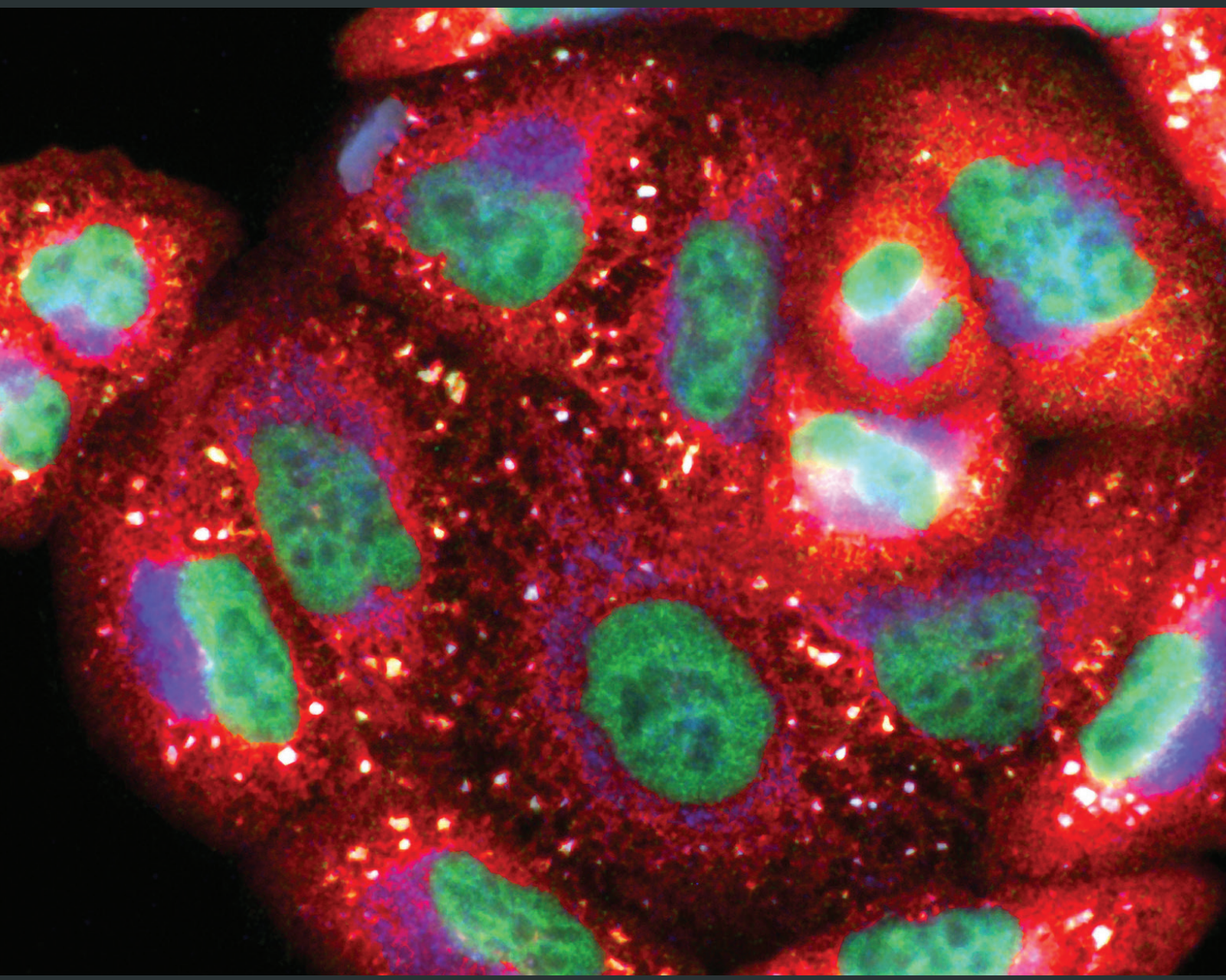
Oxidative Medicine and Cellular Longevity

New Insights into the Benefits of Physical Activity and Exercise for Aging and Chronic Disease

Lead Guest Editor: Patricia C. Brum
Guest Editors: James Turner and Vitor A. Lira



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Editorial Board

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Editorial

New Insights into the Benefits of Physical Activity and Exercise for Aging and Chronic Disease

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Diabetes mellitus, cardiovascular disease, and cancer are among the leading causes of morbidity and mortality globally. Strategies to prevent and treat diseases associated with aging and lifestyle have become a priority for health science researchers, guideline groups, and policymakers. The most effective intervention to prevent and, in many cases, treat chronic disease is the adoption of an active lifestyle; however, the cellular and molecular mechanisms by which physical activity and exercise bring about their effects remain poorly understood. This knowledge gap hinders the development of alternative strategies and therapies that could benefit individuals who cannot adhere to structured exercise regimens or make substantial changes to other aspects of their lifestyle. This special issue consists of five original scientific studies and two review articles that investigate or summarise the cellular and molecular processes implicated in chronic disease and their interaction with exercise training. These articles improve our understanding of mechanisms that underpin the preventative or therapeutic effects of interventions for countering disease and raise awareness of current thinking and controversies in aging and lifestyle research.

Two investigations in this special issue focus on cardiomyopathy—hypertrophy and fibrosis of the myocardium that usually causes heart failure—in rat models of obesity (A. C. Silveira et al.) and type I diabetes mellitus (U. Novoa et al.). Exercise is among the most effective interventions for preventing cardiomyopathy. Mechanisms include whole-body systemic processes (e.g., improvements in inflammatory and metabolic profiles) but also exercise-induced alterations that are intrinsic to cardiac tissue. A. C. Silveira et al. demonstrate, in male, five-month old obese Zucker rats exhibiting cardiac hypertrophy, that exercise (60 minutes swimming, +4% body mass applied, 5 times per week, for 10 weeks) increases cardiac expression of microRNA-29c and reduces expression of microRNA-1. Since microRNA-29c targets collagen genes reducing their expression, these findings suggest that exercise may prevent cardiac fibrosis, at least partially, by increasing microRNA-29c levels. In addition, microRNA-1 reduces the expression of the *NCX1* gene encoding a sodium-calcium exchanger, also referred to as solute carrier family 8 member 1 (SLC8A1). Low cardiac NCX1/SLC8A1 expression is common in obesity, and this protein is essential for myocardium relaxation enabling Ca^{2+} release through the sarcolemma. An exercise-dependent reduction of microRNA-1 may improve cardiac function in obesity by restoring gene expression for NCX1/SLC8A1. Thus, microRNA-29c and microRNA-1 might form the basis of new therapies for obesity-associated cardiomyopathies.

In the study by U. Novoa et al., cardiomyopathy was investigated in male, three-month old, Sprague-Dawley rats with type I diabetes induced by Alloxan—a compound with

selective toxicity to pancreatic beta cells. Diabetic cardiomyopathy is associated with both type I and type II diabetes independently of confounding factors such as hypertension and coronary artery disease. Multiple cellular and molecular mechanisms have been implicated, including overproduction of reactive oxygen species causing oxidative stress. U. Novoa et al. examined whether high-intensity exercise (10–15 minutes treadmill running, 80% maximum capacity, 5 times per week, for 4 weeks) reversed cardiac remodeling and had the potential to limit cardiac oxidative stress. Exercise training reduced cardiomyocyte hypertrophy and cardiac collagen deposition. However, there was a substantial increase in cardiac apoptosis, which occurred in parallel with uncoupling of endothelial nitric oxide synthase (NOS), and increased gene and protein expression for NADPH oxidase 2 (NOX-2), both potential sources of free radicals and reactive oxygen species. These results suggest that although high-intensity exercise training leads to beneficial effects on cardiac structure, this form of exercise is also associated with cardiac oxidative stress, justifying future studies that examine the effects of different exercise intensities.

The consequences of obesity- and diabetes-associated cardiomyopathy include heart failure with preserved ejection fraction (HFpEF) or reduced ejection fraction (HFrEF). Successful treatments, such as beta blockers, that prolong survival in patients with HFrEF do not improve the prognosis of HFpEF. In this special issue, the review article by A. B. Gevaert et al. advocates a new "whole systems" approach, moving focus from the cardiomyocyte to the endothelium, proposing that exercise interventions could limit or reverse endothelial dysfunction in HFpEF. First, A. B. Gevaert et al. summarise the molecular mechanisms underlying endothelial dysfunction. Second, the characteristics of endothelial dysfunction in HFpEF are described for the vascular beds of different organs, including skeletal muscle, the heart, lungs, and kidneys. Finally, evidence for aerobic exercise training counteracting endothelial dysfunction in HFpEF is presented, with a discussion of possible mechanisms, including increased nitric oxide availability, exercise-induced anti-inflammatory and antioxidative stress effects, and a mobilization of endothelial progenitor cells and angiogenic T cells.

Exercise-induced improvements in nitric oxide availability have clear implications for countering hypertension, but effects beyond the vascular bed also contribute to mitochondrial biogenesis and modulation of bioenergetic pathways in various cell types. Nitric oxide is produced enzymatically by three NOS enzymes: neuronal-NOS (NOS-1), inducible-NOS (NOS-2), and endothelial-NOS (NOS-3). Polymorphisms in NOS genes have been linked to hypertension, NOS enzyme activity, and expression level, but a potential influence on exercise adaptations is poorly understood. In this special issue, A. A. Trapé et al. prescribed multicomponent exercise training to 52 middle-aged overweight and obese women, characterised for three NOS-3 polymorphisms. The 12-week programme consisted of dynamic stretching and strengthening activities plus moderate-to-vigorous aerobic exercise, undertaken for 90 minutes, twice a week. Exercise brought about improvements to various measurements of physical functioning, systolic and diastolic blood pressure, nitrite concentration, and some biomarkers of oxidative stress. However, women within individual NOS3 polymorphism groups exhibited trends towards a smaller magnitude of change for systolic and diastolic blood pressure and nitrite concentration. Women with all three NOS3 polymorphisms did not exhibit exercise-induced improvements in blood pressure or nitrite concentration compared to women without polymorphisms; however, this result should be interpreted with caution due to low sample size. Although confirmation of these results is warranted in larger studies examining different forms of exercise, and the molecular mechanisms remain to be established, some NOS3 polymorphisms might result in impaired exercise-induced enzymatic nitric oxide synthesis. Individuals less responsive to exercise might benefit from dietary supplements rich in nitrate (NO_3^-) and/or nitrite (NO_2^-), which can lead to nonenzymatic nitric oxide production.

Many studies have examined the effectiveness of interventions for countering obesity-associated changes in adipose tissue mass, inflammation, and metabolic dysfunction. In one contribution to this special edition, D. B. Bartlett et al. examine the utility of a novel composite biomarker—GlycA—for assessing the anti-inflammatory effects of an exercise-based lifestyle intervention. GlycA is a nuclear magnetic resonance spectroscopy signal derived from acute-phase proteins, including α1-acid glycoprotein, haptoglobin, and transferrin. GlycA positively correlates with body mass index, markers of metabolic syndrome, and disease activity in auto-immune conditions and is associated with cardiovascular disease and type II diabetes mellitus. D. B. Bartlett et al. randomly assigned 169 overweight or obese men and women with prediabetes, to one of four intervention groups. Three groups undertook different volumes and intensities of exercise prescribed over six months, and one group combined exercise with a calorically restricted diet regimen. On average across the cohort, GlycA concentration decreased significantly by 2% over six months. Slightly larger changes were reported among those undertaking vigorous exercise or moderate exercise plus caloric restriction, but there were no statistically significant differences between groups. Reductions in GlycA occurred in parallel with improvements in body composition and fasting insulin. Thus, GlycA could be a promising biomarker for assessing anti-inflammatory effects of lifestyle interventions, but further work is required to fully understand the influence of exercise dose, plus, or minus diet (e.g., by inclusion of a nonintervention control group).

Overweight and obese individuals seem to exhibit impaired immune function, demonstrated by a greater risk of viral and bacterial infections, smaller immune responses to vaccination, and accumulations of immune cell subsets—some dysfunctional—that are linked to aging. As addressed by two articles in this special issue, exercise training is a potential countermeasure that stimulates immune function, perhaps limiting or delaying the age-associated decline in immune competence, also referred to as immunosenescence. Focusing on innate immunity in a second contribution to this special issue, D. B. Bartlett et al. examined whether different forms of exercise training, in 27 young and middle-aged adults, most classifying as overweight, cause similar

immuno-stimulatory effects. Participants were randomised to undertake three supervised group exercise classes each week for 10 weeks, consisting of either high-intensity interval training (18–25 minutes per session, >90% maximum heart rate) or moderate-intensity continuous training (30–45 minutes per session, 70% maximum heart rate). A major finding was that both forms of exercise improved to a similar extent, the capacity of neutrophils to ingest *E. coli* by phagocytosis and subsequently produce reactive oxygen species as part of killing. Thus, by improving innate immune function, both moderate and vigorous exercise training might reduce the risk of bacterial infections in overweight and aging populations. As reviewed by J. Turner and P. Brum in this special issue, the immuno-stimulatory effects of exercise also generalise to the adaptive immune system. J. Turner and P. Brum summarise the mechanisms underlying possible anti-immunosenescence effects of exercise, with a primary focus on the age- and infection-associated accumulation of late-stage differentiated T cells. In addition, studies that have investigated whether immunosenescence influences the risk of developing cancer and affects the treatment of patients with a cancer diagnosis are presented. Finally, it is discussed whether reduced cancer risk and more successful cancer treatment outcomes exhibited by regularly active people are driven by exercise limiting or delaying immunosenescence.

We hope that this special issue is successful in providing new insights into the benefits of physical activity and exercise for aging and chronic disease. As a result, we hope to stimulate new research ideas, investigations, and collaborations, which might, one day, reduce morbidity and mortality from diseases linked to aging and lifestyle.

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We would like to express our special gratitude to the expert reviewers that provided invaluable feedback to all manuscripts submitted to this special issue.

James E. Turner
Vitor A. Lira
Patricia C. Brum

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Research Article

Effect of Multicomponent Training on Blood Pressure, Nitric Oxide, Redox Status, and Physical Fitness in Older Adult Women: Influence of Endothelial Nitric Oxide Synthase (NOS3) Haplotypes

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The purpose of this study was to verify the influence of the genotype or haplotype (interaction) of the NOS3 polymorphisms [-786T>C, 894G>T (Glu298Asp), and intron 4b/a] on the response to multicomponent training (various capacities and motor skills) on blood pressure (BP), nitrite concentration, redox status, and physical fitness in older adult women. The sample consisted of 52 participants, who underwent body mass index and BP assessments. Physical fitness was evaluated by six-minute walk, elbow flexion, and sit and stand up tests. Plasma/blood samples were used to evaluate redox status, nitrite concentration, and genotyping. Associations were observed between isolated polymorphisms and the response of decreased systolic and diastolic BP and increased nitrite concentration and antioxidant activity. In the haplotype analysis, the group composed of ancestral alleles (H1) was the only one to present improvement in all variables studied (decrease in systolic and diastolic BP, improvement in nitrite concentration, redox status, and physical fitness), while the group composed of variant alleles (H8) only demonstrated improvement in some variables of redox status and physical fitness. These findings suggest that NOS3 polymorphisms and physical training are important interacting variables to consider in evaluating redox status, nitric oxide availability and production, and BP control.

1. Introduction

Population aging is occurring in all regions of the world; however, in recent years, it has been progressing more rapidly in developing countries [1]. By definition, human aging is defined as a dynamic and progressive process in which there are morphological, functional, biochemical, and psychological alterations, causing greater vulnerability

and a greater incidence of pathological processes [2]. Among these changes, it is worth noting the decrease in physical fitness [3] and greater vulnerability to chronic diseases, especially cardiovascular diseases [4, 5].

These decreases in physical fitness over the aging process are directly associated with impairment in functional capacity [2]. It is important to emphasize that the regular practice of physical exercises can be considered as one of the main measures that counteract these factors. A multicomponent intervention seems to be appropriate [6] as the physical alterations related to the aging process affect various motor skills and abilities. The most recent official position of the American College of Sports Medicine (ACSM) [3, 7] provides indications for the practice of physical exercises, including training of the various capacities and motor skills (aerobic capacity, muscular strength, flexibility, coordination, agility, and balance).

Regarding the greater vulnerability to chronic diseases related to the aging process, there is an increase in the incidence of cardiovascular disease risk factors [5], with hypertension highlighted among the modifiable risk factors [4].

The etiology of hypertension is multifactorial and may involve genetic, environmental, and psychological aspects. Humoral factors are considered as a mechanism that controls blood pressure (BP), and certain alterations could result in elevation or a decrease in BP levels [8]. Among these, reduction in the production of nitric oxide (NO) may be related to aging, a sedentary lifestyle, and some genetic polymorphisms; besides, the reduction of bioavailability of NO may also be related to high exposure to oxidative stress. Reduced NO bioavailability is directly related to impairment in vasodilation, causing an increase in peripheral vascular resistance and raising BP values. There is a decrease in antioxidant activity, which may contribute to damage from exposure to oxidative stress [9–14]. High oxidative stress, a decrease in antioxidant activity, and low concentrations of NO are associated with increased BP values, especially in the context of the aging process [9, 11, 15].

It is known that endothelial cells are responsible for the synthesis, metabolism, and release of a wide variety of mediators that regulate vascular tone, with NO of paramount importance due to its role in BP control. NO production by nitric oxide synthase (NOS), which is made from the amino acid L-arginine, is responsive to physical exercise as shear stress in vessel walls promoted by blood flow is considered one of the most important physical stimuli for the endothelial cells to produce NO [9, 11, 15]. Therefore, improvement in physical fitness may increase NO concentration and decrease reactive oxygen species production. However, improvement in BP values is still controversial and different behavior can be found among individuals [15–17]. Although lifestyles with significant physical training and nutrition control have a huge influence on BP control, the genetic influence has been receiving special attention. Therefore, it is important to investigate the relationships and interactions between genetic polymorphisms, aging, and changes in lifestyle, such as the response to physical exercise [18, 19].

Among the many existing polymorphisms, some studies point out that genetic variants in the gene encoding nitric oxide synthase 3 (NOS3) -786T>C, 894G>T (Glu298Asp), and intron 4b/a may potentially explain the difference of declines characteristic of aging [19] and help to explain why some people demonstrate more benefits from physical training than others [18].

Some meta-analyses have shown the association between the NOS3 polymorphisms and hypertension, highlighting the intron 4b/a and 894G>T polymorphisms [20–22]. However, associations of NOS3 polymorphisms with hypertension and the response to physical training remain unclear, as well as the interaction among these polymorphisms, added to which, the previously mentioned study performed aerobic exercise.

Thus, the purpose of this study was to verify the influence of the genotype or haplotype (interaction) of the NOS3 polymorphisms [-786T>C, 894G>T (Glu298Asp), and intron 4b/a] on the response to multicomponent training for BP, nitrite concentration, oxidative stress, antioxidant activity, and physical fitness in older adult women. The hypotheses of the present study were that individuals carrying these NOS3 polymorphisms (the allele "C" of the gene -786T>C, the allele "a" of the gene intron 4, and GluAsp or AspAsp genotypes of the gene Glu298Asp) could present a worse response to physical training compared to ancestral carriers.

2. Methods

2.1. Ethical Review. This project was approved by the Ethics Committee of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto of the University of São Paulo (CAAE 24579513.4.0000.5407). All participants signed a free and informed consent form after having all questions answered by the researcher in charge and before starting the study. Although the present study only includes female participants that did not practice exercises in any other physical exercise program during the previous six months, male participants and individuals who were already training also participated in the interventions and evaluations, thus obtaining a return regarding health parameters. Only women were included in the analysis; this is justified by the fact that in physical training programs for the elderly, in general, there is a predominance of women, varying from 70 to 90% [14].

2.2. Study Design. The flow of participants and study design are illustrated in Figure 1.

2.2.1. Sample Selection. All participants in the Physical Education program for the elderly, an extension project of the School of Physical Education and Sports of Ribeirão Preto (University of São Paulo), were invited to participate in this research. A convenience sample was obtained from this extension project. The subject's medical history was reviewed on their first visit. Inclusion criteria were being female and aged between 50 and 80 years. Exclusion criteria were the presence of any medical, mental, or musculoskeletal conditions that could prevent performance of the motor tests and physical training program; body mass index $> 35\,kg/m^2$, maximal systolic BP > 160 mmHg, and maximal diastolic BP > 100 mmHg; and participation in any other physical

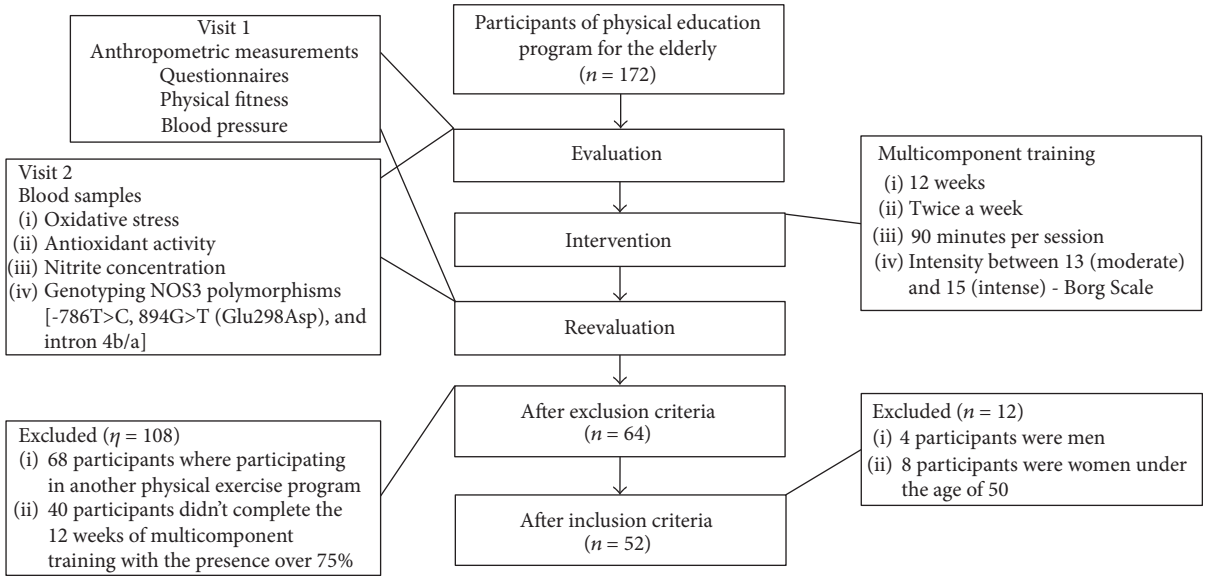

Figure 1: Flow chart: study design and sample selection.

exercise program in six months prior to or during the intervention proposed by this study and presence <75% in the activities proposed by the intervention.

2.2.2. Intervention and Evaluations. The duration of the intervention was 12 weeks, twice a week, on nonconsecutive days and each session lasted 90 minutes. All sessions were conducted by a physical education professional. The sessions were divided into four parts: (1) warm-up, including dynamic stretching exercises, coordination, and/or balance (about 20 to 30 minutes), (2) strength exercises performed in the form of a circuit using elastics, free weights, and body weight (about 30 to 40 minutes), (3) aerobic and ludic activities (dances or games) (about 20 to 30 minutes), and (4) "back to calm," relaxation, massage, and stretching exercises (about 10 minutes). The intensity of the training was controlled by the Borg Scale [23], maintaining an intensity between 13 (moderate) and 15 (intense). Evaluations were performed before and after 12 weeks of intervention. The participants were submitted to the following assessments:

(1) Anthropometric Measurements. Body mass and height measurements were performed using a scale (graduation of 50 g) with a stadiometer (precision of 1.0 mm) (Welmy W200ALCD), which allowed calculation of the body mass index (BMI) using the equation weight/height^2.

(2) Questionnaires. Level of physical activity was evaluated using the International Physical Activity Questionnaire (IPAQ) (interview that assesses frequency in days and duration in minutes of activities performed for more than ten minutes continuously in a normal week, classified as intense, moderate, and walking) [24] and assessment of nutritional status by the Food Consumption Marker Form (indicates the frequency of consumption of 10 food groups on the days of a regular week) [25]. The Brazilian Economic Classification Criterion is an instrument to estimate the purchasing power of urban families, leading to a classification of socioeconomic status. The scale presents a score that takes into account the possession and quantity of materials such as cars, refrigerators, and televisions, the schooling of the head of the family, and the presence of a domestic helper [26]. Although not included in the objectives, these three instruments were used to control for confounding factors.

(3) Physical Fitness. These tests are specific for older and elderly adults, validated and with normative reference values. Aerobic capacity was evaluated using the six-minute walk test (distance traveled on a rectangle route measuring 4.57 m × 18.28 m—the participant was required to walk as fast as possible, but without running), strength of the upper limbs was evaluated by the elbow and extension flexion test (the highest number of complete repetitions of elbow flexion and extension with the dominant arm in 30 seconds with the participant seated and using a dumbbell of 2.27 kg), and the lower limbs was evaluated by the sit and stand up test (the highest number of complete repetitions in 30 seconds of sitting and standing up from a chair—the participant was required to keep their arms crossed at the front of the trunk and touch the chair with the gluteus in each movement) [27].

(4) Blood Pressure. BP was measured using an automatic arm digital pressure gauge (OMRON brand, model HEM-7113), which uses the oscillometric measurement method. The measurement was performed at the first contact, with the participant remaining at rest for at least five minutes, according to the Brazilian Guidelines for Hypertension VII [4].

(5) Blood Analysis. Blood samples were drawn, and plasma was separated by 2000g centrifugation for 4 min at 24°C. Plasma samples were used for the analysis of oxidative stress, antioxidant activity, and nitrite concentration. Whole blood was used for the genotyping.

(5.1) Nitrite (NO2) Concentrations. Nitrite concentrations in plasma were used for the indirect determination of NO. Nitrite is the first product of the reaction of nitric oxide with oxygen. Plasma aliquots were analyzed in duplicate for their nitrite content using ozone-based chemiluminescence. Briefly, 300 μl of plasma samples was injected into a solution of acidified tri-iodide, purging with nitrogen in line with a gas-phase chemiluminescence NO analyzer (Sievers Model 280 NO Analyzer, Sievers, USA). The data were analyzed using the Origin Lab 6.1 program [28].

(5.2) Malondialdehyde (MDA). Malondialdehyde is one of the most abundant aldehydes resulting from tissue lipid peroxidation and can be considered a marker of global oxidative stress. In addition, it is related to the aging process [29]. This analysis was carried out according to the method proposed by Gerard-Monnier et al. [30], with some adaptations. For the determination of MDA in the plasma, 100 μl of plasma was used. To this, 300 μl of 10 mM solution of 1-methylphenylindole in acetonitrile and methanol (2 : 1, *v*/*v*) and 75 μl HCl PA (37%) were added. Soon after, the tubes were vortexed and incubated in a water bath at 45°C for 40 minutes. After the bath, the samples were cooled on ice and then the tubes were centrifuged at 4000 rpm for 10 minutes. From the supernatant, absorbance was read in an apparatus (SpectraMax M3, Molecular Devices, USA) with a wavelength of 586 nm. The concentration of MDA was calculated using a hydrolyzed 1,1,3,3-tetramethoxypropane (TMP) curve [31].

