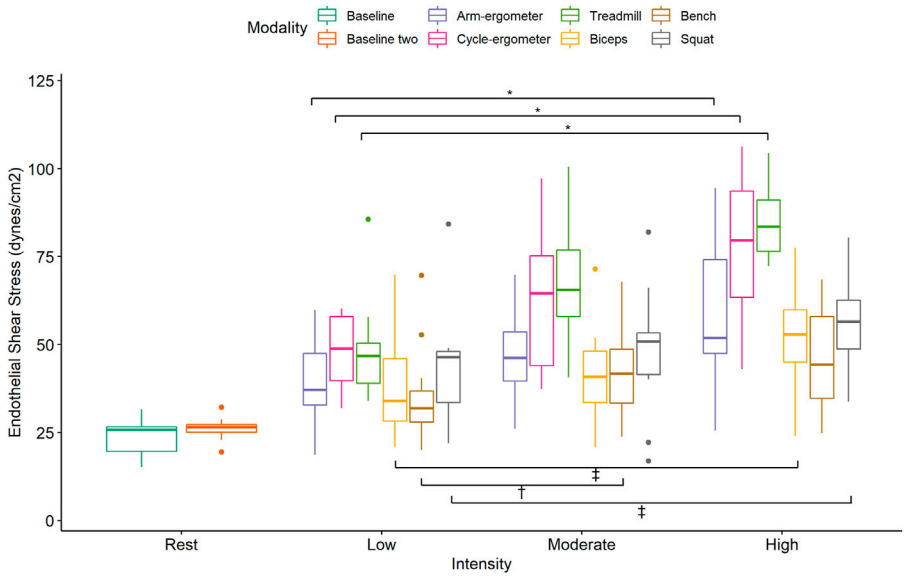

FIGURE 4 | Boxplot of Endothelial Shear Stress (ESS) by exercise modality and intensity with comparisons within modalities at each intensity. *, $p < 0.05$ low vs. moderate, low vs. high, and moderate vs. high; †, $p < 0.05$ low vs. moderate Bench press; ‡, $p < 0.05$ low vs. high and moderate vs. high; †, $p < 0.05$ low vs. moderate Biceps and Squat.

TABLE 4 | Pairwise comparisons within exercise modality by intensity for endothelial shear stress (ESS).

Modality	Intensity 1	Intensity 2	t	p.adj	Hedges g	Effect
Cycle-ergometer	Low	Moderate	−4.93	0.00	−1.24	large
Cycle-ergometer	Low	High	−8.61	0.00	−2.16	large
Cycle-ergometer	Moderate	High	−7.14	0.00	−1.80	large
Arm-ergometer	Low	Moderate	−3.68	0.01	−0.93	large
Arm-ergometer	Low	High	−5.07	0.00	−1.28	large
Arm-ergometer	Moderate	High	−3.11	0.01	−0.78	moderate
Treadmill	Low	Moderate	−4.58	0.00	−1.15	large
Treadmill	Low	High	−8.18	0.00	−2.06	large
Treadmill	Moderate	High	−3.60	0.00	−0.91	large
Squat	Low	Moderate	−1.17	0.26	−0.29	small
Squat	Low	High	−3.79	0.00	−0.95	large
Squat	Moderate	High	−4.02	0.00	−1.01	large
Bench	Low	Moderate	−4.67	0.00	−1.18	large
Bench	Low	High	−2.08	0.12	−0.52	moderate
Bench	Moderate	High	−0.81	0.43	−0.20	small
Biceps	Low	Moderate	−2.19	0.05	−0.55	moderate
Biceps	Low	High	−5.81	0.00	−1.46	large
Biceps	Moderate	High	−5.24	0.00	−1.32	large

Visual analysis of the Re plot using mean and error plot indicates that all exercise modalities from low to high intensity resulted in turbulent flow (Re > 2000) (**Figure 5**).

DISCUSSION

The purpose of the current study was to determine the effects of different exercise modalities at three different exercise intensities on endothelial shear stress (ESS) and blood flow patterns (presence of turbulent flow) at the carotid artery. Our primary hypothesis was confirmed as exercises involving larger and more muscle groups, like running on a treadmill, at higher intensities produced greater ESS and turbulent blood flow than other exercise modalities with less muscle recruitment, like Biceps curls, and lower intensities. However, exercise duration could be a confounding factor. In addition, the results of the present study confirmed that ESS increases in an intensity-dependent manner regardless of the exercise modality and that blood flow is mainly turbulent regardless of exercise modality or exercise intensity.