(5.3) Antioxidant Activity. Antioxidant molecules prevent or inhibit the harmful reactions of reactive oxygen species. Plasma concentrations of different antioxidants can be measured in the laboratory separately, but the measurements are time-consuming, costly, labor-intensive, and often require complicated techniques. As the effect of these different antioxidants is additive, an alternative is to measure the total antioxidant capacity (TAC). This variable was measured using a method based on 2,2-azinobis 3-ethylbenzthiazoline-6-sulfonate (ABTS) by absorbance reading [32]. The other variable related to the antioxidant profile was glutathione (GSH), which plays a central role in the defense of cells against oxidative stress. This tripeptide is found intracellularly at high concentrations, primarily in all aerobic organisms. Glutathione is the most abundant low molecular weight cellular thiol. This analysis was performed using the method described by Costa et al. [33]. The concentration of glutathione was calculated using a reduced standard glutathione curve. Absorbance of these two experiments was also read in the same apparatus (SpectraMax M3, Molecular Devices, USA).

(5.4) Genotyping. The process of DNA extraction adopted was salting out [34]. The purity and DNA concentration of the sample were evaluated by spectrophotometry (BioDrop μlite PC). The ratios 260/230 and 280/260 were evaluated, and the level of purity adopted as a satisfactory minimum was 1.7 for each of the ratios.

(5.4.1) -786T>C. The NOS3 polymorphisms at position -786T>C (rs2070744) were determined by real-time PCR (qPCR), as previously described. The reaction was carried out using Custom TaqMan allele discrimination assay (resynthesis part number AH5I790, Thermo Fisher, USA) and TaqMan genotyping master mix (Applied Biosystems, USA). Preparation of the reactions was performed according to the manufacturer's specifications for each sample: 1x of the master mix, 1x of TaqMan genotyping assay, and 50 ng of template DNA in 10 μl final volume. Real-time PCR was performed on StepOnePlus equipment (Applied Biosystems, USA) and analyzed with the manufacturer's software [35].

(5.4.2) 894G>T (Glu298Asp). Genotypes for the 894G>T (Glu298Asp) (rs1799983) polymorphisms were amplified by the PCR, as previously described, using the following flanking primers—sense: 5′-CATGAGGCTCAGCCCCAGA AC-3′ and antisense: 5′-AGTCAATCCCTTTGG TGCTCAC -3′. The amplicon was digested overnight at 37°C using 2 U *Mbo*I enzyme followed by electrophoresis for 3 h on 2.5% agarose gel. The G allele yields a fragment of 248 bp, and the T allele yields fragments of 190 and 58 bp [36].

(5.4.3) Intron 4. Genotypes for the variable number of tandem repeats (VNTR) polymorphism in intron 4 were determined by PCR, as previously described, using the primers 5′-AGG CCC TAT GGT AGT GCC TTT-3′ (sense) and 5′-TCT CTT AGT GCT GTG GTC AC-3′ (antisense) and fragment separation by electrophoresis for 3 h in 8% polyacrylamide gels. Fragments of 393 and 420 bp correspond to the endothelial nitric oxide synthase (eNOS) alleles 4a and 4b, respectively [37].

2.3. Statistical Analysis. The data analysis performed to achieve the proposed objectives for association and comparison among the interested variables was the Fisher's exact test to verify the statistical association of categorical variables with time (food intake) and linear mixed-effects models (random and fixed effects) adjusted for age, level of physical activity, and socioeconomic status. The classes of linear mixed-effects models are extensions of linear regression models for data collected and summarized in groups. These are used in the analysis of data in which the answers are grouped (repeated measures for the same individual); as we had information for the same individual in the pre and post time and the assumption of independence between observations in the same group is not adequate [38]. This model assumes that the residue obtained by means of the difference between the values predicted by the model and the observed values has a normal distribution with mean zero and constant variance. In these analyses, a level of significance of 5% was considered and the analysis was performed in SAS software (version 9.2) using the PROC MIXED.

3. Results

3.1. General Data. The mean age of the participants was 61.9 (8.7) (38.5% of the participants were between 50 and 59 years old and 61.5% were between 60 and 80 years old). Regarding the weekly frequency of intake of various

Table 1: Distribution of the genotypes of participants.

Genotypes	($n = 52$)	%
-786T>C		
TT	25	48.1
TC	23	44.2
CC	4	7.7
894G>T (Glu298Asp)		
GluGlu	30	57.7
GluAsp	20	38.5
AspAsp	2	3.8
Intron 4b/a		
4b4b	35	67.3
4b4a	17	32.7
4a4a	0	0

foods [25], it was verified that there were no differences between the values before and after the multicomponent physical training intervention, showing that food habits remained similar during the intervention. In the same way, percentages of individuals using hypertension drugs were homogeneous among the groups for independent genotypes [-786: TT = 32% and TC + CC = 40.7%; Glu298Asp: GluGlu = 36.6% and GluAsp + AspAsp = 36.4%; and intron 4: bb = 34.3% and ba + aa = 41.2%] and haplotypes (interaction) [H1 = 33.33%; H2 = 25%; H3 = 33.33%; H6 = 30.76%; H7 = 44.44%; and H8 = 40%].

Table 1 summarizes the frequency distribution of NOS3 genotypes. The distribution of the genotypes of participants across groups indicates that no participant was classified as 4a4a for intron 4b/a. The distribution of genotypes for the three polymorphisms showed no deviation from Hardy-Weinberg equilibrium ($p > 0.05$) with allele frequencies of 0.70 and 0.30 for T and C allele at position -786T>C, respectively. The allele frequencies for G and 4b at position 894G>T (Glu298Asp) and intron 4b/a were 0.77 and 0.84, respectively. Correspondingly, the allele frequencies for T and 4a were 0.23 and 0.16, respectively.

Multicomponent training for 12 weeks decreased body mass [71.1 (11.2); 69.4 (10.9)—kg; $p < 0.05$] and BMI after the intervention compared to baseline [28.4 (4.7); 27.7 (4.8)—kg/m^2; $p < 0.05$]. Table 2 shows that multicomponent training was effective in promoting a reduction in systolic and diastolic BP and malondialdehyde, as well as an increase in nitrite concentration, total antioxidant capacity, the flexion elbow test, sit and stand up test, and six-minute walking test. No changes were found in glutathione.

For the NOS3 genotype analysis, participants were divided into ancestral genotype groups [-786: TT ($n = 25$), Glu298Asp: GluGlu ($n = 30$), and intron 4: bb ($n = 35$)] and variant genotype groups [-786: TC + CC ($n = 27$), Glu298Asp: GluAsp + AspAsp ($n = 22$), and intron 4b/a: 4b4a + 4a4a ($n = 17$)] for each polymorphism.

3.2. Genotype Analyses. Figures 2, 3, and 4 present the effects of 12 weeks of multicomponent training on systolic and diastolic BP and nitrite concentration of participants for the

Table 2: Effect of multicomponent training on blood pressure, nitrite concentration, oxidative stress, antioxidant activity, and physical fitness of 52 older adult women.

	Before	After	Δ%
Age (years)	61.9 (8.7)		
SBP (mmHg)	132 (15)	124 (14)*	−6.3
DBP (mmHg)	83 (9)	78 (8)*	−6.1
NO_2 (nM)	112 (55)	141 (69)*	26.3
MDA (μM)	4.9 (1.6)	2.6 (1.2)*	−46.6
GSH (μM)	3.5 (0.6)	3.6 (0.8)	2.8
TAC (μM)	0.32 (0.11)	0.39 (0.12)*	21.9
EFT (reps)	17.1 (3.3)	20.1 (4.1)*	17.7
SS (reps)	14.4 (3.4)	17.8 (4.8)*	23.1
6 min WT (m)	512 (62)	559 (60)*	9.3

Data are reported as means (SD) before and after 12 weeks of multicomponent training for 52 women. SBP = systolic blood pressure; DBP = diastolic blood pressure; NO_2 = nitrite concentration; MDA = malondialdehyde; GSH = glutathione; TAC = total antioxidant capacity; EFT = elbow flexion test; SS = sit and stand up; 6 min WT = six-minute walk test. *$p < 0.05$ compared with before intervention (the same group). Linear mixed-effects models.

NOS3 polymorphisms at positions -786T>C, Glu298Asp, and intron 4b/a, respectively. Variables did not differ between groups under basal conditions. Multicomponent training supported a statistical and significant reduction in both systolic and diastolic BP in all groups (-786T>C, Glu298Asp, and intron 4b/a), but the variant genotype groups descriptively presented trends towards a smaller magnitude of improvement (Δ%) than the ancestral genotype groups. Similarly, all ancestral and variant genotype groups at position -786T>C and Glu298Asp presented improved nitrite concentration, but the variant genotype groups descriptively presented trends towards a smaller magnitude (Δ%). In the intron 4b/a groups, 4b4b improved the nitrite concentration while multicomponent training did not affect this variable in the variant genotype group (4b4a+4a4a).

Tables 3, 4, and 5 show the effects of 12 weeks of multicomponent training on oxidative stress, antioxidant activity, and physical fitness of participants for the NOS3 polymorphisms at positions -786T>C, Glu298Asp, and intron 4b/a, respectively. MDA decreased in all groups (-786T>C, Glu298Asp, and intron 4b/a) in a similar fashion (ranging between 38.2 up to 51.8%). Regarding the antioxidant activity, all groups, with the exception of the 4b/a variant genotype group (4b4a+4a4a), increased total antioxidant capacity; for glutathione, only the ancestral genotype group showed improvement (-786: TT, Glu298Asp: GluGlu, and intron 4: 4b4b). Furthermore, 12 weeks of multicomponent training affected physical fitness in all groups (-786T>C, Glu298Asp, and intron 4b/a) which presented better results after the intervention in the elbow flexion, sit and stand up, and six-minute walking tests.

3.3. Haplotype Analysis. The haplotype frequencies and effects of 12 weeks of multicomponent training on the

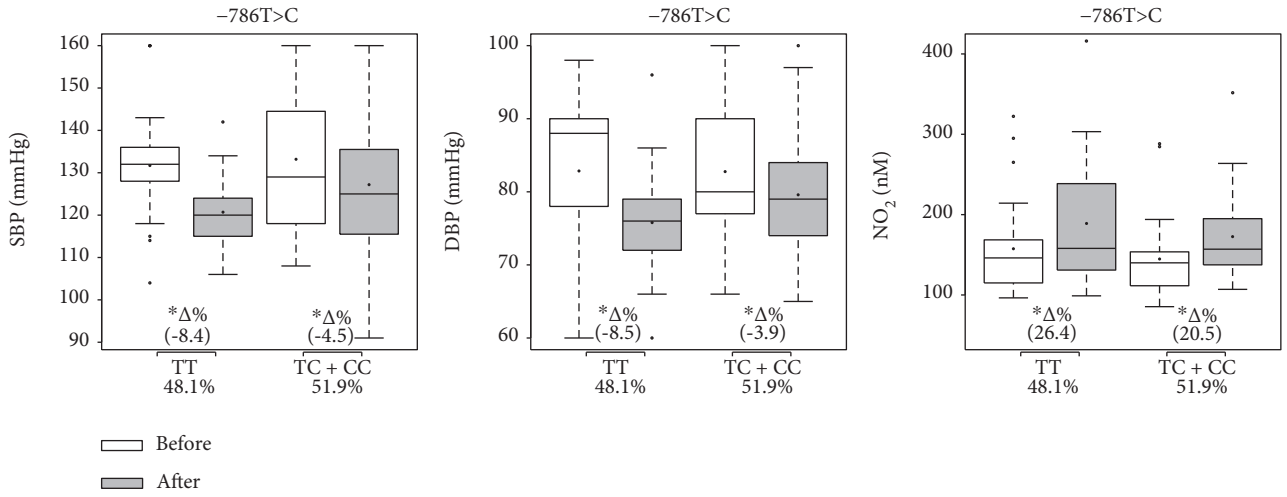

Figure 2: Effects of 12 weeks of multicomponent training on the systolic blood pressure (SBP), diastolic blood pressure (DBP), and nitrite concentration (NO_2) of 52 older adult women with or without variant genotypes for the eNOS gene at position -786T>C. $^*p < 0.05$ compared with before intervention (the same group). Linear mixed-effects models.

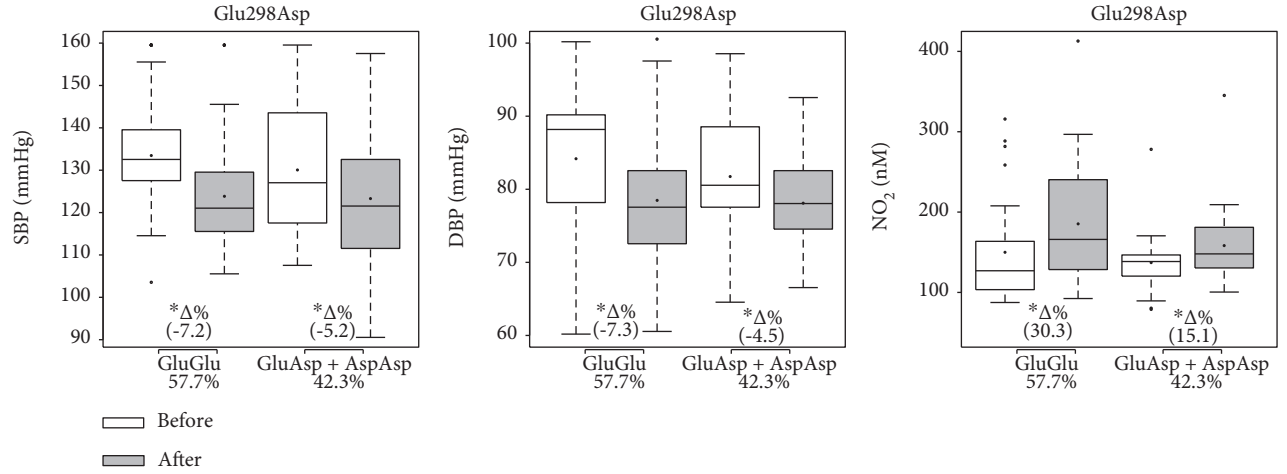

Figure 3: Effects of 12 weeks of multicomponent training on the systolic blood pressure (SBP), diastolic blood pressure (DBP), and nitrite concentration (NO_2) of 52 older adult women with or without variant genotypes for the eNOS gene at position 894G>T (Glu298Asp). $^*p < 0.05$ compared with before intervention (the same group). Linear mixed-effects models.

systolic and diastolic BP and nitrite concentration of participants grouped by haplotype (H1–H8) are illustrated in Figures 5, 6, and 7, respectively. There were no participants in H4 or H5. No differences between groups were found in nitrite concentration or systolic and diastolic BP before the intervention. The H1 group (all ancestral alleles) was the only group that presented a decrease in systolic and diastolic BP and an increase in nitrite concentration. Groups H2, H3, and H7 only presented a reduction in systolic BP, while group H6 presented a decrease in both systolic and diastolic BP. H7 also resulted in increased nitrite concentration. It is important to note that haplotypes that descriptively demonstrated trends towards the lowest magnitudes of SBP decrease, H6 and H7, presented two variant alleles. Multicomponent training did not affect the results of the H8 group.

Table 6 presents the haplotype analysis showing the effect of multicomponent training on oxidative stress and antioxidant activity. At baseline, all groups were different from the H3 group for malondialdehyde. All groups presented decreased MDA values after the intervention. For antioxidant activity, only the H1 group presented improvement in the two variables (total antioxidant capacity and glutathione). The H6 group increased only the total antioxidant capacity.

As in the isolated analysis of each polymorphism, the analysis grouped by haplotypes did not influence the effects of multicomponent training on physical fitness. The H1, H2, H3, H6, H7, and H8 presented similar improvements in the motor tests performed (data not shown) indicating that the multicomponent training was effective.

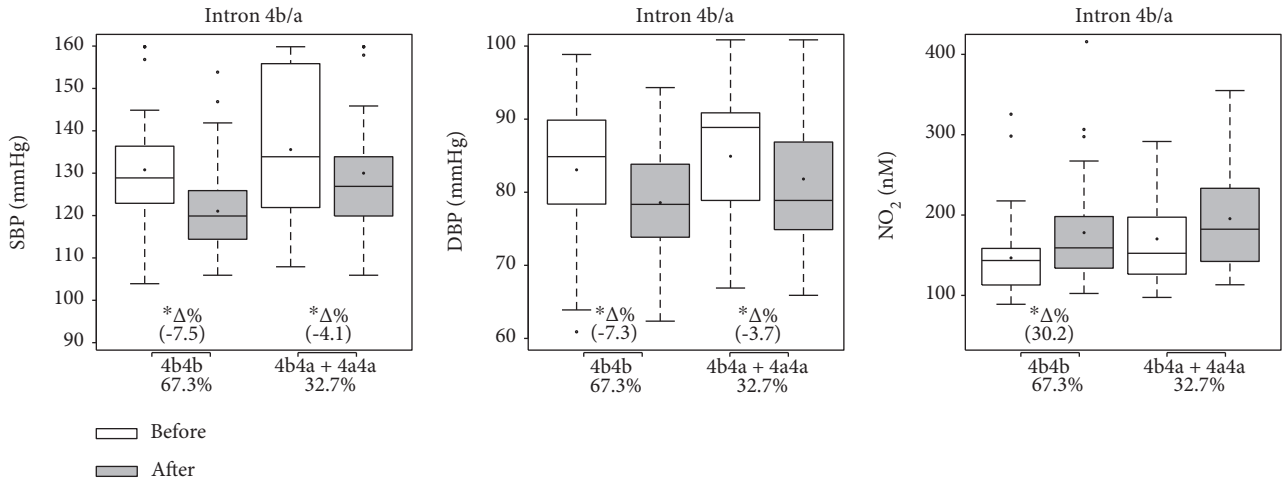

Figure 4: Effects of 12 weeks of multicomponent training on the systolic blood pressure (SBP), diastolic blood pressure (DBP), and nitrite concentration (NO_2) of 52 older adult women with or without variant genotypes for the eNOS gene intron 4b/a. $^*p < 0.05$ compared with before intervention (the same group). Linear mixed-effects models.

Table 3: Effects of 12 weeks of multicomponent training on oxidative stress, antioxidant activity, and physical fitness of 52 older adult women with or without variant genotypes for the eNOS gene at position -786T>C.

	TT (25)			TC + CC (27)		
	Before	After	Δ%	Before	After	Δ%
Age (years)	63.7 (9)			60.7 (8.4)		
MDA (μM)	5 (1.83)	2.7 (1.5)*	−45.2	4.9 (1.4)	2.5 (0.8)*	−47.7
GSH (μM)	3.6 (0.5)	3.8 (0.9)*	6.1	3.5 (0.7)	3.5 (0.8)	−0.7
TAC (μM)	0.32 (0.11)	0.38 (0.11)*	18.8	0.35 (0.12)	0.40 (0.12)*	14.3
EFT (reps)	16.5 (3.1)	19.6 (3.6)*	18.7	17.6 (3.4)	20.6 (4.5)*	16.8
SS (reps)	14 (3.6)	18.1 (5.3)*	28.8	14.8 (3.2)	17.5 (4.4)*	18
6 min WT (m)	508 (70)	559 (75)*	10.1	516 (56)	560 (43)*	8.6

Data are reported as means (SD) before and after 12 weeks of multicomponent training for 52 women. MDA = malondialdehyde; GSH = glutathione; TAC = total antioxidant capacity; EFT = elbow flexion test; SS = sit and stand up; 6 min WT = six-minute walk test. $^*p < 0.05$ compared with before intervention (the same group). Linear mixed-effects models.

4. Discussion

4.1. General Data. The general results of this study demonstrated a positive effect of training on systolic and diastolic blood pressure, nitrite concentration, redox status, and physical fitness, independent of the genotype (Table 2). Multicomponent training was chosen for the intervention as physical changes related to the aging process affect various motor skills and abilities. In addition, multicomponent training includes the training of various capacities and motor skills (aerobic capacity, muscular strength, flexibility, coordination, agility, and balance) and is in accordance with ACSM guidelines [3]. This training protocol has been demonstrated to be more effective in promoting significant improvements in physical fitness than other investigated exercise protocols (aerobic, concurrent training, and strength) [6].

Physical training can increase eNOS activity and antioxidant activity and decrease values of BP [9, 14]. However, the influence of physical exercise on plasma nitrite concentration is still controversial [36]. Some studies demonstrate an increase in NO bioavailability and production in response to acute and chronic physical exercise [36, 39], while others indicate that plasma nitrate/nitrite levels are unchanged after long-term aerobic exercise training in older adults [16]. In the present investigation, it was possible to observe that 12 weeks of multicomponent training was effective for increasing nitrite concentration and lowering BP, besides improving oxidative stress, antioxidant activity, and physical fitness.

4.2. Genotype Analyses. The main finding of the genotype analysis was that in the blood pressure response, nitrite concentration and antioxidant activity seemed to be associated with the genotype. In the current study, it was possible to verify in the isolated analysis that the groups with the variant genotypes did not present improvement, or when they did present improvement, descriptively it was of a trend towards a lower magnitude (Δ%) of decrease in systolic and diastolic BP and increase in nitrite concentration compared with the ancestral genotype groups [-786T>C, 894G>T (Glu298Asp), and intron 4b/a]. Only

Table 4: Effects of 12 weeks of multicomponent training on oxidative stress, antioxidant activity, and physical fitness of 52 older adult women with or without variant genotypes for the eNOS gene at position 894G>T (Glu298Asp).

	GluGlu (30)			GluAsp + AspAsp (22)		
	Before	After	Δ%	Before	After	Δ%
Age (years)	62 (8.4)			61.8 (9.2)		
MDA (μM)	5 (1.7)	2.8 (1.4)*	−43.1	4.8 (1.5)	2.3 (0.7)*	−51.8
GSH (μM)	3.6 (0.5)	3.7 (0.8)*	4.8	3.5 (0.7)	3.5 (0.8)	0
TAC (μM)	0.34 (0.12)	0.40 (0.12)*	17.6	0.32 (0.08)	0.39 (0.12)*	21.9
EFT (reps)	17.5 (3.1)	20.4 (4.2)*	16.8	16.6 (3.5)	19.7 (3.9)*	18.9
SS (reps)	15.3 (3.6)	18.6 (4.9)*	21.5	13.2 (2.7)	16.6 (4.5)*	25.4
6 min WT (m)	515 (66)	562 (67)*	9.1	507 (58)	556 (49)*	9.6

Data are reported as means (SD) before and after 12 weeks of multicomponent training for 52 women. MDA = malondialdehyde; GSH = glutathione; TAC = total antioxidant capacity; EFT = elbow flexion test; SS = sit and stand up; 6 min WT = six-minute walk test. *$p < 0.05$ compared with before intervention (the same group). Linear mixed-effects models.

Table 5: Effects of 12 weeks of multicomponent training on oxidative stress, antioxidant activity, and physical fitness of 52 older adult women with or without variant genotypes for the eNOS gene intron 4b/a.

	4b4b (35)			4b4a + 4a4a (17)		
	Before	After	Δ%	Before	Before	Δ%
Age (years)	61.7 (8.8)			62.1 (8.7)		
MDA (μM)	4.9 (1.5)	2.4 (0.8)*	−50.8	4.9 (1.9)	3.1 (1.7)*	−38.2
GSH (μM)	3.6 (0.6)	3.8 (0.9)*	5.3	3.4 (0.5)	3.3 (0.5)	−2.4
TAC (μM)	0.31 (0.09)	0.40 (0.13)*	29	0.34 (0.14)	0.38 (0.10)	11.8
EFT (reps)	16.7 (3)	19.8 (3.6)*	18.1	17.8 (3.7)	20.8 (4.9)*	16.9
SS (reps)	13.9 (3.2)	17.7 (5)*	27.4	15.6 (3.4)	17.9 (4.4)*	15.1
6 min WT (m)	511 (66)	558 (63)*	9.3	514 (57)	563 (55)*	9.4

Data are reported as means (SD) before and after 12 weeks of multicomponent training for 52 women. MDA = malondialdehyde; GSH = glutathione; TAC = total antioxidant capacity; EFT = elbow flexion test; SS = sit and stand up; 6 min WT = six-minute walk test. *$p < 0.05$ compared with before intervention (the same group). Linear mixed-effects models.

the groups with ancestral genotype in the three NOS3 polymorphisms studied showed improvement in glutathione, while the group with variant genotypes of intron 4b/a (4b4a+4a4a) was the only group that did not present improvement in total antioxidant capacity among all the studied groups; all groups improved malondialdehyde values. Few studies have been carried out seeking to evaluate similar aspects to the present study, and the findings are contradictory. Esposti et al. found similar improvements after eight weeks of aerobic exercise training in systolic and diastolic BP in all groups [with and without variants in -786T>C, 894G>T (Glu298Asp), and intron 4b/a]. However, they did not find improvement in malondialdehyde or nitrite/nitrate concentration [16]. Silva et al. investigated the individual and combined effects of three variants of the eNOS gene (-786T>C, Glu298Asp, and intron 4b/a) on vascular reactivity before and after exercise in male and female adults. The authors showed that participants with the Glu298Asp variant had lower vascular reactivity than wild counterparts [40]. These contradictory results can be explained by differences in the populations studied, ages of participants, analyses performed, and type of exercise program.

4.3. Haplotype Analysis. In the haplotype analysis, as in the genotype analysis, the response to multicomponent training in decreasing blood pressure or increasing nitrite concentration and antioxidant activity was also associated with the haplotypes. The H8 group, with the three variant alleles, showed improvement only in malondialdehyde and did not demonstrate an increase in nitrite concentration or antioxidant activity (total antioxidant capacity and glutathione) or a reduction in systolic and diastolic BP. However, the H1 group, carrying all three ancestral alleles, demonstrated improvement in nitrite concentration, glutathione and total antioxidant capacity, and a reduction in malondialdehyde and systolic and diastolic BP. The abovementioned study by Silva et al. regarding the combined impact of three variants in the eNOS showed that participants carrying Glu298Asp variants reduced the exercise-mediated increase in vascular reactivity, particularly when it occurred concomitantly with the -786T>C variants [40]. Zago et al. found reduced nitrite concentration in the group with eNOS genotype variants (-786: TC + CC and Glu298Asp: GluAsp + AspAsp); however, this group increased nitrite concentration and decreased BP, after eight weeks of aerobic exercise training [36]. Furthermore, Sponton et al. showed that the NOS3

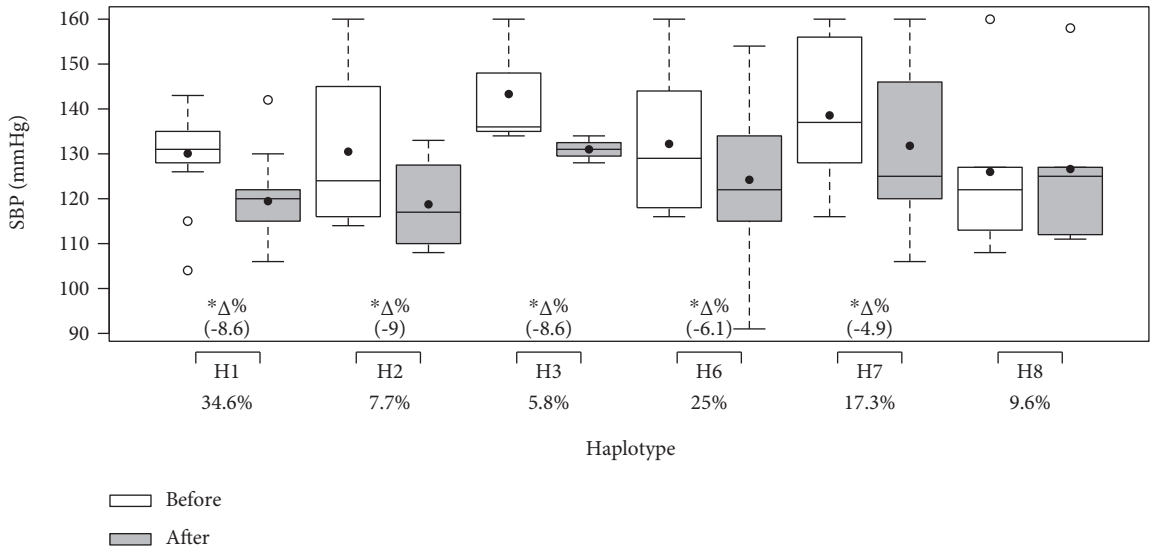

Figure 5: Effects of 12 weeks of multicomponent training on the systolic blood pressure (SBP) of 52 older adult women grouped by haplotype (H1–H8). $^{*}p < 0.05$ compared with before intervention (the same group). Linear mixed-effects models.

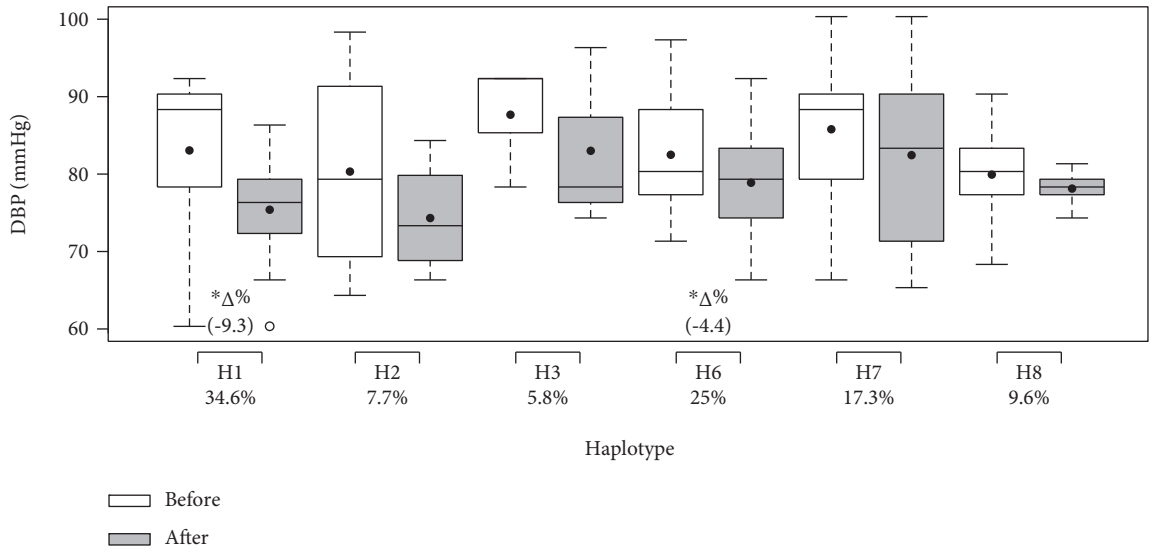

Figure 6: Effects of 12 weeks of multicomponent training on the diastolic blood pressure (DBP) of 52 older adult women grouped by haplotype (H1–H8). $^{*}p < 0.05$ compared with before intervention (the same group). Linear mixed-effects models.

polymorphism for intron 4b/a mitigated the beneficial effects of aerobic exercise training for systolic and diastolic BP but was not associated with nitrite/nitrate levels or redox state. Paradoxical responses were found for positions -T786T>C and G894G>T (Glu298Asp) for the NOS3 polymorphisms. Their data showed that two haplotype groups, H3 and H5, were unresponsive in lowering systolic and diastolic BP in response to exercise training in both office measurements and ambulatory BP monitoring [17].

Similarly to the results in the current study, Sponton et al. presented a similar response in groups H1 and H6, without any of the three variants and with two variants (-786T>C and Glu298Asp), respectively, showing improvement in systolic and diastolic BP and antioxidant activity [17]. However, in contrast, the current study also demonstrated improvement in nitrite concentrations after the intervention in groups H1 and H7 and improved levels of malondialdehyde in all groups.

Few studies have investigated the influence of NOS3 polymorphisms on the response to a physical training program [16, 17, 36]. While da Silva et al. [15] have already established an interaction of the three polymorphisms (haplotypes) with basal nitrite concentration values, Sponton et al. [17] investigated the response of physical training program in association with the NOS3 haplotypes.

The novelty of the current study is the use of a multicomponent program in the physical training intervention, and, besides the isolated analysis of each

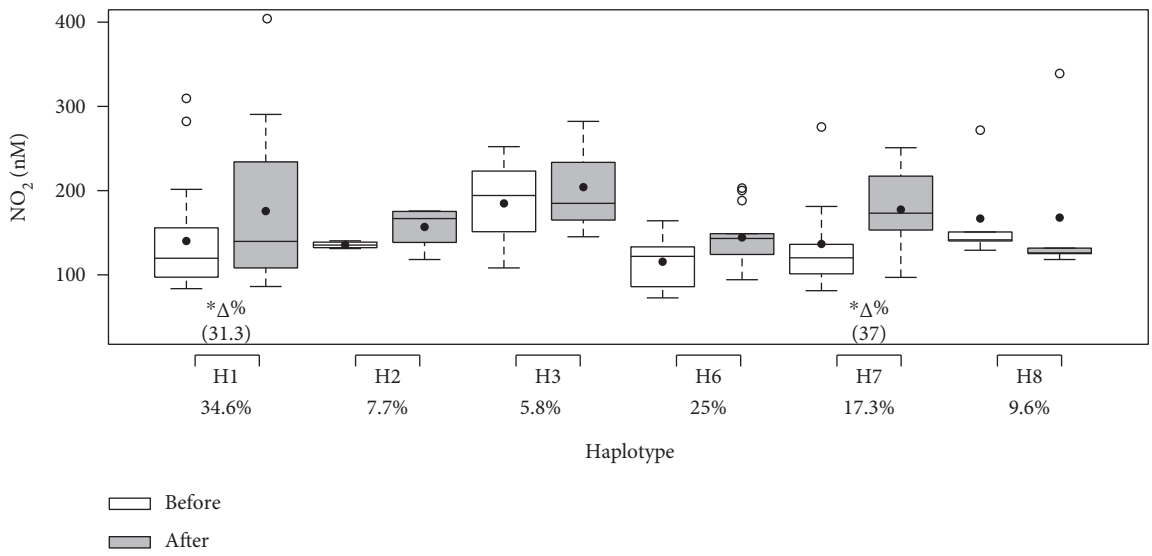

Figure 7: Effects of 12 weeks of multicomponent training on the nitrite concentration (NO_2) of 52 older adult women grouped by haplotype (H1–H8). $^*p < 0.05$ compared with before intervention (the same group). Linear mixed-effects models.

Table 6: Effects of 12 weeks of multicomponent training on oxidative stress and antioxidant activity of 52 older adult women grouped by haplotype (H1–H8).

	Haplotypes			MDA (μM) before	MDA (μM) after	Δ%	GSH (μM) before	GSH (μM) after	Δ%	TAC (μM) before	TAC (μM) after	Δ%
	-786T>C	Intron 4	Glu298 Asp									
H1	T	4b	Glu	4.8 (1.5)[a]	2.5 (0.8)*	−78.8	3.6 (0.5)	3.9 (1)*	8.3	0.33 (0.10)	0.40 (0.12)*	21.2
H2	T	4b	Asp	4 (1.2)[a]	1.9 (0.5)*	−53.4	3.7 (0.4)	3.8 (0.8)	1.3	0.30 (0.05)	0.35 (0.02)	16.7
H3	T	4a	Glu	7.3 (3.2)	5.5 (2.7)*	−24.4	3.6 (0.7)	3.6 (0.7)	0	0.33 (0.19)	0.36 (0.15)	9.1
H4	T	4a	Asp	—	—	—	—	—	—	—	—	—
H5	C	4b	Glu	—	—	—	—	—	—	—	—	—
H6	C	4b	Asp	5.3 (1.6)[a]	2.5 (0.8)*	−52.7	3.6 (0.8)	3.7 (0.9)	1.9	0.33 (0.09)	0.42 (0.15)*	27.3
H7	C	4a	Glu	4.5 (1.1)[a]	2.7 (1.1)*	−40.7	3.4 (0.4)	3.4 (0.6)	−0.3	0.37 (0.14)	0.41 (0.11)	10.8
H8	C	4a	Asp	4.3 (1.2)[a]	2.2 (0.3)*	−47.4	3.2 (0.4)	3 (0.2)	−8.1	0.29 (0.10)	0.35 (0.08)	20.7

Data are reported as means (SD) before and after 12 weeks of multicomponent training for 52 women. MDA = malondialdehyde; GSH = glutathione; TAC = total antioxidant capacity. $^*p < 0.05$ compared with before intervention (the same group). [a]$p < 0.05$ versus H3 before intervention. Linear mixed-effects models.

polymorphism, we performed the interaction analysis (haplotypes) among polymorphisms and analyzed the influence on the response to multicomponent training—as aforementioned, a previous study performed aerobic exercise. Our results showed that the presence of polymorphisms in the isolated analysis of each polymorphism affected the response of decreased systolic and diastolic BP and improved nitrite concentration and antioxidant activity. In the interaction (haplotypes) analysis, the group composed of all ancestral alleles (H1) was the only one to show improvement in all variables studied (systolic and diastolic BP, nitrite concentration, redox status, and physical fitness), while the group composed of all variant alleles (H8) only showed improvement in some variables of redox status and physical fitness. Therefore, preventive actions to change lifestyles for women's health are crucial since women live longer than men and effective prevention could decrease the high cost to the health care system for this population.

In conclusion, our data demonstrated few differences between the groups at baseline in both analyses, in each polymorphism alone and in the interaction between them (haplotypes). However, it was possible to observe a positive effect of training on the studied health parameters and the response to decreased blood pressure and improved nitrite concentration and antioxidant activity seemed to be associated with the genotype. Studies in this area are important as it could be possible in the future to consider genetic variants when choosing a more suitable exercise training program for each individual.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Exercise Training Restores Cardiac MicroRNA-1 and MicroRNA-29c to Nonpathological Levels in Obese Rats

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We previously reported that aerobic exercise training (AET) consisted of 10 weeks of 60-min swimming sessions, and 5 days/week AET counteracts CH in obesity. Here, we evaluated the role of microRNAs and their target genes that are involved in heart collagen deposition and calcium signaling, as well as the cardiac remodeling induced by AET in obese Zucker rats. Among the four experimental Zucker groups: control lean rats (LZR), control obese rats (OZR), trained lean rats (LZR + TR), and trained obese rats (OZR + TR), heart weight was greater in the OZR than in the LZR group due to increased cardiac intramuscular fat and collagen. AET seems to exert a protective role in normalizing the heart weight in the OZR + TR group. Cardiac microRNA-29c expression was decreased in OZR compared with the LZR group, paralleled by an increase in the collagen volumetric fraction (CVF). MicroRNA-1 expression was upregulated while the expression of its target gene NCX1 was decreased in OZR compared with the LZR group. Interestingly, AET restored cardiac microRNA-1 to nonpathological levels in the OZR-TR group. Our findings suggest that AET could be used as a nonpharmacological therapy for the reversal of pathological cardiac remodeling and cardiac dysfunction in obesity.

1. Introduction

Obesity results from a combination of excessive food energy intake, lack of physical activity, and genetic susceptibility [1–4]. Data from the World Health Organization (WHO) in 2014 showed that 1.9 billion people worldwide are overweight and 600 million are obese, causing 2.8 million deaths annually [5]. Obesity induces systemic inflammation and contributes to the development of atherosclerosis and cardiovascular diseases, which cooperate with the pathological cardiac hypertrophy (CH) phenotype [6, 7].

Cardiac remodeling induced by obesity is a compensatory adaptation to volume overload and/or continuous pressure imposed on the heart [8]. Studies in obese Zucker rats show an increase in left ventricular mass accompanied by pathological CH molecular markers such as β-myosin heavy chain (β-MHC), atrial natriuretic factor (ANF), α-skeletal actin; cardiac dysfunction; and ultimately, heart failure [8–10]. The diastolic dysfunction in obesity is induced both by increased collagen content and by damage to calcium signaling pathways mediated by proteins of intracellular calcium removal, such as SERCA-2a and the sodium/calcium exchanger NCX1 [11, 12].

Aerobic exercise training (AET) is a nonpharmacological strategy for preventing and treating obesity and cardiovascular disease [4, 13–16]. We have recently reported that AET reverses pathological cardiac remodeling in hypertensive and obese rats [4, 17, 18]. AET induces physiological CH by increasing the ratio of α/β-MHC and decreasing cardiac collagen content, improving ventricular compliance [19, 20]. Furthermore, AET leads to the restoration of normal

calcium handling protein levels, potentially contributing to physiological cardiac hypertrophy and improved cardiac function [21].

MicroRNAs are regulators in various physiological and pathological processes, such as cardiac remodeling [22]. MicroRNAs are small endogenous RNAs that negatively regulate the expression of their target genes [23]. Previous data reported by our group showed that physiological CH induced by different amounts of AET is related to reduced cardiac collagen expression via elevated cardiac microRNA-29c levels in healthy rats [24]. In addition, Melo et al. [25] showed that AET restored the levels of microRNA-29c in infarcted rats, contributing to a reduction in cardiac collagen content. Interestingly, studies have shown the involvement of microRNAs in the regulation of calcium signaling pathways in the heart, indicating NCX1 as a target of microRNA-1 [26–28]. However, the effects of AET on cardiac microRNAs and cardiac remodeling in obesity are not fully established.

We investigated whether obesity increases cardiac collagen deposition and calcium handling proteins regulated by microRNAs and if AET restores these parameters, consequently contributing to the conversion of pathological into physiological CH in obesity.

2. Materials and Methods

2.1. Experimental Groups. Twenty male Zucker rats (20 weeks of age) were assigned to four groups ($n = 5$ each): control lean Zucker rats (LZR), trained lean Zucker rats (LZR + TR), control obese Zucker rats (OZR), and trained obese Zucker rats (OZR + TR). The animals were housed in cages, and food and water were provided ad libitum. The room temperature was 23°C, and an inverted 12 : 12 h dark-light cycle was maintained throughout the experiment.

All protocols and surgical procedures used were in accordance with the guidelines of the Brazilian College for Animal Experimentation and were approved by the Ethics Committee (1023/07) of the Biomedical Science Institute of the University of Sao Paulo.

2.2. Exercise Training Protocol. Swimming training was performed as described previously [4]. Animals were trained in a swimming apparatus specially designed to allow individual exercise training of rats in warm water at 30–32°C. Physical training consisted of swimming sessions of 60-min duration, five times a week, for 10 weeks, with 4% of body weight workload hold in tail [tail weight − % body weight (BW)]. All animals were weighed once a week and the workload was adjusted according to BW variations. LZR and OZR were placed in the swimming apparatus for 10 minutes twice a week without applying a workload. This protocol consists of a low/moderate intensity and long training period and is effective in promoting cardiovascular adaptations and increases in muscle oxidative capacity [19].

2.3. Tissue Harvesting. Twenty-four hours after the last training session, and after twelve hours of fasting, the rats were killed by quick decapitation. The tissues and tibia were harvested, the heart (H) was weighed, and carefully, the left ventricle (LV free wall plus septum) and right ventricle (RV) were dissected. Epididymal and retroperitoneal fats were also weighed and normalized by the tibial length (TL) of each animal. The tissues were frozen at −80°C until biochemical and molecular analysis was performed.

2.4. Cardiac Morphometric Analysis. For cardiomyocyte (CMO) diameter analysis, the LV was fixed in Tissue-Tekand frozen in liquid nitrogen. The tissues were then fixed in 6% formaldehyde, embedded in paraffin, cut into 10 μm sections at the level of the papillary muscle in a cryostat (−20°C), and subsequently stained with hematoxylin and eosin for the visualization of cellular structures. Two randomly selected sections from each animal were visualized by light microscopy using an oil immersion objective with calibrated magnification (×400). CMOs with visible nuclei and intact cellular membranes were chosen for diameter determination. The width of individually isolated cardiomyocyte displayed on a viewing screen was manually traced across the middle of the nucleus with a digitizing pad, and the diameter was estimated using a computer-assisted image analysis system (Quantimet 520; Cambridge Instruments, Woburn, MA). For each animal, ~20 visual fields were analyzed. The results were expressed as micrometers (μm).

The myocardial interstitial collagen volumetric fraction (CVF) was determined using the Picrosirius red prepared tissues, as reported previously [13]. In brief, 20 fields were selected from sections placed in a projection microscope (×200), and interstitial collagen was determined using a computer-assisted image analysis system (Quantimet 520; Cambridge Instruments). The CVF was calculated as the sum of all connective tissue areas divided by the sum of all muscle areas in all fields. Perivascular tissues (reparative fibrosis) were specifically excluded from this determination. The results were expressed as μm for area.

LV intramuscular fat was determined using oil red staining. The tissues were cut into 7 μm sections in a cryostat (−20°C) and fixed in 3.7% formalin for one hour. Subsequently, the tissues were washed with distilled water and then stained with a mixture of 12 ml working solution (500 mg oil red added to 100 ml of aqueous 60% triethyl phosphate [Fluka]) and 8 ml of deionized water for 30 seconds. The slides were assembled with the aid of glycerol [10% glycerol in 10 mM Tris-HCl, pH 8.5]. The area comprising intramuscular fat was determined using a computer-assisted image analysis system (Quantimet 520; Cambridge Instruments). The results were expressed as % of fiber area [29, 30].

2.5. Molecular Analysis

2.5.1. mRNA and MicroRNA Quantification Using Real-Time PCR. The relative expression of COLIAI, COLIIIAI, ANF, α-MHC, α-actin skeletal, β-MHC, microRNA-1, microRNA-29a, microRNA-29b, and microRNA-29c was analyzed using real-time polymerase chain reactions (real-time PCR)

as described previously [24]. Frozen heart samples (100 mg) were homogenized in Trizol (1 ml), and ribonucleic acid (RNA) was isolated according to the manufacturer's instructions (Invitrogen Life Technologies, Strathclyde, UK). Samples were quantified using a spectrophotometer at 260 nm and checked for integrity by EtBr-agarose gel electrophoresis. RNA was primed with 0.5 g/l oligo(dT) (12–18 bp) (Invitrogen Life Technologies) to generate the first strand of cDNA. Reverse transcription (RT) was performed using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies). Primers were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/). DNA sequence was obtained from GenBank, and primers were designed in separate exons to distinguish PCR products derived from cDNA from those derived from genomic DNA contaminants on the basis of their size. The mRNA expression of type I/III collagen was assessed using oligonucleotide primers as follows: for COLIA, 5′-AgA gAg CAT gAC CgA Tgg A-3′ and 5′-gAggTT gCC AgT CTg TTg g-3′; for COLIIIA, 5′-AAg gTC CAC gAg gTg ACA A-3′ and 5′-Agg gCC Tgg ACT ACC AAC T-3′. Real-time quantification of the target genes was performed with a SYBRgreen PCR Master Mix (Applied Biosystems, PE, Foster City, CA) using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The expression of cyclophilin A (5′-AAT gCT ggA CCA AAC ACA AA-3′ and 5′-CCT TCT TTC ACC TTC CCA AA-3′) was measured as a real-time PCR internal control. An aliquot of the real-time PCR reaction was used for 40-cycle PCR amplification in the presence of SYBRgreen fluorescent dye, according to the protocol provided by the manufacturer (Applied Biosystems, PE, Foster City, CA). The α-MHC and β-MHC mRNA expressions were assessed by oligonucleotide primers as follows: for α-MHC, 59-CGA GTC CCA GGT CAA CAA G-39 and 59-AGG CTC TTT CTG CTG GAC C-39); for β-MHC, 59-CAT CCC CAA TGA GAC GAA G-39 and 59-AGG CTC TTT CTG CTG GAC A-39; for α-skeletal actin, sense: 5′-ACC ACA GGC ATT GTT CTG GA-3′, antisense: 5′-TAA GGT AGT CAG TGA GGT CC-3′; and for ANF, sense: 5′-CTT CGG GGG TAG GAT TGA C-3′, antisense: 5′-CTT GGG ATC TTT TGC GAT CT-3′. The expression of cyclophilin A (59-AAT GCT GGA CCA AAC ACA AA-39 and 59-CCT TCT TTC ACC TTC CCA AA-39) was measured as an internal control for sample variation in real-time PCR reaction.

To accurately detect mature microRNAs, real-time PCR quantification was performed using primers for microRNA-1, microRNA-29a, microRNA-29b, and microRNA-29c (Life Technologies) with the TaqMan microRNA Assay protocol (Applied Biosystems, CA, USA). Samples were normalized by evaluating U6 expression. Each heart sample was analyzed in duplicate. Relative quantities of target gene and microRNA expression in the LZR, OZR, LZR + TR, and OZR + TR groups were compared after normalization using the expression values of internal controls [change in threshold cycle (ΔCT)]. Fold change was calculated using the differences in ΔCT values between the two samples ($\Delta\Delta$CT) and the equation $2^{-\Delta\Delta CT}$. The results are expressed as a percentage of the control value.

2.6. Western Blotting. The frozen hearts were thawed and homogenized in cell lysis buffer containing 100 mM Tris, 50 mM NaCl, 10 mM EDTA, 1% Triton X-100, and protease and phosphatase inhibitor cocktail [1 : 100; Sigma-Aldrich, MO, USA]. Insoluble heart tissues were removed by centrifugation at 3000g, 4°C, for 10 min. Samples were loaded and subjected to SDS-PAGE in 10% polyacrylamide gels. After electrophoresis, proteins were electrotransferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Equal loading of samples (50 μg) and even transfer efficiency were monitored with the use of 0.5% Ponceau S staining of the blot membrane. The blot membrane was then incubated in a blocking buffer [5% nonfat dried milk, 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20] at room temperature and then with a polyclonal antibody directed against SERCA-2a (ab3625), PLB (ab86930), pPLB^{ser16} (ab15000), and NCX1 (ab2869) [1 : 1000; Abcam, Cambridge, United Kingdom] overnight at 4°C. Primary antibody binding was detected with the use of peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ) and detection were performed in a digitalizing unit (ChemiDoc; BioRad, CA, USA). The bands were quantified by ImageJ software (National Institute of Health, USA). GAPDH expression levels were used to normalize the results, which were expressed as a percentage of the control values as described previously [24].

2.7. Statistical Analysis. Results are represented as means ± standard error of the mean (SEM). Statistical analysis was performed using randomized two-way ANOVA. Tukey's post hoc test was used for individual comparisons between means when a significant change was observed with ANOVA. $p \leq 0.05$ was considered as statistically significant.

3. Results

3.1. Adipose Tissue. We evaluated the effect of AET on body fat content in lean and obese groups after the training protocol (Figures 1(b) and 1(c)). As expected, the AET normalized epididymal fat content in the OZR + TR (0.04 ± 0.003 g/mm) groups compared with the control (0.126 ± 0.012 g/mm) and trained (0.022 ± 0.012 g/mm) LZR groups (Figure 1(b)). The epididymal fat content (Figure 1(b)) was decreased in OZR + TR (0.04 ± 0.007 g/mm) compared with OZR (0.58 ± 0.043 g/mm). In addition, AET was effective in reducing the epididymal fat content in LZR + TR (0.022 ± 0.006 g/mm) compared with the LZR group (0.126 ± 0.012 g/mm) (Figure 1(b)). The retroperitoneal fat content in the control OZR group was higher (0.94 ± 0.21 g/mm) than in the control LZR group (0.031 ± 0.25 g/mm; $p < 0.0004$) and LZR + TR (0.1 ± 0.007 g/mm; $p < 0.0004$) (Figure 1(c)).