Approximately 40%–60% of the beneficial effects of exercise training in preventing/treating cardiovascular disease, including stroke, are unrelated to the reduction in traditional cardiovascular risk factors (Mora et al., 2007; Green, 2009). Several authors

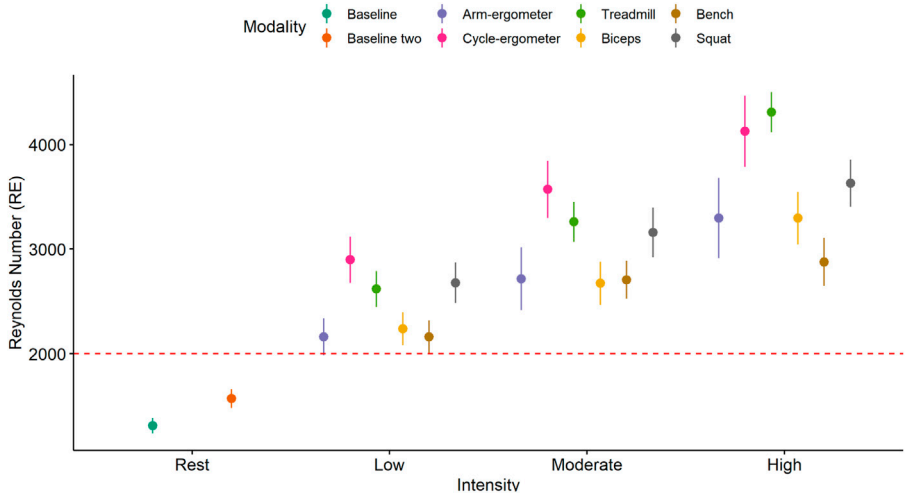

FIGURE 5 | Mean and 95% CI of Reynolds number (Re) by exercise modality and intensity with a turbulent flow threshold number of 2,000 (red dash line).

(Laughlin, 1995; Hambrecht et al., 2000; Green et al., 2002; Green et al., 2008; Laughlin et al., 2008; Green, 2009) have established a close relationship between exercise training and improvements in endothelial function. In addition, previous studies have shown that the lack of or low ESS can result in vascular inflammation, upregulation of matrix-degrading proteases, and arterial wall remodeling, which promotes the transition of stable to unstable plaque in atherosclerotic lesions (Koskinas et al., 2009). Moreover, it has been reported that low ESS and oscillatory shear stress can result in atherosclerotic lesions due to plaque formation (Cheng et al., 2006). Therefore, it is possible to speculate that the direct mechanical effects of exercise-induced blood flow patterns on the vascular endothelium could be a major mitigating factor in the prevention of cardiovascular disease.

The results of the current study showed that exercise-induced blood flow patterns are associated with exercise modality. For example, running on a treadmill or cycling at high intensity elicits more ESS than any of the resistance exercises at a comparable high intensity (**Figure 3**). This difference can be attributed to a larger muscle mass recruitment during running or cycling when compared to a single resistance exercise. In addition, ESS is intensity-dependent as almost all exercise modalities showed an increase in ESS when intensity is increased (**Figure 4**). This finding might be also associated with an increase in muscle recruitment as higher intensities would recruit a larger percentage of muscle mass. Moreover, and interestingly, it appears that all exercise modalities produced turbulent flow in the carotid artery, regardless of the exercise intensity (**Figure 5**). The presence of turbulent flow during exercise could be explained by the rather larger size of the carotid artery, compared to the brachial artery (Gurovich and Braith, 2012), and the systematic increase in blood flow velocity with the increased exercise intensity (Nichols and O'Rourke, 2005). Finally, the results of the current study showed no sex differences in any of the exercise modalities at any of the intensities. This is consistent with previous findings from our laboratory when assessing the brachial artery during cycle-ergometry at low, moderate, and high intensities (Gurovich et al., 2021b).

Even though the current study is not the first one assessing carotid blood flow during exercise (Babcock et al., 2015; Hellstrom et al., 1996; Jiang et al., 1995; Liu et al., 2015; Sato et al., 2011; Sato and Sadamoto, 2010; Wang et al., 2019), this is the first study comparing different exercise modalities and intensities. Previous studies have predominantly used walking/running on a treadmill or cycling, both upright and recumbent. For example, Jiang et al. (Jiang et al., 1995) assessed carotid blood flow velocity in eight healthy male participants during a graded exercise test on a treadmill. Unfortunately, the authors were not able to determine ESS as they found some technical difficulties with ultrasound imaging during their protocol. These technical difficulties were avoided in the current study by using a customized, patent-pending cervical probe holder placed on the participant's neck (Gurovich et al., 2021a; Morales-Acuna et al., 2020). Nevertheless, Jiang et al. found a significant increase in carotid artery blood flow velocity, up to 52% from baseline values, during exercise. Similar results were found during cycling at submaximal intensities in healthy men (Hellstrom et al., 1996), healthy women (Sato and Sadamoto, 2010), and healthy men and women (Sato et al., 2011). Similar to the findings of the present study, all these studies showed an intensity-depend increase in carotid artery blood flow, ranging from 17% to 42%, with submaximal exercise. Interestingly, Babcock, Heffernan, et al. (Babcock et al., 2015) measured carotid blood flow before and after a short bout (e.g., 30 s) of maximal exercise in 55 healthy adults, with different exercise backgrounds. Their findings showed a 15%, 19%, and 19% increase in mean carotid blood flow velocity, mean carotid blood flow, and mean shear rate, respectively. These rather smaller increases after maximal exercise could be explained by the short bout of exercise as 30 s might not be enough to elicit other vascular acute adaptations. Even though shear rate might reflect ESS, the results of the current study showed an increase in ESS from 39%, during Bench Press at low intensity, to 239%, during treadmill running at a high intensity (**Table 2**). These findings may confirm