3.2. Cardiac Hypertrophy. A previous study from our group showed pathological CH in OZR observed by echocardiography and LV mass/TL ratio [1]. Corroborating these data, we showed that the HW/TL ratio (mg/mm) was increased 29% in the OZR group compared with the LZR group,

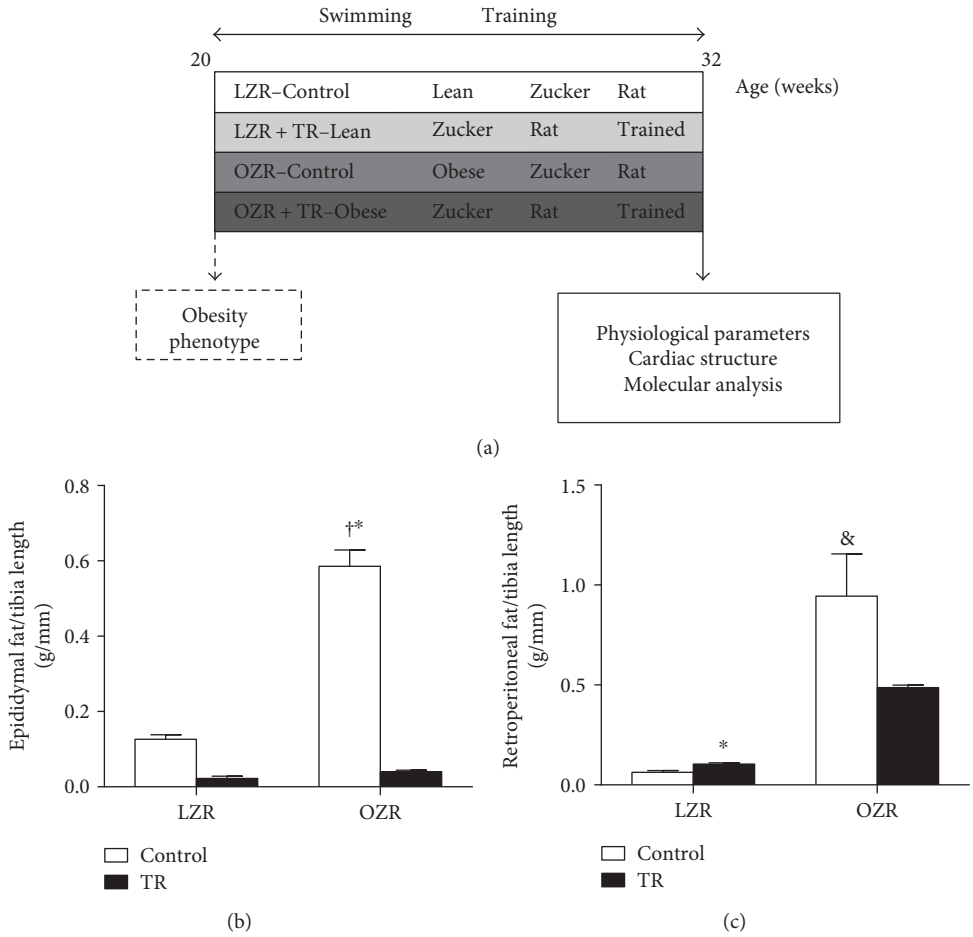

Figure 1: Effects of AET and obesity on epididymal and retroperionetal fat. Schematic panel of study design (a). Content of retroperitoneal (b) and epididymal fat (c) in LZR (control lean group), LZR + TR (trained lean group), OZR (control obese group), and OZR + TR (trained obese group). $^{\dagger}p < 0.01$ versus LZR and LZR + TR, $^{*}p < 0.05$ versus OZR + TR, and $^{\&}p < 0.001$ versus LZR + TR.

and the AET training decreased (8%) in the OZR + TR group (Figure 2(c)).

We assessed the LV intramuscular fat content and CMO diameter by histological analysis. LV intramuscular fat was increased in OZR compared with the trained groups (LZR + TR and OZR + TR) and decreased in LZR + TR compared with the LZR group (Figure 2(b)). Curiously, there were no significant differences in CMO diameter among the groups (LZR $13.8 \pm 2.7\,\mu$m; LZR + TR+ $17.7 \pm 2.1\,\mu$m OZR $17.1 \pm 0.9\,\mu$m; OZR + TR $18.2 \pm 1.1\,\mu$m) (Figure 2(a)). However, AET was effective in counteracting obesity-induced cardiac remodeling.

3.3. Molecular Markers of Pathological Cardiac Hypertrophy. Pathological cardiac remodeling induces the expression of genes commonly expressed only in the fetal period such ANF, skeletal α-actin, and β-MHC (Figure 3). The results of this study showed that obesity associated with increased β-MHC was increased in the OZR group compared with LZR, LZR + TR, and OZR + TR (Figure 3(b)). Similarly, ANF gene expression and swimming training were able to counteract it when compared with OZR + TR (Figure 3(c)). The results of this study showed that obesity and/or swimming training did not modify α-MHC gene expression (Figure 3(a)).

To confirm the involvement of obesity-regulated microRNAs in pathological CH, we analyzed the cardiac microRNA-29 family (microRNA-29a, microRNA-29b and microRNA-29c), whose expression affects collagen content. MicroRNA-29c expression was decreased in the OZR group compared with LZR, LZR + TR, and OZR + TR. AET resulted in microRNA-29c expression in the OZR + TR group approaching control levels (LZR: $100 \pm 16.2\%$; LZR + TR: $92 \pm 6.1\%$; OZR: $43 \pm 4.7\%$; and OZR + TR: $118 \pm 24.2\%$) (Figure 4(c)). The LV interstitial collagen volumetric fraction (CVF) was inversely proportional to the microRNA-29c expression level. These results show that CVF was increased in the OZR group compared with the LZR group. Interestingly, AET counteracted cardiac fibrosis in obesity, normalizing the CVF in the OZR-TR group (Figure 4(b)). However, gene expression of collagen IA and collagen IIIA did not change among the groups (Figures 4(c) and 4(d)).

3.4. Cardiac MicroRNA-1 and Calcium Signaling Proteins. MicroRNA-1 targets the NCX1 gene that is one of the most important cellular mechanisms for Ca^{2+} removal.

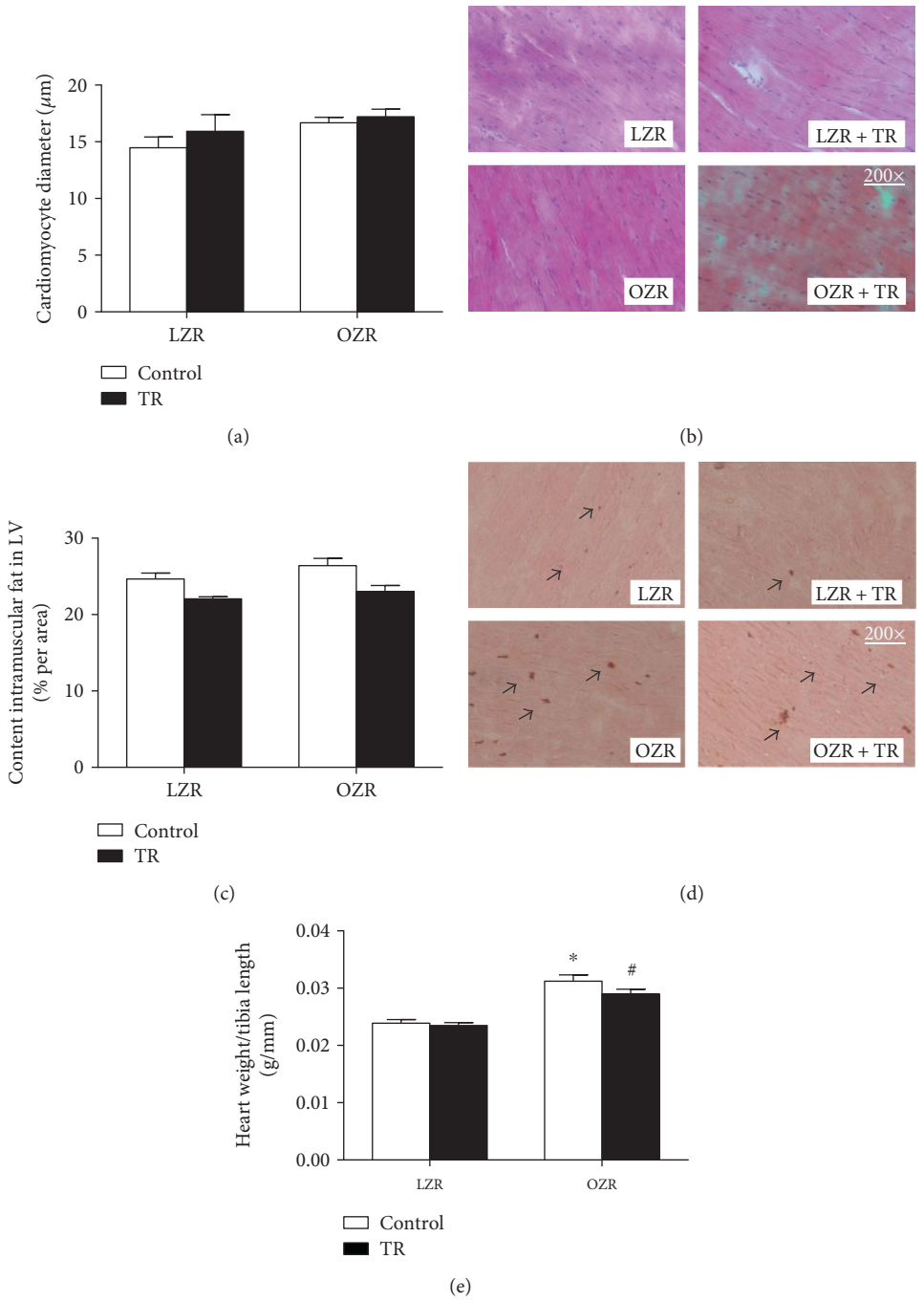

FIGURE 2: Effects of AET and obesity on cardiac intramuscular fat content and cardiomyocyte diameter. Cardiomyocyte (CMO) diameter (a). Representative histological images stained with hematoxylin and eosin (b). Cardiac intramuscular fat contents (c) were evaluated by histological analysis in LZR (lean group control), LZR + TR (lean trained group), OZR (obese group control), and OZR + TR (obese trained group). (d) Representative histological images stained with oil red for intramuscular fat. Arrows indicate fat red staining. (e) Total heart weight corrected by tibia length in LZR (lean group control), LZR + TR (lean trained group), OZR (obese group control), and OZR + TR (obese trained group). $^{\dagger}p < 0.005$ *versus* LZR + TR, $^{\&}p < 0.05$ versus OZR + TR $^{*}p < 0.0001$ versus LZR and LZR + TR, and $^{\#}p < 0.001$ versus LZR and LZR + TR.

MicroRNA-1 expression was increased in the OZR group compared with LZR, LZR + TR, and OZR + TR. Interestingly, AET was able to normalize microRNA-1 levels in the OZR + TR group. In addition, AET reduced microRNA-1 expression in LZR + TR compared with the LZR and OZR groups (Figure 5(a)). In parallel with the microRNA-1 expression, NCX1 expression was significantly reduced in the OZR group compared with LZR, LZR + TR, and OZR + TR. However, AET restored NCX1 expression in the OZR-TR group toward control levels (Figure 5(b)). The

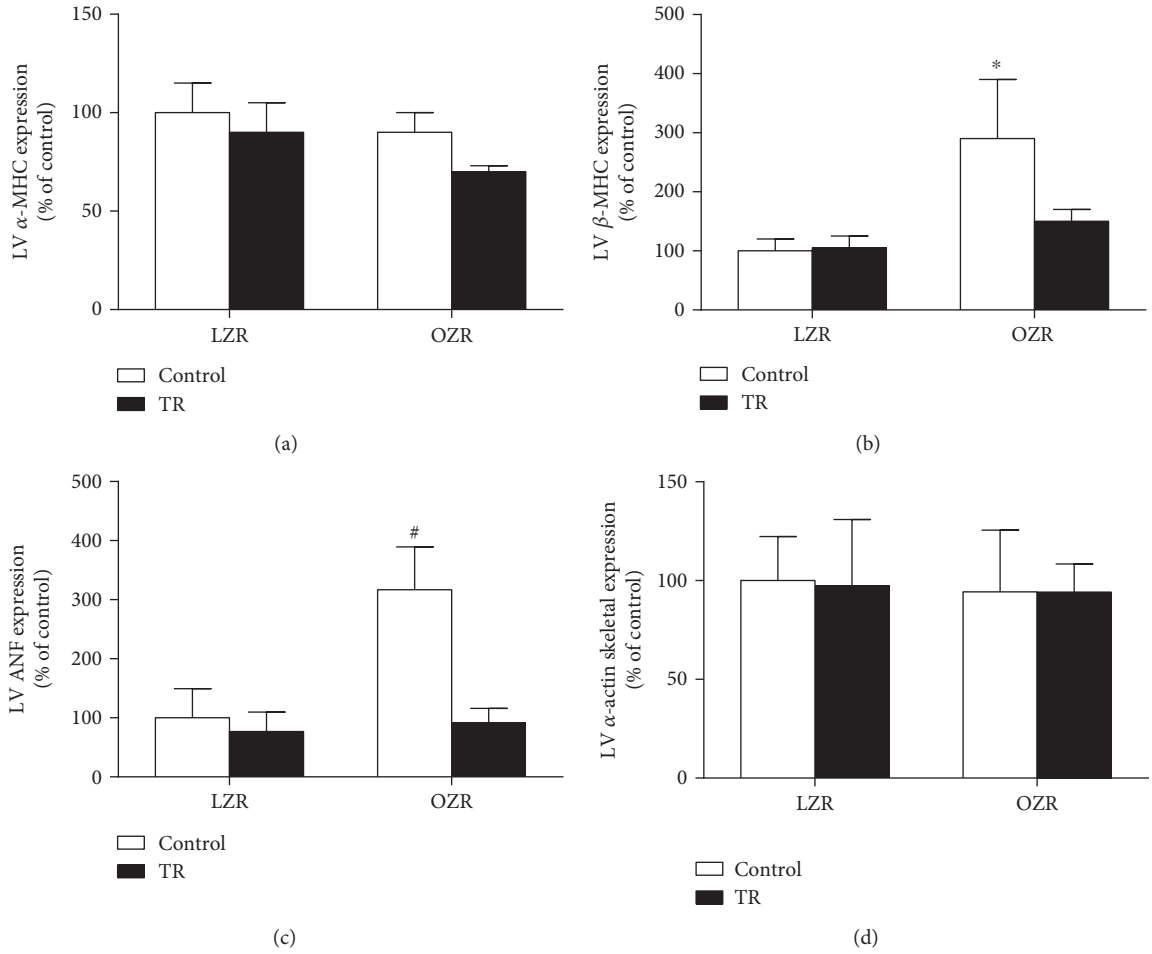

Figure 3: Effects of AET and obesity on α-MHC and β-MHC (alpha/beta-myosin heavy chain) (a, b), ANF (atrial natriuretic factor) (c), and α-actin skeletal (d) ratio in rat ventricles. Data are reported as means of 6 and SEMs of 5 animals in each group. $^{*}p < 0.05$ versus LZR and $^{\#}p < 0.03$ versus LZR, LZR + TR, and OZR + TR.

representative protein level by western blot is shown in Figure 5(c). These results show that NCX1 expression in obesity-induced pathological CR could be possibly reduced via increasing microRNA-1 expression by exercise training.

Other components of the calcium signaling pathway were also evaluated. Ryanodine (RYR2) gene expression increased in both groups (OZR + TR: 57 ± 5%; LZR + TR: 47 ± 19%) compared to the control group LZR (100 ± 9%) and also when compared with OZR (Figure 6). Figure 7(a) shows that SERCA-2a protein levels were decreased in the LZR + TR group compared with the LZR group; there were no significant differences in PLB and $pPLB^{ser16}$ protein levels among the groups (Figures 7(b) and 7(c)). The representative protein level by western blot is shown in Figure 7(d).

4. Discussion

Obesity is a chronic disease that results from a convergence of genetic, psychological, and social factors. It is a risk factor for the development of cancer, diabetes, and cardiovascular diseases that induce pathological CH [2, 4, 29, 30]. This study evaluated molecular mechanisms of pathological cardiac remodeling induced by obesity and investigated whether AET reverses and/or prevents cardiac remodeling. Our results show that obesity-induced pathological cardiac remodeling leads to an increase in cardiac pathological hypertrophy markers and downregulation of microRNA-29c expression, which can be associated with the increase in the LV collagen volumetric fraction. In addition, obesity upregulated microRNA-1, which targets NCX1. NCX1 was decreased in the OZR group. In contrast, AET restored the pathological expression of microRNA-1 and microRNA-29c and their target genes, which likely counteracted the pathological cardiac remodeling and cardiac dysfunction in obesity.

As shown in a previous study from our group, AET was efficient in producing cardiovascular changes in OZR, such as a reduction in heart rate due to vagal hypertonia in the trained groups [4]. Barretti et al. [4] demonstrated that obesity leads to increased LV mass in OZR and that AET prevents this increase. Soci et al. [24] demonstrated that different intensities of swimming training lead to different magnitudes in the expression of microRNA-29c levels. Animals trained on the same protocol as the current study showed that the microRNA-29c levels decreased by 52% and that COLIAI and COLIIIAI expressions

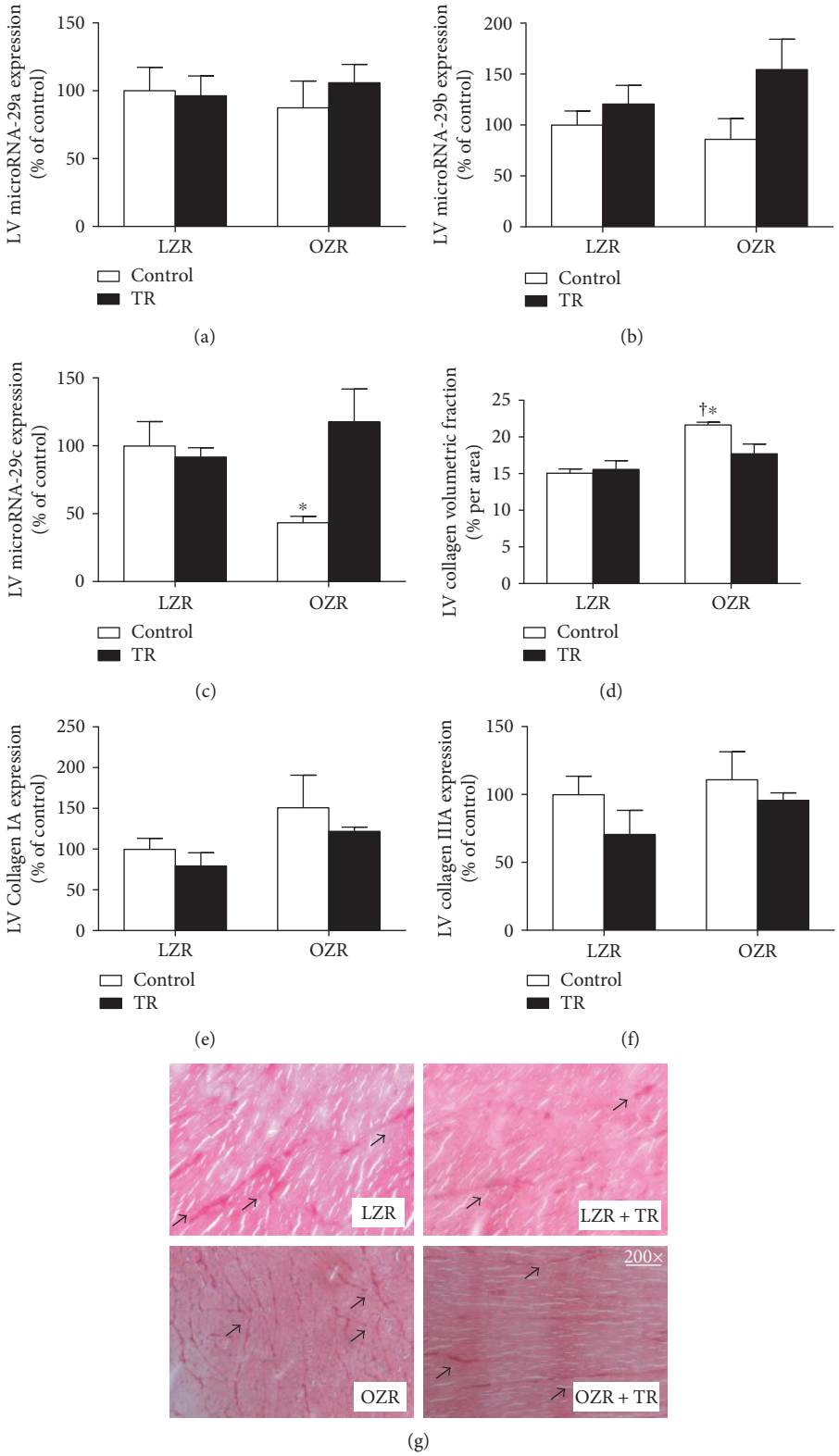

Figure 4: Effects of AET and obesity on cardiac microRNA-29 family expression, interstitial collagen volumetric fraction (CVF), and collagen expression. Cardiac microRNA-29a, microRNA-29b, and microRNA-29c expressions were evaluated by real-time PCR (a–c). LV interstitial CVF was evaluated by histological analysis, staining with Picrosirius red (d). Left ventricle (LV) collagen IA (COLIA) and IIIA (COLIIIA) gene expression was evaluated by real-time PCR (e, f). Representative histological images stained with Picrosirius red for CVF. Arrows indicate collagen red staining (g). LZR (lean group control), LZR + TR (lean trained group), OZR (obese group control), and OZR + TR (obese trained group). $^{*}p < 0.05$ versus OZR + TR and $^{\dagger}p < 0.01$ versus LZR and LZR + TR.

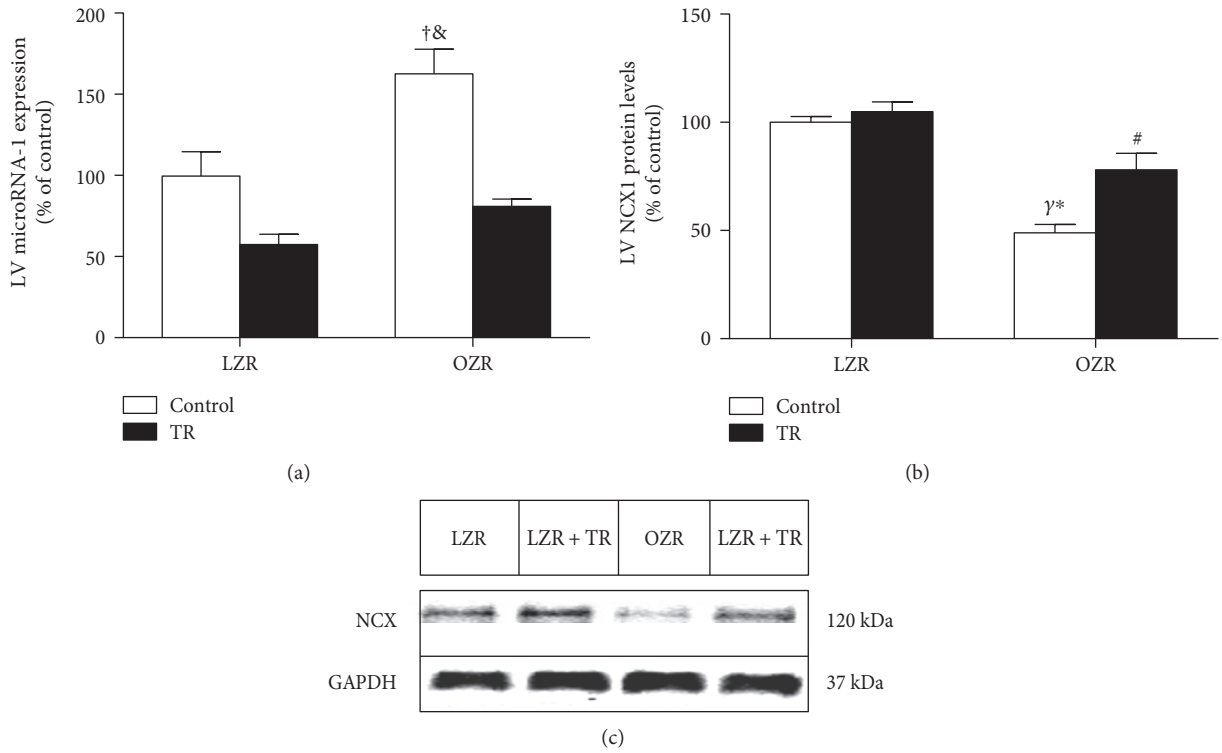

Figure 5: Effects of AET and obesity on microRNA-1 expression analyzed by real-time PCR (a) and NCX protein levels analyzed by western blot (b) in the left ventricle (LV). (c) Representative blots of NCX1 and GAPDH in LZR (lean group control), LZR + TR (lean trained group), OZR (obese group control), and OZR + TR (obese trained group). $^{\#}p < 0.05$ versus LZR, $^{\dagger}p < 0.01$ versus LZR, $^{\&}p < 0.001$ versus LZR + TR and OZR + TR, $^{\Upsilon}p < 0.0001$ versus LZR and LZR + TR, and $^{*}p < 0.05$ versus OZR + TR.

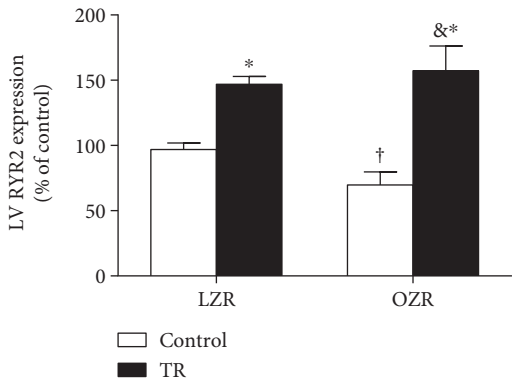

Figure 6: Ryanodine receptor 2 (RYR2) gene expression was evaluated by real-time PCR in the left ventricle (LV). LZR (lean group control), LZR + TR (lean trained group), OZR (obese group control), and OZR + TR (obese trained group). $^{\dagger}p < 0.01$ versus LZR + TR, $^{*}p < 0.05$ versus LZR, and $^{\&}p < 0.001$ versus OZR.

decreased by 27% and 38%, respectively. Animals trained on a higher intensity protocol presented an increase of 123% in microRNA-29c expression and decreases of 33% and 48% for COLIAI and COLIIIAI, respectively [24].

In the present study, as expected, AET was effective in decreasing epididymal and retroperitoneal fat. The OZR + TR group had a lower body fat content than the OZR group, as also shown by Disanzo and You who found that obesity led to an increase in endothelial growth factor A (VEGF-A) that is responsible for stimulating angiogenesis in adipose tissue counteracting glycolytic metabolism in this tissue and contributing to their decrease by exercise [31].

The OZR group presented pathological CH [4]. We quantified the CMO diameter; however, there were no differences among the groups, which suggest that the increase in cardiac mass in the OZR group is due to increased LV intramuscular fat and/or cardiac collagen. Moreover, to corroborate with the pathological cardiac hypertrophy phenotype, obesity induced an increase of fetal gene expressions, such as ANF and β-MHC.

Here, we showed that LV intramuscular fat was higher in the OZR group compared with LZR, LZR + TR, and OZR + TR. In fact, the reduced cardiac fat in the OZR-TR group caused by AET can be explained as part of the 13% reduction in LV mass or even the 7% reduction in total heart weight compared with that in OZR. Some studies suggest that this increased fat content in the myocardium leads to heart dysfunction and predisposition to chronic diseases [32, 33]. These findings reinforce the importance of AET as a preventive tool against cardiovascular pathologies.

The microRNA-29 family has been described to negatively regulate collagen content and to be highly responsive to AET [22, 24, 25]. Studies have shown that AET increases microRNA-29 expression in the heart and consequently decreases collagen expression and protein levels [24, 25]. In

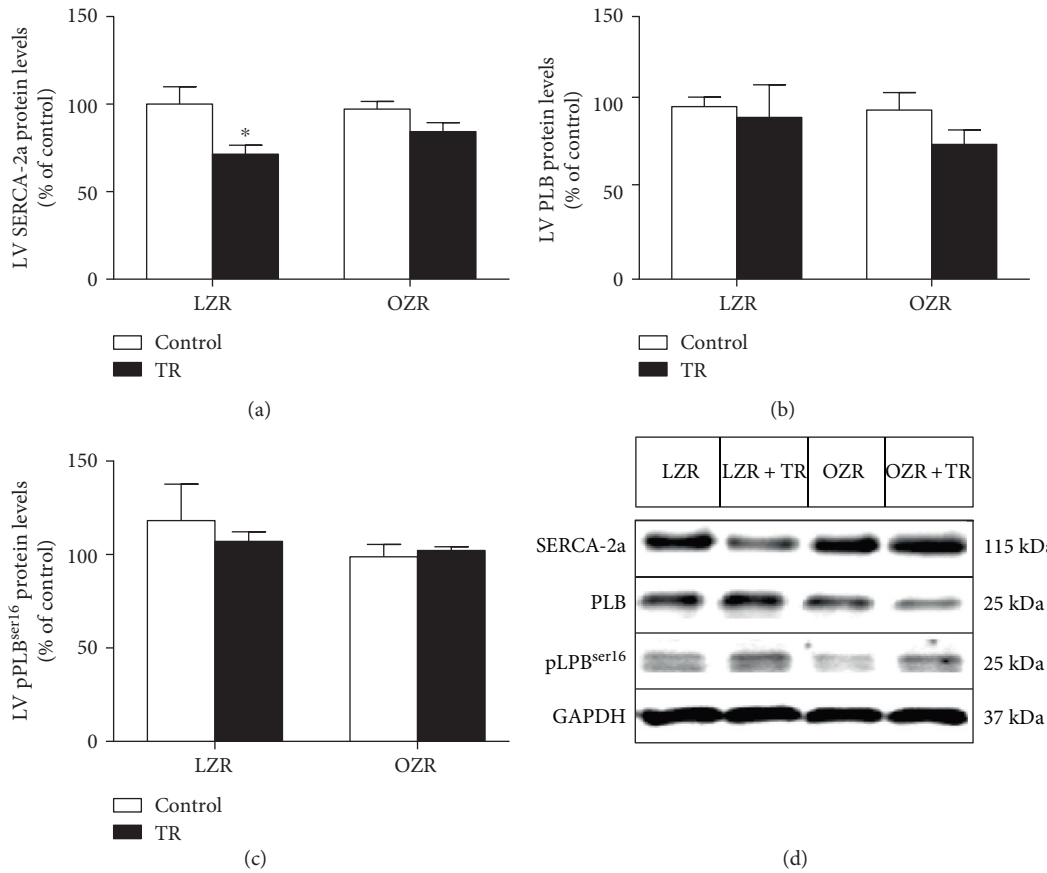

Figure 7: Effects of AET and obesity on calcium signaling proteins. SERCA-2a (a), phospholamban (PLB) (b), and phospholamban phosphorylated on serine16 (pPLB^{ser16}) (c) protein levels were evaluated by probing western blots of left ventricle (LV) proteins. (d) Representative blots of SERCA-2a, PLB, pPLB^{ser16}, and GAPDH in LZR (lean group control), LZR + TR (lean trained group), OZR (obese group control), and OZR + TR (obese trained group). $^{*}p < 0.05$ versus OZR and LZR.

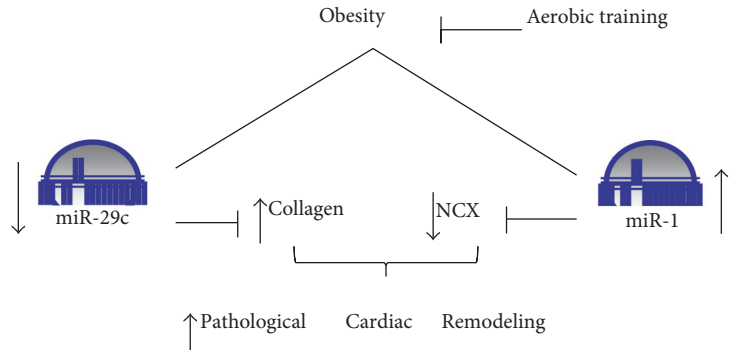

Figure 8: Schematic representation of the effects of AET on obesity-induced pathological cardiac remodeling via the involvement of microRNA-1 and microRNA-29c. AET is a powerful stimulus modulating microRNA-1 and microRNA-29c that regulates their target genes (NCX1 and collagen), thereby counteracting the pathological CH phenotype.

the present study, obesity decreased the cardiac microRNA-29c expression in OZR by 47% compared with LZR, which induced an increase in the cardiac CVF. Thus, AET was able to normalize cardiac microRNA-29c expression and CVF in OZR + TR, and these results suggest that AET has a cardioprotective effect against pathological CH as shown in Figure 8.

In our previous study, although there was no statistical difference ($p = 0.07$), a 25% reduced time E/A wave ratio was found when OZR was compared with untrained LZR [4], suggesting damage in the contractile myocardium. In the present study, we demonstrated that the cardiac collagen content in OZR could induce impaired compliance. Dong et al. [12] showed reduced compliance in isolated CMO from

obese mice. In our study, we investigated molecular mechanisms involved in diastolic dysfunction induced by obesity. We evaluated the levels of calcium transporter proteins involved in contractile mechanisms. MicroRNA-1 targets the NCX1 protein and is an important regulator of calcium mechanisms in the heart [26]. MicroRNA-1 was significantly increased in OZR compared with LZR, LZR + TR, and OZR + TR, in contrast to previous studies that have shown a reduction in microRNA-1 in pathological cardiac remodeling caused by others pathologies [22, 24, 26]. Thus, we hypothesized that cardiac remodeling induced by obesity is a milder compensatory response than that found in other pathologies, such as CH due to ischemic diseases [22]. Interestingly, AET caused downregulation of microRNA-1 expression in OZR + TR compared with OZR, showing that it could be an important tool against the pathological phenotype caused by obesity. AET was also able to reduce the expression of microRNA-1 in LZR + TR compared with LZR, data that reinforces the profile observed in the previous AET studies [24].

The NCX1 protein, which is the direct target of microRNA-1, was downregulated in OZR compared with the other groups (LZR, LZR + TR, and OZR + TR); this data indicates a possible antagonism between NCX1 and microRNA-1 expressions [34]. In contrast, AET was effective in restoring NCX1 levels in OZR + TR compared with OZR.

In the present study, there was an increase in the RYR2 receptor expression in both trained groups (LZR + TR and OZR + TR) compared with their controls (LZR and OZR). Our findings were different from those found in a study with rats submitted to AET and food restriction, where no significant change in RYR2 receptor expression was found [35, 36]. This could be because the swimming training was most effective to promote this adaptation in obesity phenotype. Increased RYR2 receptor expression improves the release of sarcoplasmic Ca^{2+}, which could lead improvements in cardiac contractility [20, 34–36].

We also observed that SERCA-2a expression was decreased in LZR + TR compared with LZR and a tendency in OZR + TR compared with OZR. While SERCA-2a expression decreased, the RYR2 expression was increased that could be causing an imbalance in the sarcoplasmic Ca^{2+} content. However, it is known that SERCA-2a function is dependent on the phosphorylation of the PLB protein [11] and there were no differences in total PLB and $pPLB^{ser16}$ expressions. Thus, NCX1 could be contributing to maintain intracellular normal Ca^{2+} concentration, at least in OZR + TR compared with OZR, in these trained animal models. These findings and the results concerning the upregulation of microRNA-1 can be associated with the downregulation of NCX1 in OZR which suggest that cardiac contractile dysfunction was prevented in OZR + TR improving these mechanisms, thus improving the cardiac function [4].

Our study demonstrates for the first time that AET was efficient in restoring the microRNA-1 and microRNA-29c to nonpathological levels in obesity, as well as its targets NCX1 and collagen, respectively.

Despite the strong association between microRNAs and their target genes, we do not demonstrate a direct proof of concept between them. However, the genes were validated to these microRNAs by other authors [34, 37]. Thus, further studies are needed to assess whether modulation of the microRNA-1 and microRNA-29c in vivo in the obesity phenotype would play a key role in preventing pathologic cardiac remodeling.

In conclusion, obesity downregulated microRNA-29c in OZR possibly leading to increased cardiac collagen content. Conversely, microRNA-1 levels were upregulated, and their target gene NCX1 was decreased in OZR, maybe causing diastolic dysfunction in these animals as we showed before [4]. Figure 8 shows a schematic representation of these data. One implication of our findings is that AET protects the heart against an aberrant increase of extracellular matrix components and prevents calcium-signaling pathway dysfunction in the cardiac remodeling phenotype caused by obesity through microRNA modulation.

Disclosure

An earlier version of this work was presented as an oral abstract presentation at the journal Hypertension (Session Title: Concurrent XV B: Obesity and Diabetes).

Conflicts of Interest

No conflicts of interest are declared by the authors.

Authors' Contributions

André C. Silveira, Tiago Fernandes, Úrsula P. R. Soci, Diego L. Barretti, and Edilamar M. Oliveira conceived and designed the study. André C. Silveira, Tiago Fernandes, Úrsula P. R. Soci, João L. P. Gomes, Glória G. F. Mota, and Edilamar M. Oliveira collected the data and performed the experiments. André C. Silveira, Tiago Fernandes, Úrsula P. R. Soci, João L. P. Gomes, Carlos Eduardo Negrão, and Edilamar M. Oliveira interpreted and analyzed the data. André C. Silveira, Tiago Fernandes, Úrsula P. R. Soci, Carlos Eduardo Negrão, and Edilamar M. Oliveira drafted the manuscript. André C. Silveira, Tiago Fernandes, João L. P. Gomes, Úrsula P. R. Soci, Carlos Eduardo Negrão, and Edilamar M. Oliveira edited and revised the manuscript.

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Review Article

Does Regular Exercise Counter T Cell Immunosenescence Reducing the Risk of Developing Cancer and Promoting Successful Treatment of Malignancies?

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Moderate intensity aerobic exercise training or regular physical activity is beneficial for immune function. For example, some evidence shows that individuals with an active lifestyle exhibit stronger immune responses to vaccination compared to those who are inactive. Encouragingly, poor vaccine responses, which are characteristic of an ageing immune system, can be improved by single or repeated bouts of exercise. In addition, exercise-induced lymphocytosis, and the subsequent lymphocytopenia, is thought to facilitate immune surveillance, whereby lymphocytes search tissues for antigens derived from viruses, bacteria, or malignant transformation. Aerobic exercise training is anti-inflammatory and is linked to lower morbidity and mortality from diseases with infectious, immunological, and inflammatory aetiologies, including cancer. These observations have led to the view that aerobic exercise training might counter the age-associated decline in immune function, referred to as immunosenescence. This article summarises the aspects of immune function that are sensitive to exercise-induced change, highlighting the observations which have stimulated the idea that aerobic exercise training could prevent, limit, or delay immunosenescence, perhaps even restoring aged immune profiles. These potential exercise-induced anti-immunosenescence effects might contribute to the mechanisms by which active lifestyles reduce the risk of developing cancer and perhaps benefit patients undergoing cancer therapy.

1. Introduction and Overview

Since the first observations of the immune system being modulated by bouts of vigorous exercise in the 1890s, a substantial body of evidence has accumulated showing that most aspects of immune function are sensitive to exercise-induced change [1]. Many of these changes have been interpreted as being beneficial, and this view has led to aerobic exercise training being advocated as a method to prevent, limit, or delay the age-associated decline in immune function referred to as immunosenescence [2–4]. It has even been suggested that aerobic exercise training might rejuvenate aged immune profiles [2, 3], which is attractive given our ageing population, and the potential implications that immunosenescence has for increasing susceptibility to infections and increasing the risk of developing cancer.

The purpose of this article is to summarise the effects that aerobic exercise training and physical activity can have on aspects of immune function with an emphasis on how some of these changes could be considered as countering immunosenescence. Few studies have investigated changes to immune function brought about resistance exercise (e.g., lifting weights) or high-intensity sprint interval exercise; therefore, these activities will remain largely undiscussed. In addition, this article will summarise studies that have investigated whether immunosenescence influences the risk of developing cancer or affects the treatment of patients with a cancer diagnosis. Readers are directed towards articles that

have reviewed these concepts in more depth, including the effects of exercise on immune function [1, 5, 6], the influence of chronological age and infection history on immunosenescence [7–9], the effects of aerobic exercise training on immunosenescence [2–4, 10], and the links between immunosenescence and cancer risk or treatment [11–13].

2. Characteristics of an Ageing Immune System

The profile of the innate and adaptive immune system is markedly different between young and elderly individuals. For example, with ageing, there is an increase in systemic inflammatory activity referred to as inflammageing [14, 15]. Age-associated alterations are also observed with the number, phenotype, and function of innate immune cells [9]. Neutrophils exhibit diminished phagocytosis and have an impaired capacity to control their direction of migration (chemotaxis), but their speed of movement (chemokinesis) is maintained. Mast cells decline in number but not function, whereas the number of eosinophils and basophils, along with their function, remains largely unchanged. Monocyte numbers in peripheral blood are stable with ageing, but classical cells (CD14++CD16−) decline, and intermediate (CD14+CD16+) and nonclassical (CD14+CD16++) cells increase [9, 16]. These numerical changes coincide with altered signalling via some toll-like receptors resulting in impaired cytokine production. Changes in monocytes due to age per se are thought to be mirrored by the phenotype and functional properties of tissue macrophages, whereby classically activated M1 cells decline and alternatively activated M2 cells accumulate [17]. However, the composition of tissue-resident cells is complicated by adipose tissue accumulation and dysfunction—dominated by M1 macrophages [18, 19]—and the M1/M2 paradigm is likely to be an oversimplification [20, 21]. It is unclear if ageing affects the number of dendritic cells in peripheral blood, but numbers decline in the skin and mucosal membranes, and antigen processing and presentation, cytokine production, costimulatory capacity, and migration are impaired. The number of natural killer cells increases with ageing, but this change varies by subtype; cytotoxic cells accumulate and regulatory cells decline; however, overall cytokine production and cytotoxicity are less on a per cell basis. With ageing, there is a decline in the number of invariant natural killer T cells (iNKT cells)—innate lymphocytes, which represent <1% of the T cell pool and recognise tumours or infected cells via CD1d-presented glycolipids [22]. It is possible that some of these age-associated changes are driven by an increase in myeloid-derived suppressor cells (MDSCs)—a heterogenous population of granulocytes, macrophages, and dendritic cells that suppress aspects of immune function by producing reactive oxygen species and inhibitory cytokines [23].

Within the adaptive immune system, a classical hallmark of ageing that predicts mortality in the elderly is impaired mitogen-induced T cell proliferation [24]. Many other age-associated changes to T cell numbers and phenotypes have been reported. For example, among T cell subpopulations, such as CD4+ T-helper (Th) cells, ageing is associated with a predominance of cells with a Th2 cytokine profile (i.e., IL-4 and IL-10 producing cells), whereas there is a decline in cells with a Th1 profile (i.e., IFN-γ and TNF-α producing cells) [25]. Another functional shift in T-helper cell profile includes an accumulation of IL-17 producing cells that are associated with autoimmunity and inflammatory disease [26]. Other characteristic observations among T cells include decreased numbers and proportions of CD4+ and CD8+ naïve T cells and increased numbers and proportions of late-stage differentiated effector memory CD4+ and CD8+ T cells [7, 8]. These changes are largely driven by a combination of three factors: first, lower numbers of haematopoietic stem cells which also exhibit intrinsic damage and a phenotype skewed towards the myeloid lineage; second, thymic involution and reduced output of antigen-naïve T cells; and third, infection with latent herpes viruses, in particular, *Cytomegalovirus* (CMV) [7, 27]. Among the less abundant T cell populations, natural regulatory T cells (nTREGs) increase with ageing whereas inducible TREGs decrease [28]. It is unclear whether the suppressive capability of these cells is altered with ageing: an increase could promote immune suppression and cancer, whereas a decrease could promote immune activation and autoimmunity [28]. T cells that express natural killer cell-associated cell-surface proteins (NKT-like cells) increase with ageing and exhibit similar changes to their phenotype, functional properties, and specificities as with the broader populations of CD4+ and CD8+ T cells [22]. With ageing, there is a decline in the number of $\gamma\delta$ T cells; however, age per se, in the absence of chronic infections, is associated with a decline in Vδ2 cells (60–80% of $\gamma\delta$ T cells), whereas Vδ1 (15–20% $\gamma\delta$ T cells) remain stable [29]. As with T cells, ageing is associated with a decline in the numbers and proportions of naïve B cells, an accumulation of memory B cells with limited specificities, and less robust antibody production by plasma cells in response to novel antigens [30]. Some of these parameters, when clustered together, have been shown to predict all-cause mortality in studies of octogenarians and nonagenarians [31, 32]. These characteristics, which were referred to as the Immune Risk Profile (IRP), included low numbers and proportions of B cells, high numbers and proportions of late-stage differentiated CD8+ T cells, poor T cell proliferation in response to mitogens, and a ratio of CD4+ T cells to CD8+ T cells less than 1.0 [31, 32]. Infection with CMV was part of the IRP [33], and later work showed that plasma IL-6 was associated with frailty, cognitive decline, and mortality [34, 35].

Many features of an ageing immune system appear to be driven by CMV, and infection with this virus has been linked to poor vaccine responses in both young and elderly individuals [36–39]. The effects of CMV on the effectiveness of vaccination remains a contentious issue, however, with some studies showing no effect in the elderly [38, 40, 41] and others even showing vaccine-boosting effects of CMV in young individuals [42]. Irrespective of CMV serostatus, elderly individuals, exhibiting other established hallmarks of an ageing immune system, generally mount less robust immune responses to vaccines compared to young individuals [43]. Aside from influencing responses to novel antigens, CMV infection has also been directly associated with frailty [44]

and cognitive impairment [45], and some studies have shown that infection results in earlier mortality [46–48].

3. Anti-Immunosenescence Effects of Exercise: General Observations

The idea that aerobic exercise training could influence immunosenescence developed in the late 1990s, almost two decades after the field of exercise immunology, was established [49]. Many studies have investigated how the immune system responds acutely (i.e., over minutes, hours, and days) to different modes, durations, and intensities of single and repeated exercise bouts. Generally, exercise bouts are considered "immunostimulatory" partly due to the rapid exercise-induced leukocytosis that was observed in early studies [50–52]. Among lymphocytes, for example, and in particular T cells and natural killer cells, subpopulations that exhibit strong effector function rapidly and substantially increase in blood during exercise [1, 53, 54]. Subsequently, in the hours following exercise, the same effector cells migrate to tissues, leaving blood to search for cells infected with viruses, bacteria, or those that have undergone a malignant transformation [55, 56]. It is likely that these principles also translate to other cells of the innate immune system, such as monocytes and neutrophils, but these cells exhibit different exercise-induced kinetics compared to lymphocytes. Considering that these processes might reduce the risk of infections [57] and facilitate the detection and elimination of tumours [58], then these exercise-induced changes could be interpreted as countering some aspects of immunosenescence. In addition to the short-term "immunostimulatory" effects of exercise bouts, long-term aerobic exercise training interventions, in both young and elderly individuals, elicit strong anti-inflammatory effects that might counter inflammageing [6]. It seems that these exercise-induced effects may not have their roots in immune cells though and may be due to other cytokine producing cells (e.g., myocytes, adipocytes, fibroblasts, endothelial, and epithelial cells) considering that long-term changes to the function and phenotype of most immune cells are not brought about by regular exercise training [6, 10].

Other studies have examined basal or resting immune function in participants of cross-sectional or longitudinal studies, categorising individuals as being sedentary, inactive, physically active, or extremely active using self-report questionnaires. A more recent approach has been objectively measuring the volume and intensity of free-living activities using wearable activity monitors. However, the data generated by these devices is complicated to interpret using globally recommended physical activity guidelines (e.g., 150 minutes of moderate-to-vigorous intensity physical activity each week *on top* of normal activities of daily living) [59–61]. Wearable activity monitors capture activity accumulated both purposefully (e.g., through exercise) and through daily living, but because they do not discriminate between the two, it has been suggested that a total of ~1000 minutes of moderate-to-vigorous intensity activity per week is a more appropriate target to assess with these devices [62]. Despite these important nuances, regularly active individuals appear to exhibit better overall immune function than inactive individuals, even in the elderly [10]. For example, one of the most robust tests of immune competence is the antibody or cell-mediated response to novel antigens, often administered experimentally by vaccination. Both humoral and cell-mediated responses to vaccination appear to be stronger in active compared to inactive individuals and those who have engaged in structured exercise in the months leading up to vaccine administration [5, 63]. Indeed, different forms of exercise appear to amplify vaccine responses, facilitating long-term immunity in the months after vaccination [5, 63]. These effects have been shown by studies that have not controlled for the degree of immunological ageing (e.g., stratifying by CMV serostatus) suggesting powerful effects of exercise.

Although many aspects of immune function change with ageing, and some of these alterations can be bolstered or restored transiently by different forms of exercise, it remains to be determined whether clinically meaningful and long-term exercise-induced changes to the established hallmarks of immunosenescence can be observed. However, evidence is beginning to accumulate showing that some of these parameters are at least sensitive to change by aerobic exercise training. In addition, theories have been put forward for mechanisms that might underlie possible anti-immunosenescence effects of exercise. So far, this work has largely focused on an ageing adaptive immune system with an emphasis on T cells.

4. Anti-Immunosenescence Effects of Exercise: Proposed Mechanisms

It has been suggested that aerobic exercise training might prevent or delay immunological ageing by limiting the expansion of senescent late-stage differentiated T cells, which is a hallmark of immunosenescence [2, 3]. In this hypothesis, it is proposed that three exercise-induced processes bring about this effect. First, cells of a late-stage differentiated phenotype are mobilised into peripheral blood during single bouts of exercise. Second, these cells extravasate from blood, homing to peripheral tissues, where they are exposed to proapoptotic stimuli (e.g., reactive oxygen species, glucocorticoids, and cytokines). Third, assuming that the "size" of the adaptive immune system is fixed, the naïve T cell repertoire expands in response to the immunological "space" that has been created, initiated by a hypothetical negative feedback loop that governs the number of naïve and memory cells and perhaps bolstered by exercise-induced thymopoiesis and/or extrathymic T cell development in tissues such as the liver.

Several parts of this hypothesis are supported by robust findings in exercise immunology. For example, many investigations have shown that late-stage differentiated T cells are mobilised into peripheral blood during bouts of moderate- and vigorous-intensity aerobic exercise, followed by an assumed, in humans at least, extravasation to peripheral tissues [53, 54]. In addition, there is evidence from studies in mice that lymphocyte apoptosis occurs postexercise in tissues that are thought to be the homing destination of mobilised cells [64]. This process occurs in parallel with an increase of

haematopoietic stem cells in the bloodstream and at these homing sites, which seems to be an effect of apoptotic cells and cell debris [65]. Such apoptosis-induced haematopoietic stem cell mobilisation might result in trafficking to the thymus (or potentially extrathymic sites) stimulating the development of naïve T cells [66]. Finally, contracting skeletal muscle secretes IL-7 [67] which might increase thymic mass and promote the emergence of recent thymic emigrants [68]. Recent studies have provided insight into whether the numbers and proportions of late-stage differentiated T cells in peripheral blood are sensitive to change with long-term exposure to aerobic exercise training. These studies have been discussed in detail elsewhere [4] but are summarised below.

5. The Effects of Aerobic Exercise Training on Late-Stage Differentiated T Cells

There are no randomised and controlled trials that have examined whether aerobic exercise training reduces the numbers and proportions of late-stage differentiated T cells resulting in clinically meaningful changes to overall immune function. However, several cross-sectional studies have provided initial evidence that the characteristics of the T cell pool vary between individuals who have been exposed to different volumes and intensities of aerobic exercise training over periods of their life. In addition, limited evidence suggests that the characteristics of the T cell pool are sensitive to modulation over several months when the volume and intensity of aerobic exercise training are changed.

To summarise, it has been shown that individuals with a high cardiorespiratory fitness, which largely reflects an active lifestyle, exhibit lower proportions of late-stage differentiated T cells and higher proportions of naïve T cells in resting blood samples [69]. The effects of cardiorespiratory fitness appeared to be largely independent of age, body composition, and CMV serostatus [69]. Similar observations have been made in young adults who engage in structured exercise that is typical of training for competitive team sports [70, 71]. In the elderly, however, an active lifestyle only appears to limit the accumulation of late-stage differentiated T cells and does not appear to substantially affect the proportions of naïve T cells [72]. Thus, these findings largely support an anti-immunosenescence effect of aerobic exercise training, particularly in young adults. However, individuals undertaking a much higher volume of endurance training appear to exhibit exaggerated signs of T cell immunosenescence compared to less active age-matched controls [73, 74]. Examples of these changes include a greater accumulation of late-stage differentiated T cells, fewer naïve T cells, and reduced thymic output, as shown by lower levels of T cell receptor rearrangement excision circles in resting blood samples [73, 74]. These effects appear to be most prominent in younger compared to older athletes [74]. Thus, these findings could be interpreted as being a proimmunosenescence effect of very prolonged and repeated aerobic exercise bouts. In support, it has been shown that over six months, when very prolonged endurance training was undertaken by both younger and older athletes, the numbers and proportions of late-stage differentiated T cells increase and the numbers and proportions of naïve T cells decrease [75, 76].

To conclude, it seems likely that individuals who meet or exceed by less than approximately five times, the recommendations for physical activity (i.e., accumulating 150 minutes of moderate intensity or 75 minutes of vigorous-intensity activity on a weekly basis) exhibit less marked immunosenescence than age-matched controls who are not active [4]. However, individuals who take part in regular very prolonged or extreme aerobic exercise over their lifetime may exhibit changes in the T cell pool that are indicative of exaggerated immunosenescence [4]. It seems that both the anti-immunosenescence effects of typical aerobic exercise training and the proimmunosenescence effects of extremely prolonged endurance training are most prominent in young individuals. This indicates that the magnitude of effect brought about by aerobic exercise training is relatively modest, or the effects are at least masked within individuals who already exhibit an aged immune profile. Thus, the limited evidence to date suggests that anti-immunosenescence effects of aerobic exercise training might have most utility in delaying the onset of immunological ageing, rather than restoring aged immune profiles. Although there is some evidence for an anti-immunosenescence effect of aerobic exercise training on late-stage differentiated T cells, some concepts that underlie the possible biological mechanisms are debated. Indeed, many questions remain unanswered, such as how many late-stage differentiated T cells truly classify as being senescent, whether these cells can and should be removed, and whether the "size" of the immune system is fixed. These themes are summarised below with a commentary on whether some anti-immunosenescence effects of aerobic exercise training could be brought about indirectly.