that longer exercise bouts could elicit different acute vascular responses and that shear rate should not be considered as a surrogate for ESS (Gurovich and Braith, 2012). The ESS data shown in the current study are in agreement with previous reports (Liu et al., 2015; Wang et al., 2019). Both Wang et al. (2019) and Liu et al. (2015) reported an increased ESS, from 40% to 100%, during exercise. In addition, Wang et al. (2019) showed an intensity-dependent increase in ESS from resting to moderate and high intensity cycling exercise (~50 dynes/cm^2 vs. ~75 dynes/cm^2 vs. 100 dynes/cm^2, respectively) that are comparable to the cycling ESS in the current study (resting: 26.5 ± 3.3 dynes/cm^2, low: 48.0 ± 10.8 dynes/cm^2, moderate: 62.6 ± 19.4 dynes/cm^2, and high: 77.5 ± 20.3 dynes/cm^2). Interestingly, the resting data from the current study is very similar to data obtained with echo particles imaging velocimetry (PIV) (Gates et al., 2018); however, exercise PIV data has yet to be determined.

Even though the current study was designed to assess blood flow patterns during an acute bout of exercise, these findings can be associated with adaptations such as endothelial function and atherosclerotic plaque vulnerability. For example, there are some conflicting results when comparing endothelial function before and after resistance and aerobic exercises (Iwamoto et al., 2018; Boeno et al., 2019). Boeno et al. (2019) showed no improvement in endothelial function, measured *via* flow-mediated dilation, with a single session of repeated knee extension exercise at a moderate and high intensity. In contrast, Iwamoto et al. (2018) showed that endothelial function improved in an intensity-dependent manner after low intensity (50%–55% of HRmax) and high intensity (75%–80% HRmax) cycle-ergometry. Similarly, Spence et al. showed that 6 months of aerobic and resistance training induced significant changes in the carotid artery size and function (Spence et al., 2013). In addition, there is some evidence that turbulent flow can improve the strength of atherosclerotic plaque (Cheng et al., 2006; Koskinas et al., 2009). Both Cheng et al. (2006) and Koskinas et al. (2009) using very elegant study designs, showed that turbulent flow in pro-atherosclerotic vascular areas can induce stable lesions by mobilizing smooth muscle cells. Altogether, the significant increase in ESS and turbulent flow at higher intensities observed in the current study, if applied chronically (i.e., exercise training), may elicit beneficial effects to treat and prevent cardiovascular diseases.

Limitations

The present study is not exempt from limitations. Our study was limited to the sample size. Our between-subjects comparison analyzes could have been compromised by the low sample size (males = 7, females = 7). However, because of inexistence differences between males and females in blood flow patterns, our overall sample size was 14 participants, which was enough to show differences in responses through the standardized mean difference as denoted by the effect size. Moreover, each of the pairwise comparisons (exercise modality by intensity) yielded a possible 42 comparisons. Thus, in order to avoid the increased chance of committing type 1 (false positive) and type 2 (false negative) errors, we utilized a Holm-Bonferroni correction (Eichstaedt et al., 2013). Another possible limitation is the difference in the exercise duration of each exercise modality. Future studies should use standardize time/volume of each exercise bout to make it more comparable.

The inferences derived from this investigation can only be extrapolated to a similar population (healthy young male and female participants), and the effects of different exercise modalities and intensities on ESS and Re for clinical populations (i.e., CV problems) remain unknown. Moreover, it is unknown if other alternative exercise modalities such as plyometrics (jumping), boxing, agility training, balance, Taichi, Yoga, etc,. would affect (short or long term) endothelial shear stress and function, and as such, researchers should investigate these. Finally, our study was cross-sectional, and only acute interaction of exercise modality and intensity and ESS or Re can be inferred. Thus, the differences between exercise modalities and intensities on ESS or Re at short and long-term exercise remains unknown.

CONCLUSION

Blood flow patterns during exercise in the carotid artery show that flow is mainly turbulent, independent of the exercise modality and intensity and that ESS is dependent on exercise intensity regardless of the exercise modality. In addition, activities engaging larger and more muscle groups, like running or biking, at a high intensity yield the greatest ESS. Thus, clinicians should take into consideration exercise-induced blood flow patterns at the carotid artery during the different exercise intensities and modalities.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://github.com/Samuelmontalvo/Modalities.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institution Review Board at The University of Texas at El Paso. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AG and FM-A contributed to conception and design of the study. SM, MG, AL, SA, and LR collected the data. SM performed the statistical analysis. SM and AG wrote the first draft of the manuscript. MG wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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