6. What Are the Most Likely and Desirable Anti-Immunosenescence Effects of Exercise?

A theme of the original idea for how aerobic exercise training might counter immunosenescence has been the removal of senescent late-stage differentiated T cells [2, 3]. This process is considered desirable for two primary reasons: first, because it is thought some of these cells are unable to function and second, because these senescent cells are thought to be a waste of "space" assuming the "size" of the immune system is fixed.

The concept of a finite amount of immunological space came about, in part, because it has traditionally been assumed that thymic output of naïve T cells is negligible after adolescence [14]. This implies that there is an upper limit to the number of T cells in the immune system and a fixed number of naïve cells capable of mounting responses to novel antigens [14]. Thus, it has been proposed that antigen-naïve cells could be "used up" due to ongoing differentiation into memory cells that "fill up" immunological "space" [14]. In addition, it has been suggested that the accumulation of virus-specific T cells over a lifetime may lead to a "squeezing out" of T cells targeting less dominant viruses or nonpersistent infections, potentially leading to loss of viral control [77]. Although studies continue to support the age-

associated reduction in thymic output, evidence points towards a gradual decline, whereby thymic function persists, albeit reduced, up until around 70 years of age [78]. Moreover, the concept of a fixed amount of immunological space has been strongly debated [79, 80]. For example, CMV-seronegative recipients of a CMV-infected kidney demonstrate an "enlargement" of the CD8+ T cell compartment, shown by an appearance and expansion of CMV-specific T cells, but a maintenance (and not deletion) of pre-existing Epstein Barr virus-specific and influenza-specific T cells over time [79]. Although it remains up for debate if there is a limit to the "size" of the immune system and the number of naïve T cells within in it, the assumption that low numbers of naïve cells increase the susceptibility to infection has not been tested experimentally [8]. However, a removal of late-stage differentiated cells may be desirable for other reasons, as for example, these cells might contribute to the age-associated increase in inflammatory activity [14].

Assuming late-stage differentiated T cells remain functional, it is likely that they have an important role in controlling latent viruses such as CMV. These cells, which do not express CD27, CD28, CD62L, and CCR7, but express CD45RA, CD57, and KLRG1 [7], almost exclusively accumulate due to CMV infection and reactivation and do not accumulate in elderly CMV-seronegative individuals [7, 81, 82]. Thus, a targeted removal of these cells by exercise is probably only relevant to individuals infected with CMV, but this is a large proportion of the worldwide population (30–90% depending on age, ethnicity, socioeconomic status, and geographical location) [83]. It is however well established that in CMV-seropositive individuals, approximately 10% of the CD4+ and CD8+ T cell pool becomes specific for this virus [84]. Although such large accumulations of CMV-specific T cells are often interpreted to be deleterious, it is unknown what proportion of the T cell pool needs to be specific for CMV to ensure adequate viral control [85]. The importance of fully functioning late-stage differentiated T cells in controlling latent viruses is highlighted by the complications that arise from CMV reactivation and CMV disease in patients receiving solid organ or stem cell transplants, which in part is due to loss or suppression of cell-mediated immunity [86]. Indeed, protocols have been developed to adoptively transfer CMV-specific CD8+ T cells to prevent viral reactivation in transplant recipients [87]. Even in healthy individuals, evidence points towards an important role for CMV-specific immune surveillance. For example, in a longitudinal analysis of elderly individuals, it has been shown that a lower proportion of naïve CD8+ T cells, a higher proportion of memory CD8+ T cells, combined with a robust proinflammatory response to CMV, is associated with survival [88].

Along with uncertainty over how many CMV-specific late-stage differentiated T cells are required for antiviral control, it is unclear what proportion of these cells classify as being truly senescent. The assumption that many of these cells are senescent came about because early studies showed that the proportion of antigen-stimulated IFN-γ producing cells among elderly donors was lower than in young donors [89]. However, the cumulative IFN-γ production was higher in the elderly due to greater absolute numbers of these cells compared to the young [89], and this could therefore be a mechanism to limit excessive inflammatory activity when controlling CMV in the elderly. Most CMV-specific cells are multifunctional, producing IFN-γ, IL-2, and TNF-α, and have telomeres of intermediate length; therefore, do not classify as senescent [90]. Although some CMV-specific cells express programmed death-1 (PD-1), this cell-surface protein is thought to better represent exhausted cells that have impaired effector function and are not truly senescent [91, 92].

Although it might be desirable to remove truly senescent late-stage differentiated T cells, it is likely that a biological explanation exists for why these cells accumulate [14]. For example, in vitro experiments have shown that senescent CD8+ T cells, defined as cells being incapable of cell division, are resistant to apoptosis [93]. Conversely, other in vitro work has shown that senescent T cells, defined by expression of cell-surface proteins that have been associated with senescence such as CD57 and KLRG1, are *more* susceptible to H_2O_2-induced apoptosis than naïve cells [94, 95]. When examining CMV-specific CD8+ T cells in vitro, it has been shown that rather than being resistant to apoptosis, these cells are equally as susceptible to apoptosis as the broader pool of CD8+ T cells [96]. However, in vivo experiments show that peripheral blood CMV-specific CD8+ T cells exhibit high levels of the antiapoptotic protein Bcl-2, potentially rendering them insensitive to Fas-L/Fas-R-induced death [97]. Currently, it remains unclear whether CMV-specific late-stage differentiated T cells, some of which may classify as being senescent, are resistant to apoptosis in vivo. Thus, it is not certain if these cells would be susceptible to exercise-induced apoptosis as part of countering an ageing immune system, perhaps implicating other indirect mechanisms.

If aerobic exercise training invokes anti-immunosenescence effects by limiting the accumulation of CMV-specific late-stage differentiated T cells, it could be that these changes are brought about indirectly, by limiting viral reactivation, improving redox balance, and countering inflammageing. For example, many individuals who accumulate large volumes of sedentary behaviour and do not engage in physical activity are likely to be overweight or obese, and it is possible that CMV will reactivate frequently because adiposity drives chronic systemic inflammation and oxidative stress [18, 98]. In turn, proinflammatory cytokines and reactive oxygen species reactivate CMV directly [99, 100]. Aerobic exercise training decreases visceral and subcutaneous adipose tissue [101], providing a potent anti-inflammatory stimulus that helps maintain redox balance [6, 102]. Exercise-induced improvements in redox balance may also prevent dysfunction of late-stage differentiated T cells, considering that activation of the p38 mitogen-activated protein kinase signalling pathway by excess reactive oxygen species prevents T cell proliferation [103]. Thus, aerobic exercise training might delay the accumulation of late-stage differentiated CMV-specific T cells by reducing viral reactivation, or preventing T cell dysfunction, and by limiting adipose tissue accumulation and dysfunction that drives inflammation and oxidative stress.

In support of the idea that sedentary behaviour and low levels of physical activity might exacerbate immunosenescence, perhaps via dysregulated adipose tissue, overweight or obese individuals are at a greater risk of viral and bacterial infections, have longer stays in hospital, and exhibit more frequent and prolonged complications, such as antibiotic treatment failure [104–106]. Further, obese individuals exhibit diminished antibody responses to vaccination [107–109], impaired lymphocyte proliferation to mitogens [110], and shorter peripheral blood leukocyte telomere length [111]. In addition, it has been shown that obesity is associated with an accumulation of nTREGs and Th2-phenotype cells that is also seen with ageing [112]. Other reports have shown that obese individuals have large expansions of late-stage differentiated $\alpha\beta$ T cells and $\gamma\delta$ T cells, with the latter exhibiting impaired antiviral function [113–115]. Thus, aerobic exercise training might counter immunosenescence indirectly by limiting adipose tissue accumulation and dysfunction that appear to exacerbate ageing processes.

In summary, the proposed anti-immunosenescence effects of aerobic exercise training that focus on limiting the age-associated expansion of late-stage differentiated T cells are desirable based on several assumptions. First, some of these cells are classified as being senescent, failing to adequately control latent viruses, but meanwhile contributing to inflammageing. Second, the "size" of the immune system is fixed and the capacity to produce antigen-naïve T cells is limited, and this process increases the risk of infections and cancer in the elderly.

7. Immunosenescence and Cancer: An Overview

In 2015, there were 17.5 million cases of cancer worldwide causing 8.7 million deaths, implicating malignancies as the second most common cause of mortality behind cardiovascular disease [116]. The incidence of cancer is increasing worldwide, as shown by a 33% rise in cases between 2005 and 2015, largely driven by an ageing population [116]. This age-associated increase in cancer incidence occurs in parallel with the age-associated decline in immune function. However, the idea that immunosenescence causes or at least increases susceptibility to cancer is controversial, even when considering the observed relationships between immune function and cancer risk or disease progression. For example, there is a high incidence of cancer in people who are immunosuppressed such as organ transplant recipients [117]. Supporting the concept that a fully functioning immune system hinders the development of cancer, a longitudinal study over 11 years, has shown that individuals with high natural killer cell cytotoxicity, measured in cells isolated from peripheral blood, exhibit a lower incidence of cancer than individuals with less cytotoxic natural killer cells [118]. Indeed, clinical reports of "spontaneous" cancer regression, "disappearance" of tumours, and patients living with "dormant" cancer for up to 20 years provide anecdotal evidence for the importance of anticancer immunity [119]. However, strong evidence proving links between the age-associated decline in immune competence and the development or progression of cancer is lacking, despite advances in the scientific understanding of tumour biology, the development of disciplines that focus entirely on the immunology of cancer, and the subsequent growth of cancer therapies that manipulate aspects of immune function.

It is now almost universally accepted that the immune system has a fundamental role in the detection and elimination of malignant cells, although some aspects of immune function have also been linked to tumour progression [117]. Indeed, the relationship between these processes and malignancy is referred to as the cancer-immunity cycle [120] and it is thought that when anticancer immune surveillance becomes impaired, tumour masses become detectable and clinically relevant [117, 121]. Subsequently, research has led to extensive immunological characterisation of tumours, with signature tumour-associated antigens established and prioritised for the development of cutting-edge treatments [122]. Even with conventional cancer therapies, such as chemotherapy and radiotherapy, it is known that the immune system facilitates some of their effects [123]. Some recent cancer therapies stimulate aspects of immune function, for example, by targeting immune check point inhibitory circuits, and many treatments involve administering immune products such as cells, antibodies, or cytokines [124–126]. Finally, some immunological variables predict clinical outcomes in patients with cancer, such as tumour infiltrating leukocytes ([127, 128] this extensive and complex literature will not be discussed). Despite advances in our understanding of the development, detection, and treatment of malignancies, the quest to investigate whether causal or correlational relationships exist between immunosenescence and cancer is hampered by several unanswered and fundamental questions. First, do individuals who go on to develop cancer already exhibit immunosenescent profiles, perhaps due to low grade inflammation and chronic stimulation with viral antigens? Second, does the development of cancer itself cause immunosenescence, perhaps due to tumour-derived inflammation and chronic stimulation with tumour-associated antigens? Third, do processes involved in both cancer and immunosenescence interact to exacerbate immunological decline in patients with malignancies?

In principle, many characteristics of an ageing immune system could contribute towards cancer risk and lead to poor outcomes in patients. For example, chronic inflammation is tumour promoting [13] and the age-associated changes to the cellular composition and functional capability of the immune system might impair the ability to detect and eliminate cancer cells [11, 12, 129]. Candidate examples include defects in the ability of innate immune cells to recognise malignant transformation, such as reduced natural killer cell cytotoxicity or defective toll-like receptor signalling in macrophages and dendritic cells, with the latter exhibiting a reduced ability to effectively activate T cells [11, 12, 129]. Ineffective priming of T cells by dendritic cells may lead to a weakened or anergic T cell response, which might be exacerbated by reduced thymic output of naïve T cells and an accumulation of exhausted T cells that have been chronically stimulated by virus or tumour antigens [11, 12, 129]. Thus, these effects may limit the ability to respond to novel antigens, such as those expressed by malignant cells. Age-

associated reductions in less abundant T cell populations, such as $\gamma\delta$ T cells and CD1d-restricted iNKT cells, may also impair cell-mediated tumour surveillance, and a decline in the number and function of B cells may lead to a less effective antitumour humoral response [11, 12, 129]. Finally, enhanced suppressive mechanisms with ageing may contribute, such as the accumulation of MDSCs, nTREGs, and Th2 cells producing suppressive or inhibitory cytokines such as IL-10 and TGF-β [11, 12, 129]. Indeed, some of these inhibitory mechanisms may have their roots within tumour masses, for example, as part of immune evasion strategies adopted by malignant cells or the process of immunoediting, whereby less immunogenic tumour cells are selected for and subsequently persist [130]. Although it seems intuitive that the age-associated decline in immune function could increase cancer risk and hinder cancer therapy, the links between malignancy and biomarkers of immunosenescence remain a debated topic of research. Recent studies examining links between immunosenescence and cancer are summarised in the next section.

8. Do Immunosenescent Profiles Increase the Risk of Cancer and Predict Poor Clinical Outcomes in Patients with Malignancies?

If immunosenescence increases the risk of developing cancer, then it might be expected for epidemiological studies to have repeatedly shown relationships between markers of an ageing immune system and the incidence of cancer or cancer-specific mortality. Relationships of this kind are perhaps difficult to detect and interpret however. Only a few characteristics of an ageing immune system have been linked to all-cause mortality, and most studies have either not examined or have failed to detect associations with cancer-specific deaths [31, 33–35, 46–48, 131]. Thus, it is unclear whether classical biomarkers of immunosenescence can estimate the chance of developing cancer or reflect the capacity to detect and eliminate tumour cells. However, other measurements of immune function that have not typically been considered hallmarks of immunosenescence may provide some insight. For example, a broad marker of immune competence, salivary S-IgA, *has* been independently linked to cancer. In a 19-year analysis of mortality data for 639 adults aged 63 years at the time of measurement, there was a significant negative association between the secretion of S-IgA into saliva and all-cause mortality, driven by an underlying association with cancer-specific mortality and in particular with cancers other than lung cancer [132]. Importantly, these relationships withstood adjustment for sex, occupation, smoking, medication use, and self-reported health [132]. Although it is well established that the incidence of cancer increases with age, coinciding with a decline in immune competence, many other factors, including physical activity level, body composition, and diet, which also change with ageing, likely interact. Thus, even when predicting cancer risk with accepted hallmarks of immunosenescence, such as plasma inflammatory activity—a common parameter to have been examined for associations with cancer, the results are likely to be influenced by multiple lifestyle variables and infection history, which have not always been assessed or controlled robustly [133–135]. In the context of inflammation predicting cancer risk, panels assessing multiple biomarkers may have better predictive utility than individual inflammatory variables, but the process of ageing and inflammageing is highly variable between individuals and very closely related to physical functioning [136]. The search for biomarkers that predict cancer risk, cancer-specific mortality, and disease progression in patients has spanned numerous immunological, inflammatory, and genetic variables. It is beyond the scope of this article to provide a thorough discussion of studies examining these general measurements of ageing. Thus, the remainder of this section will summarise relationships between cancer and the most established, robust, and immunosenescence-specific biomarkers.

8.1. Infection with CMV and Risk of Developing Cancer. Considering that many characteristics of an ageing immune system are driven by infection with CMV, if immunosenescence can be linked to cancer, it might be expected that CMV-seropositive individuals would demonstrate increased cancer-specific mortality. Links between CMV and mortality are contentious, with some studies reporting that CMV-seropositivity predicts greater all-cause mortality [47, 131] and others showing no effect of infection [44, 137]. In addition, the investigations implicating CMV in mortality mostly demonstrate that this relationship is driven by death from cardiovascular disease rather than by cancer [46, 48, 138]. However, in an analysis of 13,090 immunocompetent individuals aged 40–79 years at recruitment, which showed CMV-seropositivity was associated with increased all-cause mortality over a period of approximately 14 years (age- and sex-adjusted hazard ratio 1.16), cause specific analyses showed that in addition to cardiovascular disease (hazard ratio 1.06), cancer also contributed to the overall associations (hazard ratio 1.13) [139].

A small body of research has attempted to firmly establish links between CMV infection and the risk of developing specific forms of cancer in healthy people. For example, it has been hypothesised that late or recent exposure to CMV might cause breast cancer [140]. This idea was formed on the basis that breast cancer incidence is higher in countries where exposure to CMV occurs later in life, compared to countries where almost the whole population is exposed during childhood [140]. In testing this hypothesis however, there were no differences in CMV serostatus between women who developed breast cancer compared to controls [141]. Yet, in CMV-seropositive women, CMV-specific IgG was higher among breast cancer patients compared to controls, which was interpreted as reflecting recent exposure to CMV [141]. Similarly, it has been shown in CMV-seropositive women that an increase in CMV-specific IgG, measured in serum samples collected at least one year apart and approximately four years prior to breast cancer diagnosis, appears to precede tumour development [142]. However, high or rising CMV-specific IgG could also represent prolonged infection due to exposure early in life, viral reactivation, or superinfection with multiple virus strains [143, 144]. Indeed,

associations between the sero-epidemiology of CMV infection and breast cancer incidence are not consistent with measurements of viral DNA in breast tumours [145].

Other literature examining whether CMV increases the risk of cancer has focussed on mortality after organ transplantation. For example, it has been shown in a large study of 22,461 recipients of kidney, heart, liver, or lung transplants that mortality over 10 years, from a variety of causes, was greater when organs from a CMV-seropositive donor were transplanted into a CMV-seronegative recipient [146]. A greater incidence of posttransplantation cancer was observed among CMV-seropositive recipients compared to CMV-seronegative recipients (irrespective of donor CMV serostatus) [146]. Importantly, these associations were lost when controlling statistically for age and sex, challenging the links between CMV infection and cancer risk [146]. Other smaller studies have reported conflicting results. For example, in a study of 455 kidney transplant recipients, it was shown that pretransplant exposure to CMV and posttransplant CMV replication was associated with an increased incidence of cancer [147]. Compared to organ recipients who remained disease free, those who developed cancer also exhibited other signs of immunosenescence, such as large expansions of CD8+CD28− T cells [147]. In contrast, a study of 105 kidney transplant recipients showed that CMV-naïve individuals, compared to recipients who were exposed to CMV pre- or posttransplant, demonstrated a *greater* risk of cancer, which was attributed to antitumour activities of $\gamma\delta$ T cells that accumulate with CMV infection [148]. The relationships between CMV infection and the risk of developing cancer are unclear, and further research is required. However, it may be that infection with this virus per se does not increase the risk of developing cancer, and rather it is the consequences of infection that are more important. For example, in a study of 117 kidney transplant recipients identified as being at high risk of cutaneous squamous cell carcinoma, the age- or CMV-associated accumulation of CD8+CD57+ T cells was a strong predictor of cancer development, rather than viral infection directly [149].

8.2. Infection with CMV and Tumourigenesis. The majority studies examining links between CMV and malignancy have searched for viral DNA, RNA, and proteins in tumour cells from individuals with a confirmed cancer diagnosis or have examined the effects of infecting cancer cell lines with CMV in vitro [150–152]. Most research implicates CMV in tumourigenesis, rather than being a factor that increases the risk of developing cancer, and a common view is that CMV is "oncomodulatory," infecting tumour cells and modulating their malignant properties [150–152]. Changes include increasing chromosome instability, stimulating proliferation by dysregulation of the cell cycle, enhancing resistance to apoptosis, facilitating invasion, migration and endothelial adhesion, promoting angiogenesis, and contributing to immune evasion [150–152].

The cancer diagnosis to have received most attention in the context of CMV are malignant gliomas—the most common primary brain tumours in adults—comprising both astrocytoma and glioblastoma multiform [152, 153]. It was first established in 2002 that a high percentage of malignant glioma tumours are infected with CMV and multiple gene products are expressed that likely contribute to oncogenesis [154]. These findings have been confirmed by multiple studies [153]. It has also been shown that there is a negative association between the number of tumour cells infected with CMV and length of survival [155]. In addition, treating glioma patients with Valganciclovir, which limits CMV reactivation, improves two-year survival rate (by 72%) extending median survival by approximately 43 months [156]. CMV has subsequently been detected in tumour cells from patients with cervical cancer [157, 158], breast cancer [145, 159], colorectal cancer [160], prostate cancer [161], and gastric cancer [162]. The specific role(s) that CMV might have in oncogenesis and whether this virus negatively influences treatment outcomes is uncertain for most of these cancers, and the direction of effect may even be surprising. For example, despite the complications that CMV can have during the treatment of haematological malignancies, CMV reactivation is associated with a *decreased* chance of cancer relapse in acute myeloid leukaemia [163, 164].

8.3. Relationships between Cancer and Cellular Markers of Immunosenescence. In addition to CMV infection, other hallmarks of immunosenescence have been examined in patients diagnosed with different forms of cancer, and comparisons have been made to healthy individuals, or relationships with survival have been explored. For example, compared to healthy controls, patients with glioblastoma multiform exhibit lower percentages of total T cells, an accumulation of $\gamma\delta$ T cells, and expansions of $\alpha\beta$ T cells with CD4+CD28− and CD4+CD57+ phenotypes [165]. In addition, higher levels of CD4+CD28− and CD4+CD57+ cells measured three weeks after surgery in these patients were associated with shorter survival [165]. Similar accumulations of CD28− and CD57+ cells have been observed within the CD8+ T cell pool in patients with lung cancer [166, 167]. These expansions of CD8+CD28− cells were linked to broader aspects of immune function and clinical outcomes, such as the efficacy of a therapeutic cancer vaccine, assessed by length of survival following administration [167]. Other immune parameters, such as the frequency of CD57+ NKT-like cells in gastric cancer patients, have been reported as being comparable to healthy controls [168]. However, among the most advanced-stage patients, higher proportions of CD57+ NKT-like cells were associated with shorter survival [168]. Likewise, analysis of PD-1 expression on T cells from patients with acute myeloid leukaemia revealed no differences compared to healthy controls at the time of diagnosis, but PD-1 expression increased substantially at the time of relapse [169]. Thus, it seems that the timing of blood sampling and stage of disease are critical for interpreting these measurements. Other classical hallmarks of immunosenescence have also been examined, with reports of low CD19+ B cell numbers in patients with lung cancer, along with a decreased ratio of CD4+ T cells to CD8+ T cells, which has also been shown in patients with late-stage melanoma

[166, 167, 170]. These late-stage melanoma patients also exhibited marked changes to the composition of the $\gamma\delta$ T cell pool, exhibiting a decline in the number of Vδ2 cells, no change in Vδ1 cells, and a subsequent increase in the ratio of Vδ1 to Vδ2 cells [170]. A higher frequency of Vδ1 cells was negatively associated with survival, driven by Vδ1 cells with an early differentiated phenotype (as with CD8+ early differentiated $\alpha\beta$ T cells), but Vδ2 cells were not linked to clinical outcomes [170].

It is possible that the alterations within the T cell pool reported in patients with cancer are treatment- or disease-induced acceleration of normal ageing processes, such as reduced thymic output. For example, it has been shown that patients with breast cancer, compared to healthy controls, exhibit lower levels of T cell receptor rearrangement excision circles and lower percentages of recent thymic emigrants, observed in parallel with fewer CD8+ naïve T cells and shorter telomeres assessed in peripheral blood mononuclear cells [171]. Indeed, studies have reported incremental changes to biomarkers of immunosenescence following repeated administration of chemotherapy, including lower ratios of CD4+ T cells to CD8+ T cells and accumulations of CD28− and CD57+ cells in patients with breast and lung cancer [166, 172]. Both murine and human studies have shown that common chemotherapeutic drugs induce a senescence-associated secretory phenotype in peripheral blood T cells [173–175]. These so-called therapy-induced senescent cells, which accumulate and contribute to local and systemic chronic inflammation, can be identified by elevated expression of $p16^{INK4a}$, a tumour suppressor [173–175]. In T cells, $p16^{INK4a}$ is a marker of molecular ageing in healthy individuals, and its expression positively correlates with age and physical inactivity [174, 176]. In patients with cancer, T cell $p16^{INK4a}$ expression increases in a dose-dependent manner with chemotherapy administration [174, 175]. Interestingly, removal of $p16^{INK4a}$ expressing T cells increased physical activity and strength in tumour bearing mice [173]. Considering that in humans, $p16^{INK4a}$ T cell expression correlated with self-reported fatigue in the context of breast cancer [173], and that exercise is recommended for its fatigue-countering effects [177, 178], then it might be that some of the exercise-induced benefits for patients with cancer are, in part, brought about by interaction with $p16^{INK4a}$. Moreover, removal of $p16^{INK4a}$ expressing T cells in mice has been shown to reduce cancer recurrence [173]. Thus, $p16^{INK4a}$ seems to be an important marker of T cell senescence that not only interacts with exercise but might also be linked with survival, perhaps due to better anticancer immunity. In support, it has been demonstrated that IL-15 enhances the activity of tumour-specific T cells via delaying or reversing senescence, as shown by lower expression of $p16^{INK4a}$ among other markers of senescence [179]. Indeed, recent research has indicated that the capacity of peripheral blood T cells to recognise and respond to tumour-associated antigens is a predictor of survival in several malignancies, including breast cancer [180, 181], colon cancer [182], melanoma [183, 184], and hepatocellular carcinoma [185].

9. Does Exercise Elicit Anticancer Effects by Countering Immunosenescence?

9.1. Relationships between Active Lifestyles and Cancer: An Overview. An active lifestyle reduces the risk of developing cancer. In an analysis of self-report leisure-time physical activity data from 661,137 people in six population-based prospective cohorts, it was shown that the most active individuals (who performed more than ten times the recommended minimum volume of exercise each week) had a 31% lower cancer mortality risk compared to individuals reporting no activity [186]. Further, an investigation of risk for different cancer types among 1.44 million people from twelve prospective studies showed, by comparing individuals in the 90th and 10th percentiles for self-reported leisure-time physical activity, that very active lifestyles are associated with a lower risk of thirteen cancers, including oesophageal adenocarcinoma, liver, lung, kidney, gastric cardia, endometrial, myeloid leukaemia, myeloma, colon, head and neck, rectal, bladder, and breast [187]. The reduction in risk ranged from 10 to 42% among the different cancers, and most associations remained significant after statistical adjustment for body mass index and smoking [187].

Despite substantial reductions in cancer risk brought about by active lifestyles, the relevance and magnitude of effect for external and potentially modifiable factors influencing the development of malignancies has been debated. For example, comparing the total number of tissue-specific lifetime stem cell divisions and the lifetime risk of cancer in the same tissue has indicated that around 30% of the variation in cancer risk is due to external or inherited predispositions [188]. However, an alternative and more extensive analysis of similar data proposed that external factors contribute 70–90% of lifetime cancer risk [189]. A more conservative estimate is provided by investigations of risk factor exposure and cancer incidence. For example, approximately 43% of cancers occurring in the UK in 2010 have been attributed to suboptimal or past exposure to potentially modifiable external factors [190]. Although the most substantial cancer risk factor was tobacco, attributable to 19.4% of all diagnoses, lifestyle factors that can in principle be modified by intervention accounted for a substantial number of cases [190]. Factors included suboptimal diet (9.2%), overweight and obesity (5.5%), consumption of alcohol (4.0%), and inadequate exercise (1.0%) [190]. Other exposures implicated in the development of cancer, although potentially modifiable, are more difficult to avoid for practical, political, and societal reasons (e.g., occupation 10.0%, ultraviolet radiation 3.5%, ionizing radiation 1.8%, infections 3.1%, and reproductive factors 0.9%) [190].

Although current evidence implicates inadequate physical activity as accounting for a modest proportion of cancer diagnoses, these estimates are derived from self-reported physical activity data and the relationships that had previously been established between specific cancers and active lifestyles. Traditionally, research has linked inactive lifestyles with risk of breast, colon, and prostate cancer, with some evidence for endometrial and lung cancer [191]. However, more recent research suggests that a much larger range of cancers

are linked to inadequate physical activity, and it seems likely that this list will grow as more data become available [187]. Thus, as new evidence accumulates using objective measurements of physical activity in combination with measurements of body composition that are more sensitive than body mass index, the number of cancer cases attributable to inadequate physical activity may increase. Even if with future research, the fraction of cancers thought to be attributable to inactive lifestyles does not change; this estimate should not be discounted. Preventing just 1% of the 17.5 million cases of cancer worldwide is attractive and further justified by the increasing cancer incidence due to an expanded ageing population [116].

It should be emphasised that the relationships between cancer and exercise or physical activity are not limited to reducing the risk of developing malignancies in healthy individuals. For example, there is a large body of evidence demonstrating that aerobic exercise training can benefit patients with a cancer diagnosis. It is recommended that patients at all stages of the cancer survivorship continuum (e.g., from diagnosis, during and following treatment, to end of life) adhere to the same physical activity guidelines as for healthy individuals, so long as the mode, duration, intensity, and frequency of each exercise session are appropriate for the stage of disease and nature of treatment [177]. This recommendation has been made on the basis that exercise brings about many benefits for patients, including improvements in overall quality of life, cardiorespiratory fitness, strength, flexibility, mood, anxiety, and self-esteem [192, 193]. In addition, aerobic exercise training is an effective method for countering some of the side-effects of cancer treatment, such as fatigue [178]. Most importantly, there are strong relationships between cardiorespiratory fitness or habitual engagement in physical activity with positive treatment outcomes, including reduced disease recurrence and longer survival [192, 193]. These relationships have been demonstrated by observational studies of patients with different malignancies, including breast, prostate, colorectal, and lung cancer [194–198]. In support, the relationship between survival and aerobic exercise training during cancer therapy has been shown by a randomised and controlled trial in patients with breast cancer [199].

9.2. Relationships between Active Lifestyles and Cancer: Possible Mechanisms. The mechanisms underlying relationships between regular engagement in physical activity and the risk of developing cancer, some of which are also likely to be relevant to the positive effects that an active lifestyle can have on clinical outcomes in patients, have not been confirmed. This is despite conceptual understanding of the natural mechanisms protecting against cancer, which can be divided into two broad categories: first, defences that reduce or limit exposure to factors that promote malignant transformation and tumour growth and second, defences that target malignant cells more directly [200]. The first category of defences might be considered nonimmune mechanisms and include dietary anticarcinogenic substances, such as antioxidant vitamins; enzymes that actively remove carcinogenic substances, such as antioxidant enzymes; sensors of DNA damage that trigger repair or apoptosis; tumour suppressor genes; telomeres that limit cell division; and inhibitors of angiogenesis, cell migration, or invasion [200]. The second category of defences might be considered immune mechanisms and comprise almost all aspects of humoral and cell-mediated immunity, spanning the innate and adaptive immune compartments [200]. Thus, active lifestyles or aerobic exercise training could conceivably elicit protective effects via interaction with several of these defences [201].

Many of the exercise-induced anticancer mechanisms proposed in the literature have focussed on processes relevant to the cancers traditionally linked with reduced risk brought about by active lifestyles. For example, in the context of colon cancer, it has been suggested that regular aerobic exercise training or physical activity increases gastrointestinal transit time, reducing mucosal exposure to potentially carcinogenic substances [191]. With breast cancer, it has been proposed that regular aerobic exercise training or physical activity reduces lifetime exposure to oestrogen, perhaps due to delayed menarche and reduced ovulatory cycles [191]. Finally, the traditionally observed lower risk of prostate cancer has been attributed to an exercise-induced production of sex hormone binding globulin that reduces exposure to testosterone [191]. Other more widely applicable and commonly proposed anticancer effects of regular aerobic exercise training or physical activity include reducing oxidative stress and inflammation, limiting adipose tissue accumulation, modulating the insulin-like growth factor axis, and stimulating immune function [201, 202]. Most research has sought to identify mechanisms that could explain the lower incidence of cancer in very active individuals. However, limited research has investigated mechanisms underlying better clinical outcomes exhibited by patients who remain regularly active or engaged in structured aerobic exercise training during cancer treatment. For example, a murine model of breast cancer showed that regular wheel running improved the effectiveness of chemotherapy by promoting tumour vascularity, reducing tumour hypoxia, and subsequently enhancing tumour perfusion [203]. A factor that is likely to be relevant to both reducing the risk of developing cancer in healthy individuals, but also improving treatment outcomes in patients, is an exercise-induced enhancement of immune surveillance that might facilitate the detection and elimination of malignant cells. Research exploring this idea has so far focussed on natural killer cells. For example, in humans, it has been shown that an acute bout of vigorous-intensity exercise mobilises highly cytotoxic natural killer cells improving their ability to lyse multiple myeloma and lymphoma cell lines [204]. In addition, murine models of melanoma, lung, and liver cancer have shown that repeated bouts of wheel running stimulate an adrenaline- and IL-6-dependent mobilisation of natural killer cells into peripheral blood, which subsequently extravasate and home to implanted tumours, limiting cancer growth [58]. Indeed, it is likely that research investigating exercise, muscle, tumour, and immune cross-talk, exploring a role for both cellular and soluble mediators, will bring further insights into the most likely anticancer effects of aerobic exercise training [205].

9.3. Relationships between Active Lifestyles and Cancer: An Anti-Immunosenescence Effect of Exercise? Considering that, first, physical activity reduces the risk of developing cancer, second, cancer risk might be modulated by immunosenescence, and third, aerobic exercise training may bring about anti-immunosenescence effects, it has never been investigated whether the anticancer properties of exercise are elicited by countering immunological ageing. To emphasise, as healthy individuals who undertake a moderate volume of aerobic exercise throughout their lifetime appear to have a reduced risk of developing cancer and may exhibit less marked immunosenescence than those who are inactive, it could be hypothesised that these two observations are linked. In other words, does aerobic exercise training reduce the risk of developing cancer by limiting the age- and infection-associated accumulation of late-stage differentiated T cells along with concomitant changes in the naïve T cell pool, and perhaps overall immune function? Although worthy of investigation, this hypothesis may not be confirmed. First, in healthy individuals who are not immunosuppressed, the characteristics of the T cell pool have not been directly and robustly linked to the development of cancer. Second, although individuals undertaking extremely large volumes of endurance exercise may exhibit exaggerated signs of immunosenescence, there is generally a dose-response relationship between exercise and all-cause mortality and cancer-specific mortality [186, 187]. Despite the exception of links between ultraendurance exercise and cardiovascular risk [206], engagement in very high-volume exercise has not been linked with morbidity and mortality from other diseases, especially cancer [186, 187]. However, anti-immunosenescence effects of aerobic exercise training might have more relevance for patients who have been diagnosed with cancer and are undergoing treatment. As outlined earlier, higher cardiorespiratory fitness and regular exercise is linked to *successful* cancer treatment and *longer* survival [192–199]. In addition, exacerbated immunological decline, assessed by measuring cellular markers of immunosenescence, is linked to *unsuccessful* cancer treatment and *shorter* survival [165–168, 170]. Thus, it is possible that positive treatment outcomes, exhibited by patients with high cardiorespiratory fitness, who are physically active or undertake regular aerobic exercise training and exhibit less marked immunosenescent profiles compared to their inactive counterparts, could be driven by an anti-immunosenescence effect of exercise.

10. Conclusions

Considering that ageing results in impairments to innate and adaptive immunity and that a single bout of exercise is a powerful stimulus of immune function, it is appealing that regular aerobic exercise training might exert anti-immunosenescence effects. Evidence is accumulating in support of this idea, and these exercise-induced effects might delay the age-associated alterations to immune function. Although it is unknown whether such effects are brought about by exercise directly, such as a targeted removal of dysfunctional T cells, or indirectly, such as lower inflammatory activity and less frequent viral reactivation, it is conceivable that these changes will bring about considerable benefits to health, including reduced morbidity and mortality from infectious disease and cancer.

The links between biomarkers of immunosenescence and the risk of developing cancer in healthy individuals remain to be fully established. However, evidence is beginning to show that patients diagnosed with cancer exhibit immunosenescent profiles. Analysis of disease outcomes, in combination with measurements estimating the extent of immunosenescence, suggests that exacerbated immunological ageing is linked to unsuccessful clinical outcomes in several cancers. Considering that active lifestyles reduce the risk of developing cancer and are associated with positive treatment outcomes in patients, it is possible that some of these effects could be driven by an anti-immunosenescence effect of regular aerobic exercise training.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Targeting Endothelial Function to Treat Heart Failure with Preserved Ejection Fraction: The Promise of Exercise Training

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Although the burden of heart failure with preserved ejection fraction (HFpEF) is increasing, there is no therapy available that improves prognosis. Clinical trials using beta blockers and angiotensin converting enzyme inhibitors, cardiac-targeting drugs that reduce mortality in heart failure with reduced ejection fraction (HFrEF), have had disappointing results in HFpEF patients. A new "whole-systems" approach has been proposed for designing future HFpEF therapies, moving focus from the cardiomyocyte to the endothelium. Indeed, dysfunction of endothelial cells throughout the entire cardiovascular system is suggested as a central mechanism in HFpEF pathophysiology. The objective of this review is to provide an overview of current knowledge regarding endothelial dysfunction in HFpEF. We discuss the molecular and cellular mechanisms leading to endothelial dysfunction and the extent, presence, and prognostic importance of clinical endothelial dysfunction in different vascular beds. We also consider implications towards exercise training, a promising therapy targeting system-wide endothelial dysfunction in HFpEF.

1. Introduction

Heart failure (HF) is the most frequent cause of hospitalization in people over 65 years, and incidence is still increasing. Despite improved medical management, prognosis is grim, especially for heart failure with preserved ejection fraction (HFpEF) which has a 65% mortality rate at 5 years [1]. In contrast to heart failure with reduced ejection fraction (HFrEF), timely diagnosis of HFpEF remains a challenge and current standard therapy fails to improve prognosis [2]. Beta blockers and renin-angiotensin-aldosterone axis antagonists, drugs that mainly target the heart and have reduced mortality in HFrEF, had disappointing results in HFpEF trials [3–5]. As such, a "whole-systems" approach has been proposed, moving therapeutic focus in HFpEF away from the cardiomyocyte [6, 7].

Although HFpEF emerged as a distinct HF phenotype about three decades ago and about half of patients fall into this category, its pathogenesis remains incompletely understood. Beside advanced age, female sex, and sedentary lifestyle, HFpEF is associated with comorbidities such as arterial hypertension, diabetes, obesity, chronic obstructive pulmonary disease, and renal dysfunction [8]. Cardiac and extracardiac adjustments to these comorbidities can become maladaptive and lead to the HFpEF syndrome, with exercise intolerance as its main symptom. This maladaptation is characterized by structural changes such as myocardial hypertrophy and fibrosis, driven by a neurohormonal imbalance and systemic cytokine overexpression [9]. As a third mechanism, dysfunction of endothelial cells throughout the entire cardiovascular (CV) system has been put forward as the link between comorbidities and the pathophysiology of HFpEF.

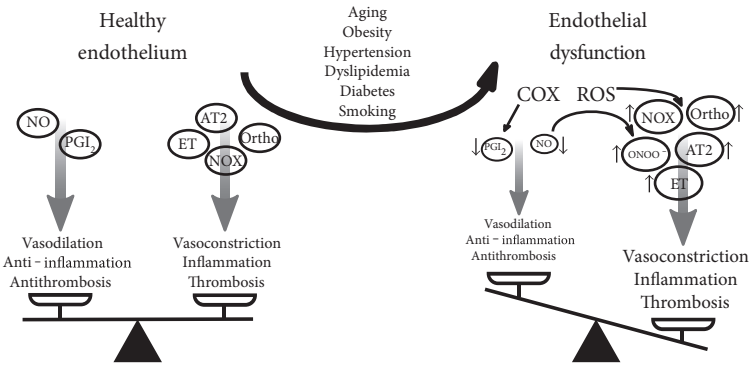

Figure 1: Pathophysiology of endothelial dysfunction. Healthy endothelium maintains a balance between vasodilating, anti-inflammatory, and anti-thrombotic factors on one side and vasoconstricting, inflammatory, and thrombotic factors on the other. In endothelial dysfunction, increased oxidative stress caused by comorbidities tips the balance over to a vasoconstricting, inflammatory, and thrombotic profile. AT2=angiotensin 2, COX=cyclooxygenase, ET=endothelin, NO=nitric oxide, NOX=nicotinamide adenine dinucleotide phosphate oxidase, $ONOO^-$=peroxynitrite, Ortho=orthosympathetic nerve activity, PGI_2=prostacyclin, ROS=reactive oxygen species.

This builds on experimental evidence by Brutsaert et al. in the 1980s that the interaction between endothelial cells and cardiomyocytes directly influences diastolic function [10, 11].

Clinical endothelial dysfunction (ED) is recognized as a precursor to many CV diseases including HF [12]. Moreover, its prognostic value is proven in cohorts ranging from an unselected general population over patients at risk for CV disease (hypertension, chronic kidney disease) to patients with established CV disease [13]. Endothelial function is an independent predictor of survival in HF patients [14]. Exercise intolerance, the cardinal symptom in HFpEF, is objectively measured by peak pulmonary oxygen uptake (VO_2peak) which is determined by the product of cardiac output and arteriovenous oxygen (O_2) difference. Hence, both O_2 delivery mechanisms (cardiac output, peripheral vascular function) as well as O_2 utilizing factors (skeletal muscle) contribute to exercise intolerance [15]. Reduced endothelial-dependent vasodilation on exertion limits systemic O_2 delivery, precipitating the switch to an anaerobic metabolism and thereby exacerbating fatigue and dyspnea [16]. ED also forms an attractive therapeutic target due to its reversibility at early stages [17]. This has shifted the search for an effective HFpEF therapy towards interventions correcting ED.

Exercise training is one of the most successful approaches to improve and even correct ED [18, 19]. Exercise-based cardiac rehabilitation programs have already earned their merit by improving symptoms and reducing mortality in various CV diseases, including HFrEF [20, 21]. The additional beneficial effects on other comorbidities and risk factors make exercise training conceptually a promising therapy for HFpEF [22].

In this review, we will focus on different aspects of ED in HFpEF. First, we briefly review the underlying molecular mechanisms leading to ED. We list the existing evidence on the presence of ED in distinct vascular beds and the clinical importance relative to HFpEF. Finally, the effects of exercise training on endothelial function are discussed, portending important implications for HFpEF treatment.

2. The Endothelium Is More than a Barrier

The endothelium was long considered a mere protective layer between the blood and different extravascular tissues. We now know that endothelial cells are dynamic, highly interacting cells regulating blood vessel function and homeostasis. The healthy endothelium prevents platelet and leukocyte adhesion and aggregation, inhibits smooth muscle proliferation, and regulates vascular tone through release of vasoactive substances, all of which are essential in organ perfusion [23]. Nitric oxide (NO) is the major effector molecule, formed from its precursor L-arginine by endothelial NO synthase (eNOS) in response to stimuli such as shear stress, cytokines, and platelet-derived factors. In endothelial cells, NO inhibits expression of leukocyte adhesion molecules, reducing vascular inflammation and atherosclerosis. By diffusing into platelets and vascular smooth muscle cells, NO stimulates the soluble guanylate cyclase—cyclic guanosine monophosphate—protein kinase G (sGC-cGMP-PKG) pathway, hereby inhibiting platelet aggregation and inducing vasorelaxation [23]. NO also diffuses to cardiomyocytes adjacent to coronary microvascular and endocardial endothelial cells, modulating cardiac function [24]. Finally, NO mobilizes stem cells and progenitor cells important for vascular homeostasis and repair [25].

In the setting of CV disease risk factors (smoking, aging, hypercholesterolemia, hypertension, hyperglycemia, and obesity), the endothelium loses these regulatory functions [26, 27]. Reactive oxygen species play an important role, reacting with NO to form toxic peroxynitrite, thereby reducing NO bioavailability. This disturbance of endothelial homeostasis can lead to a vasoconstrictory, proinflammatory, and prothrombotic phenotype at risk for CV disease [12]. The term "endothelial dysfunction" refers to these phenotypic alterations. Figure 1 summarizes the most important molecular influences on healthy and dysfunctional endothelium.

Repair of diseased endothelium does not solely depend on proliferation of existing endothelial cells. Bone marrow-derived endothelial progenitor cells can be mobilized to sites

of endothelial injury or ischemia. They are able to proliferate, exert beneficial paracrine effects through secreting vascular growth factors, and finally integrate into the endothelial layer by differentiating into endothelial cells [28, 29].

3. Evaluation of Endothelial Function

ED is recognized as the first—but still reversible—step to overt atherosclerosis. As such, several diagnostic evaluation methods have been developed, with the goal to identify high-risk populations and start preventive therapy early. At the other end of the spectrum, presence and severity of ED is related to a negative outcome in established coronary ischemic heart disease and HFrEF [30].

Usually, endothelial function is measured as vasodilation in response to an endothelium-specific stimulus. This includes drugs, such as acetylcholine, but a short period of local ischemia also elicits endothelium-specific hyperemia. The amount of vasodilation can be assessed invasively (e.g., coronary angiography, intravascular flow wires), although noninvasive methods are more widely used nowadays. The percentage dilation of the brachial artery in response to forearm ischemia, measured by ultrasound, is called flow-mediated dilation (FMD) [31]. The more recent EndoPAT™ device (Itamar Medical, Israel) uses a fingertip probe to measure arterial tone. The response to ischemia is calculated automatically and is called reactive hyperemia index (RHI) [32]. Details and advantages of these and other techniques to measure endothelial function have been reviewed previously [26, 33]. Generally, FMD is considered a measure of the response to shear stress in conduit vessels (macrovascular), which is largely NO dependent, while RHI measures microvascular dilatation to shear stress, which involves other vascular mediators in addition to NO [34].

4. Endothelial Dysfunction in HFPEF: Cause or Consequence?

Impaired coronary endothelial-dependent vasodilation was found in nonischemic dilated cardiomyopathy, highlighting the implication of the endothelium in HFrEF regardless of the presence of atherosclerosis [35]. Moreover, ED is not only limited to the coronary arteries, but is equally present in other vascular beds, indicating the systemic nature of ED in HFrEF.

In 2013, Paulus and Tschöpe hypothesized that ED plays a causal role in the development of HFpEF [10]. They postulate that the comorbid illnesses seen in HFpEF are the primary impellent of a systemic inflammatory state, leading to coronary microvascular ED. Indeed, elevated levels of inflammatory cytokines are seen in HFpEF patients [36]. In asymptomatic patients, biomarkers of inflammation predict the onset of HFpEF but not HFrEF [37]. Circulating inflammatory cytokines activate and inflame the endothelium throughout the vascular system, including the coronary microvasculature. This coronary microvascular endothelial inflammation is seen in animal models of HFpEF and in human cardiac biopsies [38, 39]. Reduced endothelium-dependent vasodilation is seen in animal models as well [40].

Reduced NO signaling from dysfunctional endothelium then influences adjacent cardiomyocytes and cardiac fibroblasts through the sGC-cGMP-PKG pathway [24]. Lower myocardial PKG content eventually leads to functional and structural cardiac changes associated with HFpEF [41]. These include delayed myocardial relaxation, increased cardiomyocyte stiffness, cardiac hypertrophy, and interstitial fibrosis [10]. Cardiac-endothelial interaction is reviewed in more detail in 6.2.

However, a two-way interaction between HFpEF and ED exists. Once HF develops, the syndrome maintains a vicious circle, further impairing endothelial function. HFpEF itself causes a systemic inflammatory state with high levels of circulating proinflammatory cytokines, increasing production of reactive oxygen species and exerting direct deleterious effects on eNOS expression [42, 43]. Neurohormonal activation in HFpEF increases oxidative stress and activates collagen synthesis [44]. Thus, HFpEF worsens system-wide ED, causing a downward spiral eventually leading to progressive HF.

5. Clinical Importance: Endothelial Dysfunction as Prognostic Marker in HFPEF

As there is no universally accepted cutoff for defining ED, the actual prevalence of ED in HFpEF is unknown. In community studies, endothelial function declines with age and presence of CV risk factors [45, 46]. Understandably, FMD and RHI values are lower in populations with established CV disease, including HF patients [14, 47]. In one of the first studies proving reduced RHI in HFpEF, Borlaug et al. estimated the prevalence of ED in HFpEF patients at 42% [48]. Of note, the cutoff to define ED in this study was arbitrarily chosen as RHI <2.0, which is substantially higher than the original reference value defined in coronary artery disease patients (RHI <1.67) [49]. The prevalence of ED found in the Borlaug study could as such be overestimated.

In the largest study to date, measuring endothelial function in 321 Japanese HFpEF patients, Akiyama et al. found that a RHI below the median predicted CV events [47]. For each decrease of 1.0 in RHI, CV risk increased 20%. The prognostic significance of ED in HFpEF patients was independent of clinical, echocardiographic, and neurohormonal factors. This was later confirmed in a smaller study by Matsue et al. [50]. Of note, both Japanese studies propose a prognostic cutoff value for RHI <1.63, close to the original reference value of RHI <1.67. Applied to the large Akiyama study, this implies an ED prevalence of 50% in HFpEF patients. Full details of studies measuring peripheral ED in HFpEF can be found in Table 1.

Given the central role of ED in the development of HFpEF, this estimated prevalence of ED of 42–50% seems low. However, to be more precise, 42–50% of HFpEF patients have *peripheral* ED *as defined by a given RHI cutoff*. In our opinion, the other 50% fail to show decreased RHI because of the following reasons. First, it takes time before microvascular inflammation is translated to clinically measureable

Table 1: Studies assessing peripheral endothelial function in HFpEF patients compared to a control population.

Reference	Technique	Outcome variable	Study design	Number of patients	Number of HFpEF patients	Control groups	Result	*P* value
Studies assessing macrovascular endothelial function								
Hundley et al., 2006 [150]	FMD (magnetic resonance)	% change in femoral artery area	Case-control	30	9	Healthy, matched for age	FMD comparable in HFpEF and healthy,(12 ± 1 versus 14 ± 2%), no difference in shear rate	ns
Haykowsky et al., 2013 [61]	FMD (ultrasound)	% dilatation brachial artery	Case-control	111	60	Young healthy group, matched for gender Old healthy group, matched for age and gender	FMD better in young healthy (6.13 ± 0.53%) FMD comparable in HFpEF and old healthy (3.64 ± 0.28 versus 4.00 ± 0.38%),	<0.001 versus young ns versus old
Farrero et al., 2014 [94]	FMD (ultrasound)	% dilatation brachial artery	Case-control	70	28	Hypertensive, matched for age	FMD significantly lower in HFpEF + PHT (1.95 [−0.81–4.92] versus 5.02 [3.90–10.12] %), no difference in shear rate ($p = 0.47$)	0.002
Kishimoto et al., 2017 [151]	FMD (ultrasound)	% dilatation brachial artery	Case-control	206	41	Subjects without heart failure, unmatched	FMD significantly lower in HFpEF (2.9 ± 2.1 versus 4.6 ± 2.7%)	0.0002
Studies assessing microvascular endothelial function								
Balmain et al., 2007 [65]	Laser Doppler; venous occlusion plethysmography	Perfusion units; mL/100mL blood	Case-control	36	12	Coronary heart disease patients, unmatched	Cutaneous blood flow lower in HFpEF patients, venous capacitance was not different versus control*	<0.001
Borlaug et al., 2010 [48]	RHI (PAT)	Ln (PAT ratio 60–120 sec)	Case-control	50	21	Hypertensive group; Healthy group; both matched for age and gender	RHI significantly lower in HFpEF versus healthy (0.85 ± 0.42 versus 1.33 ± 0.34), but not in HFpEF versus hypertensive (0.85 ± 0.42 versus 0.92 ± 0.38)	<0.05* ns
Akiyama et al., 2012 [47]	RHI (PAT)	Ln (PAT ratio 90–150 sec)	Prospective cohort	494	321	Healthy, matched for age, gender, and presence of hypertension and diabetes mellitus	RHI significantly lower in HFpEF (0.53 ± 0.20 versus 0.64 ± 0.20)	<0.001
Vitiello et al., 2014 [152]	Venous occlusion plethysmography	mL/100mL blood	Case-control	32	18	Healthy, unmatched	Venous capacitance was not different versus healthy	ns
Yamamoto et al., 2015 [64]	RHI (PAT)	Not reported	Case-control	128	64	Healthy, matched for age, gender and comorbidities	RHI significantly lower in HFpEF (1.70 [1.55 – 1.88] versus 2.01 [1.64 – 2.42])	<0.001
Studies assessing both macro- and microvascular endothelial function								
Maréchaux et al., 2016 [62]	FMD (ultrasound)	% dilatation brachial artery	Case-control	90	45	Hypertensive, matched for age, sex, and presence of diabetes mellitus	FMD significantly lower in HFpEF patients (3.6 [0.4 – 7.4] versus 7.2 [3.2 – 12.7] %)	0.001
	RHI (Laser Doppler)	Perfusion units					Cutaneous blood flow lower in HFpEF patients (135 [104 – 206] versus 177 [139 – 216] units)	0.03

Table 1: Continued.

Reference	Technique	Outcome variable	Study design	Number of patients	Number of HFpEF patients	Control groups	Result	*P* value
Lee et al., 2016 [63]	FMD (ultrasound)	% dilatation brachial artery	Case-control	48	24	Healthy, matched for age, sex, and brachial artery diameter	FMD lower in HFpEF (3.06 ± 0.68 versus 5.06 ± 0.53), but no difference in FMD when corrected for shear rate	ns
	RHI (ultrasound)	Blood flow AUC after cuff release					AUC lower in HFpEF (454 ± 35 versus 659 ± 63 mL/min)	0.03

AUC: area under the curve; FMD: flow mediated dilatation; HFpEF: heart failure with preserved ejection fraction; Ln: natural logarithm; ns: not significant; PAT: peripheral arterial tonometry; PHT: pulmonary hypertension; RHI: reactive hyperemia index; *exact numbers not reported.

disturbances in vasoreactivity. Second, the cutoff of RHI <1.63 reflects a value useful for clinical prognosis, but has not been correlated with pathophysiological changes such as endothelial inflammation and reduced NO bioavailability. Third, RHI and FMD show poor agreement which suggests different mechanisms are measured [51]. Possibly, FMD more accurately reflects reduced NO signaling, but data on FMD in HFpEF is incomplete (no large or prognostic studies), and a cutoff defining ED is not available. Perhaps, it is more correct to state that the prevalence of ED in HFpEF is hard to estimate based on current data, but almost half of patients have reduced peripheral endothelial-dependent vasodilation compared to controls, which is linked to increased CV events.

Another clinical clue to the importance of ED is the relation with exercise intolerance, objectively measured by cardiopulmonary exercise testing and determination of VO_2peak. This is related to adverse prognosis, since VO_2peak is one of the strongest predictors of mortality in HFpEF [52]. The Fick principle (VO_2 = cardiac output • arteriovenous O_2 difference) states that VO_2peak can be limited by either a central factor, cardiac output, or peripheral O_2 extraction. The latter is influenced by oxygenation of the blood in the lungs, O_2 carrying capacity of the blood, appropriate distribution of blood to the peripheral tissues, and adequate tissue O_2 extraction from the blood. A key factor is the oxygen diffusion capacity (DO_2), which can be a limiting factor in both pulmonary and skeletal muscle O_2 kinetics. Applying Fick's law of diffusion ($VO_2 = DO_2$ • (capillary pO_2 – intracellular pO_2) with pO_2 being partial oxygen pressure) in exercising muscle, where intracellular pO_2 is very low, the capillary pO_2 determines the O_2 diffusion gradient. As such, capillary pO_2 can limit VO_2 during exercise [53]. Adequate endothelial function is necessary for an appropriate exercise-induced increase in blood flow to the muscles [54]. As capillary pO_2 is determined by the instantaneous balance between VO_2 and perfusion, ED can also limit capillary pO_2 [53]. In theory, ED can thus limit VO_2 both by reducing capillary blood flow and limiting O_2 diffusion.

Reduced cardiac output on exertion was long considered the main mechanism behind exercise intolerance in HFpEF [55]. Chronotropic incompetence and reduced peak stroke volume have both been implicated as the most important factor limiting VO_2peak [56]. More recently, a peripheral limitation to exercise capacity in HFpEF has been put forward. Borlaug et al. reported reduced systemic vascular resistance and lower RHI at peak exercise in HFpEF patients compared to hypertensive and healthy controls [48]. Haykowsky et al. even suggested that a failure to increase peripheral O_2 extraction during exercise is the predominant factor limiting VO_2peak [57]. The rest-to-peak change in peripheral O_2 extraction was the strongest independent predictor of VO_2peak in their study. This was later confirmed using exercise hemodynamics and exercise echocardiography [58, 59]. Although the dominant limiting factor to VO_2peak remains controversial, clearly peripheral elements play a role in determining exercise capacity in HFpEF. We further elaborate this finding in the next section.

6. Various Vascular Beds Display Endothelial Dysfunction in HFPEF

Theoretically, many clinical findings related to the HFpEF syndrome could be explained by a system-wide ED, leading to alterations in several organ systems. In Figure 2, we postulate that systemic ED is the underlying pathophysiological mechanism by which HFpEF risk factors lead to exercise intolerance. Systemic inflammation induced by HFpEF risk factors creates oxidative stress at the level of the endothelium throughout the vasculature, reducing NO availability for adjacent cells pertaining to all organs implicated in exercise performance.

In what follows, we review the evidence of the presence, extent, and underlying mechanisms of ED in different vascular beds and the corresponding organs.

6.1. Peripheral Vasculature and Skeletal Muscle. The peripheral circulation is the preferred organ system for measuring endothelial-dependent vasodilation, because of the easy, noninvasive measurement and the good correlation with "gold standard" invasive coronary vasodilation [60]. Studies evaluating peripheral endothelial function in HFpEF are summarized in Table 1.

Evidence regarding macrovascular ED in HFpEF is conflicting. The largest study to date reported no significant difference in FMD between HFpEF patients and healthy volunteers matched for age and gender [61]. In contrast, in almost all studies assessing microvascular peripheral endothelial function through RHI measurement, HFpEF patients have evidence of microvascular ED [48, 62–65]. Also, prognostic significance for ED in HFpEF has only been proven for microvascular dysfunction [47]. Of note, many studies have different methodologies even when using the same technique for measuring endothelial function. Control groups are often heterogeneous and unmatched, few studies using FMD adhere to the most recent guidelines that state shear stimulus must be reported, [66, 67] and different cutoffs for identifying ED are used. These disparities complicate the interpretation of study results.

Besides vasodilatory dysfunction of the afferent arteries to the working muscle, reduced peripheral O_2 extraction in HFpEF can also result from skeletal muscle dysfunction. HFpEF patients indeed have abnormalities in skeletal muscle mass, composition, capillary density, and oxidative metabolism. In contrast to the high prevalence of obesity, HFpEF patients have reduced lean leg mass [68]. This could be related to adipose tissue infiltration in muscle, which shows a similar correlation with exercise capacity. A markedly lower VO_2peak indexed to lean body mass in HFpEF patients further confirms that abnormalities in skeletal muscle perfusion and/or metabolism contribute to exercise intolerance [69]. Mitochondria are important regulators of skeletal muscle metabolism. Recently, reductions in muscle mitochondrial content, oxidative capacity, and expression of key mitochondrial proteins were found in muscle biopsies of HFpEF patients [70]. These changes were related to VO_2peak, emphasizing muscle mitochondrial dysfunction is likely a limiting factor to exercise

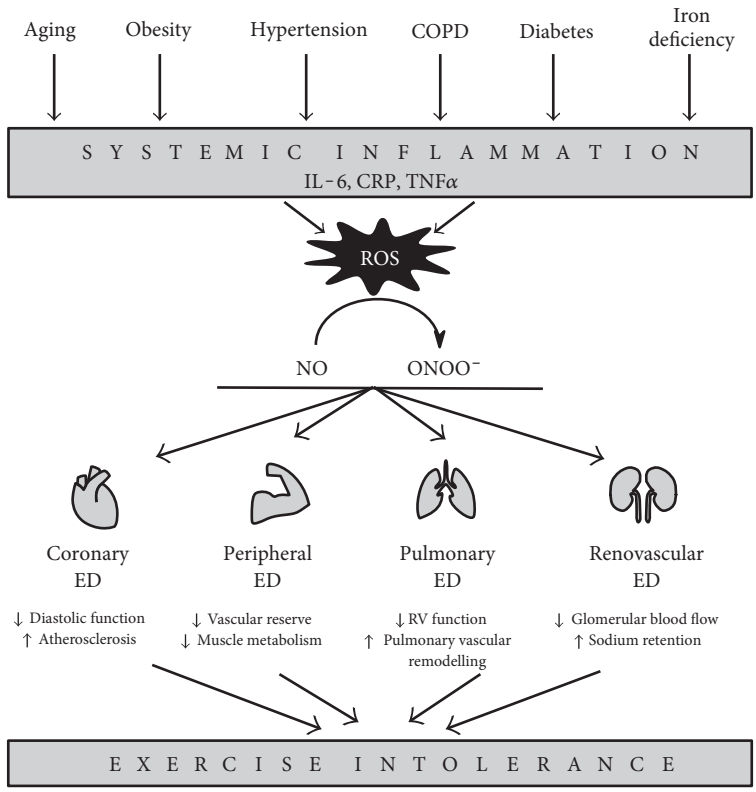

Figure 2: Role of system-wide endothelial dysfunction in HFpEF pathophysiology. Comorbidities induce systemic inflammation, creating oxidative stress in endothelial cells system-wide. Reduced NO bioavailability through reduction of NO to $ONOO^-$ causes endothelial dysfunction. In different vascular beds, endothelial dysfunction has heterogeneous effects, which manifest as the cardinal HFpEF symptom of exercise intolerance. COPD=chronic obstructive pulmonary disease, CRP=C-reactive protein, ED=endothelial dysfunction, IL-6=interleukin-6, NO=nitric oxide, $ONOO^-$=peroxynitrite, ROS=reactive oxygen species, RV=right ventricle, TNFα=tumor necrosis factor alpha.

capacity. Other possible underlying molecular changes could be a switch from oxidative slow-twitch type I fibers to glycolytic fast-twitch type II fibers which reduces oxidative capacity, increased muscle fatigability, and a reduction in skeletal muscle capillary density [71].

The latter is especially intriguing, as it links these skeletal muscle abnormalities to vascular dysfunction. Kitzman et al. demonstrated a severely reduced capillary-to-fiber ratio in muscles of HFpEF patients, related to VO_2peak [72]. A lower capillary density, and hence reduced capillary blood supply, may also underlie the muscle fiber atrophy seen in animal and human HFpEF studies [69, 71]. Also, as muscle blood flow assumes an important role in limiting VO_2 kinetics, the authors suggest a decreased O_2 diffusion to contracting muscle limits exercise capacity in HFpEF. As mentioned above, ED could play a role in this limitation of diffusive capacity by reducing the pO_2 driving gradient.

When leg blood flow is measured by ultrasound Doppler, HFpEF patients indeed have a reduced muscle blood flow during exercise compared to healthy controls, even at relatively low workloads of 10-15 W [73]. Stroke volume and heart rate were similar in HFpEF and control patients in this study, again implying a vascular (and not cardiac) limitation of exercise capacity. Also, HFpEF patients fail to augment peripheral O_2 extraction during exercise with a greater increase in blood pressure than controls [59]. This suggests that a reduced vasodilatory capacity prevents appropriate distribution of blood flow during exercise, leading to limitation of exercise capacity [55]. Possibly, microvascular ED contributes more than macrovascular ED at the level of the muscle vascular bed, as Haykowsky et al. found a peripheral limitation of exercise capacity but no decrease in FMD [61, 74].

In summary, there is evidence for microvascular ED in HFpEF, predictive of long-term CV morbidity. Reports on macrovascular dysfunction are conflicting, and all studies suffer from methodological disparities. Also, HFpEF patients suffer numerous changes in skeletal muscles which correlate with reduced VO_2peak, including mitochondrial dysfunction, fiber atrophy, and reduced oxidative capacity. Possibly, skeletal muscle abnormalities are linked to vascular dysfunction through a reduction in muscle capillary density, which limits muscle blood flow and O_2 diffusion during exercise.

6.2. Heart. Traditionally, coronary endothelial function is measured by intracoronary infusion of a vasodilating substance such as acetylcholine. Subsequently, microvascular function can be estimated by measuring coronary flow reserve (CFR), the ratio of coronary blood flow after the vasodilating stimulus over blood flow at rest. In HFrEF

patients, CFR correlates with VO_2peak, invasive and echocardiographic hemodynamics, and mortality [75–77]. Tschöpe et al. measured CFR in patients with diastolic dysfunction, showing a reduced vasodilatory response to intracoronary acetylcholine infusion even before onset of HF symptoms [78]. Furthermore, invasively measured CFR is reduced in HFpEF patients and CFR correlates with echocardiographic measures of diastolic function and LV hypertrophy [76, 78, 79]. Interestingly, two studies in HFrEF patients showed no relationship between CFR and peripheral endothelial function [75, 80]. As such, different pathophysiological mechanisms may lie at the origin of coronary and peripheral ED.

As mentioned above, reduced NO bioavailability leads to both structural and functional changes in HFpEF. Structurally, HFpEF hearts are characterized by interstitial fibrosis and both macroscopic and microscopic hypertrophy [10]. Hemodynamically, diastolic dysfunction is evident as slowed ventricular relaxation on one hand and decreased compliance due to myocardial stiffness on the other hand [9].

In the normal heart, endothelial NO bursts directly modulate relaxation in a beat-to-beat way [81]. High levels of peroxynitrite ($ONOO^-$), however, increase diastolic calcium content and thus delay cardiomyocyte relaxation [82]. Through its effects on sGC, NO is also able to modify cardiomyocyte stiffness and hypertrophy. sGC increases cGMP production, which in turn increases cellular PKG content. PKG acutely reduces cardiomyocyte stiffness through phosphorylation of the giant protein titin, the most important regulator of passive myocardial stiffness. Also, PKG functions as a brake on several pathways implicated in left ventricular hypertrophy. The sGC-cGMP-PKG pathway and its targets are indeed downregulated in HFpEF animals [83, 84]. Low PKG content has also been found in myocardial biopsies from HFpEF patients [41].

Finally, NO exerts direct antifibrotic effects in the heart by counteracting endothelin-1, angiotensin II, and aldosterone. Reduced NO bioavailability leaves profibrotic actions of these molecules unopposed, promoting proliferation of fibroblasts and myofibroblasts [85].

In summary, microvascular cardiac endothelium modulates diastolic function and development of LV hypertrophy and fibrosis. Coronary microvascular function, as measured by CFR, is reduced in HFpEF but does not relate to peripheral ED.

6.3. Lungs. Pulmonary hypertension (PHT) at rest is highly prevalent in HFpEF patients, with up to 83% affected [86]. Patients often have an exaggerated elevation of pulmonary artery pressures during exercise [87, 88]. This increased afterload on the right ventricle (RV) and the presence of common risk factors explain the high prevalence of RV dysfunction in HFpEF, which is associated with increased morbidity and mortality [89].

Passive transition of elevated end-diastolic pressure explains only part of the elevated pulmonary artery pressures in HFpEF [86]. As in patients with HFrEF and pulmonary arterial hypertension, impaired NO-dependent pulmonary vasodilation has been described in HFpEF patients. The Mayo Clinic group has spearheaded research in this field, proving abnormal RV and pulmonary artery hemodynamics both at rest and on exertion [88]. Although initially an increased pulmonary vasodilatory capacity was suggested based on dobutamine infusion [90], recent invasive measurements showed reduced exercise-induced pulmonary vasodilation in HFpEF [88].

Pulmonary arterial endothelial function was disturbed, and pulmonary artery pressures were higher in an animal infarct model of HFpEF, while aortic endothelial function and intracardiac pressures remained unaltered [91]. This could mean that pulmonary vascular ED even precedes systemic ED in HFpEF. Indeed, as the pulmonary circulation is primarily flow-driven in contrast to the pressure-driven systemic circulation, it may be more susceptible to the influence of shear stress and ED [92]. More recently, a murine model of HFpEF with PHT was established by blocking vascular endothelial growth factor receptors in obese and hypertensive rats. Oral administration of nitrite, which acts as NO donor, prevented the development of PHT but could not reverse established PHT [93]. These findings are compatible with "reversible" pulmonary ED playing an early role in the establishment of PHT, while "fixed"vascular remodeling occurs in more advanced stages.

In a cohort of 28 HFpEF patients with PHT that had severe macrovascular ED (FMD median 1.95%), Farrero et al. found a significant inverse correlation between FMD and pulmonary vascular resistance. No correlation was found with capillary wedge pressure [94]. While this does not prove a causal relationship, it is plausible that more severe HFpEF is related with more severe ED in the systemic and pulmonary vasculature, ultimately leading to PHT. This would corroborate the concept of whole-body ED in HFpEF.

PHT is also induced through reactive pulmonary vasoconstriction and vascular remodeling [95]. This process is largely mediated by NO, as pulmonary vascular reactivity is maintained by continuous local NO production [95]. A systemic reduced NO bioavailability, as found in HFpEF, causes vascular smooth muscle dysfunction in the pulmonary vasculature, paving the way for PHT [96].

Pulmonary function itself is frequently disturbed in HFpEF patients, with 59% suffering airflow limitation on spirometry [97]. As pulmonary impairment increases with symptom severity, pulmonary edema is a likely explanation. But diaphragm dysfunction may also contribute by increasing work of breathing. The diaphragm exhibits similar changes as skeletal muscle in HFpEF, including fiber atrophy, decreased oxidative capacity, impaired mitochondrial function, and increased fatigability [71]. As ED possibly underlies several skeletal muscle alterations, ED could also be a pathophysiological factor in diaphragm dysfunction, forming the link between skeletal muscle and respiratory abnormalities in HFpEF.

Pulmonary gas exchange is impaired in up to 83% of HFpEF patients, showing true O_2 diffusion limitation at rest in 59% [97]. At exercise, diffusion abnormalities are exacerbated in HFpEF patients compared to healthy individuals [98]. These findings provide further evidence that exercise

capacity is limited by O_2 diffusion in both the systemic and the pulmonary microcirculation.

In summary, PHT is a frequent and ominous finding in HFpEF patients. Vascular remodeling and reactive pulmonary vasoconstriction, caused by a reduced systemic NO bioavailability, play an important role in its development. Spirometry, diaphragm function, and pulmonary diffusion capacity are frequently impaired in HFpEF patients. Possibly, ED plays a role by impairing O_2 diffusion in the pulmonary microcirculation and causing adverse changes in diaphragm muscle composition similar to those in skeletal muscle.

6.4. Kidneys. HFpEF can induce renal dysfunction, and vice versa. Chronic kidney disease is highly prevalent in HFpEF patients (30–34% in large outcome trials) [99, 100]. Moreover, HF mortality is increased by concurrent renal impairment [101].

Clinically, endothelial function is impaired in patients with even mild chronic kidney disease, whether measured by RHI or FMD [102, 103]. Furthermore, worse endothelial function correlates with worse diastolic function on echocardiography [104]. Studies on the impact of renal disease on progression of ED in HFpEF are currently still lacking, but it is certainly an interesting field for future research [105].

HFpEF can cause renal dysfunction in different ways [106]. First, hemodynamic factors impair glomerular blood flow. Renal congestion due to elevated central venous pressure increases efferent glomerular pressure [107]. Additionally, fixed stroke volume and chronotropic incompetence reduce cardiac output on exertion, which impairs afferent blood flow [108]. The net result is decreased glomerular blood flow, leading to renovascular and glomerular injury and activating sodium retention pathways [109]. Second, the systemic inflammation that accompanies HFpEF has deleterious effects on the kidneys. Leukocyte recruitment causes renal fibrosis through transforming growth factor β-mediated fibroblast stimulation. Also, systemic inflammation reduces NO bioavailability as described above. Renal blood flow is dependent on systemic NO supply, which is reduced in HFpEF [110]. In a metabolic syndrome rat model of HFpEF, degradation of peritubular and glomerular microvasculature is linked with progressive glomerulosclerosis [111]. Interestingly, in this last study, microscopic renal damage was evident *before* onset of HFpEF.

On the other hand, renal disease can also lead to HFpEF. In long-term follow-up of >8500 chronic kidney disease patients, 34% was diagnosed with new-onset HFpEF [112]. Possible mechanisms include, again, worsening endothelial function and inducing systemic inflammation [105]. Several important feedback mechanisms, regulated by the kidney and disturbed in renal failure, induce ED: vitamin D deficiency, erythropoietin deficiency, elevated parathyroid hormone levels, and phosphorus excess [113–115]. Also, the endothelium is involved in sodium handling. Sodium retention could increase intracellular sodium, which disrupts endothelial homeostasis [116]. Asymmetric dimethyl arginine, a retention product found in kidney failure, is a competitive inhibitor of eNOS and increases endothelial oxidative stress [106].

In summary, HFpEF and chronic kidney disease are mutually influencing conditions. ED is an important risk factor for both diseases, and interesting pathophysiological links exist.

7. Exercise Training: The Silver Lining on the Cloud

Cardiac rehabilitation programs have been a mainstay of HFrEF treatment after it was discovered that training is safe and reduces hospitalizations [21]. The evidence in HFpEF, however, is still emerging. Several medium-sized single-center studies demonstrated substantial benefit of training in HFpEF patients [117–122]. Three recent meta-analyses concluded that exercise training in HFpEF increases VO_2peak and physical function scores [123–125]. Diastolic function (measured by E/e' ratio and left atrial volume) also improved with exercise in the landmark Ex-DHF trial [117]. These results have led to a class I, level of evidence A recommendation for exercise training in HF patients regardless of their ejection fraction in recent European Society of Cardiology HF guidelines [2]. Although no recommendations are made towards the intensity of exercise training, existing evidence suggests diverging effects of standard moderate-intensity aerobic training (at 60–70% of VO_2peak) and high intensity interval training (adding short intervals at 80–90% VO_2peak). In a single-center trial, high intensity training in HFrEF patients led to superior increases in VO_2peak and ejection fraction compared to moderate training [126]. Unfortunately, these findings could not be replicated in the large multicenter SmartEx trial [127]. Of note, the majority of patients exercised below the prescribed target in the high-intensity group and above target in the moderate group. A pilot study in 15 HFpEF patients showed superior effects of high intensity interval training on exercise capacity and diastolic function [128]. However, the lack of VO_2peak improvement in patients training at moderate intensity contrasts with the earlier studies.

The ongoing OptimEx study aims to study optimal exercise dose in 180 HFpEF patients with regard to aerobic capacity [129]. Also, this trial will reevaluate the effect of exercise training on FMD in HFpEF patients and add much-needed information on microvascular function.

In the contemporary "whole-systems" approach towards HFpEF therapy, ED forms an attractive target due to its systemic nature and reversibility in early stages. Improving ED in HFpEF can be achieved through correction of comorbidities, increasing NO bioavailability, or antioxidative therapy. Sadly, none of these approaches alone has thus far been successful in decreasing HFpEF-related morbidity or mortality. Exercise training integrates all three mechanisms, forming a promising systemically oriented therapy [7].

Both peripheral endothelial function and muscle metabolism are beneficially influenced by exercise. Exercise increases NO production by upregulating and phosphorylating eNOS through increased shear stress and vascular endothelial growth factor 2 release [19]. Exercise training also

reduces oxidative stress by downregulating angiotensin receptors and nicotinamide adenine dinucleotide phosphate oxidase [130]. In addition, the anti-inflammatory and permeability decreasing properties of exercise may contribute to improvement of endothelial function [22].

Circulating progenitor cells could add to these favorable changes [131]. Endothelium-repairing endothelial progenitor cells are mobilized from the bone marrow by stimuli such as ischemia and cytokine release, under control of circulating angiogenic T lymphocytes [132, 133]. Our group has shown that the number of circulating angiogenic T lymphocytes and their functional capacity increase with exercise training, both in healthy subjects and HF patients [134]. The acute exercise-induced changes in circulating angiogenic T lymphocyte function wane with exercise training, suggesting that repetitive exercise bouts progressively lead to endothelial repair [135]. Another group has recently shown increases in endothelial progenitor cell number and function in HF patients as well [136].

Molecular determinants of exercise-induced effects specific to HFpEF are still poorly investigated. In HFpEF rats, exercise training restored endothelial-dependent vasodilation measured ex vivo in organ baths [40]. Endothelial function correlated well with eNOS expression, which was reduced in HFpEF rats and recovered after exercise training. Matrix metalloproteinase activity, which is an indirect measure of extracellular matrix degradation and thus vessel wall modulation, was increased in HFpEF and blunted by exercise training while the endothelial cell layer remained intact. This suggests exercise-induced vascular changes extend beyond the endothelium.

In a secondary analysis of the Ex-DHF trial, circulating cytokines and hormones were analyzed in HFpEF patients before and after training [137]. Inflammatory cytokines (interleukins 1β, 6, and 10 and tumor necrosis factor alpha) showed no change with exercise. Interestingly, levels of the growth hormone releasing peptide ghrelin, which inhibits cardiomyocyte and endothelial cell apoptosis in vitro, increased by exercise training. Clearly, molecular determinants underlying the exercise-induced benefits in HFpEF deserve further in-depth exploration.

Clinically, peripheral endothelial function shows improvement after exercise training in patients with CV risk factors, coronary atherosclerosis, and HFrEF [138–140]. Of note, when comparing high intensity interval training to moderate training in HFrEF, endothelial function (as measured by FMD) and mitochondrial function (determined from muscle biopsies) improved only by high intensity training [126]. In HFpEF patients, Haykowsky et al. found that exercise training can increase peripheral O_2 extraction. The increase in VO_2peak was almost entirely attributable to an improvement in peripheral function (i.e., improved vascular and/or skeletal muscle functions) [141]. In a study by Fu et al., aerobic interval training increased muscle perfusion and muscle O_2 extraction in HFpEF patients. This increase in muscle vascular function was the only significant predictor of VO_2peak. Interestingly, this phenomenon was not seen in HFrEF patients, for whom improved cardiac output was the only predictor of VO_2peak [142].

Conversely, Kitzman et al. could not demonstrate an improvement of FMD after training HFpEF patients, despite an increase in VO_2peak [74]. A possible confounder could be that FMD was measured in the postprandial state in the Kitzman study, while guidelines advise to assess FMD in a fasting state because of a significant influence of food ingestion [67, 143]. In addition, the intensity of the exercise training protocol in this study was rather moderate and therefore could have failed to induce changes in macrovascular endothelial function.

There is no data regarding the effects of training on coronary, pulmonary, or renal ED in HFpEF patients. However, studies in patients with other CV diseases suggest exercise training is indeed able to improve regional endothelial function. Coronary endothelial function is improved by cardiac rehabilitation in dilated cardiomyopathy and coronary atherosclerosis [138, 144]. In patients with chronic kidney disease, changes in several molecular markers (asymmetric dimethyl arginine, glutathione, and lipid peroxidation products) suggest increased NO bioavailability through exercise training [106]. Unfortunately, Van Craenenbroeck et al. found that exercise training did not improve FMD nor cellular markers of vascular function, despite an increase in VO_2peak [102]. However, data on microvascular function is lacking.

8. Future Directions

Considering HFpEF as a multisystem syndrome rather than an isolated cardiac disease could lead us to alternative research approaches and eventually to successful therapies. The heterogeneity of the HFpEF patient population has frequently been cited as one of the reasons major trials have failed to prove a benefit for pharmacological treatment [145]. Efforts to subdivide HFpEF patients into different phenotypes have only started recently [146–148]. In the spectrum of HFpEF as a multisystem pathology, some patients seem younger and suffer less cardiac impairment, some have important metabolic disorders and more severe cardiac disease including RV and pulmonary vascular involvement, and others have a predominant renal dysfunction. Importantly, prognosis between phenotypes differs substantially [147]. The greatest challenges for future HFpEF research will be to correctly stratify patients into phenogroups and to design clinical trials accordingly. Whether endothelial function measurement could aid in identifying the correct HFpEF phenotype in patients is still unknown.

Also, a one-size-fits-all therapeutic approach is probably not the best strategy for the heterogeneous HFpEF population. A treatment algorithm based on presence of different comorbidities has recently been proposed [146]. Keeping in mind the important effects of even low-level exercise, matching or stratifying groups for physical activity seems reasonable when designing HFpEF trials, although maintaining statistical power will require a delicate balance. Rather, we support further subdividing of HFpEF based on large phenotyping studies to better characterize this heterogeneous population. Clinical trials could then be focused on a well-defined subgroup, eliminating confounding by other phenotypes.

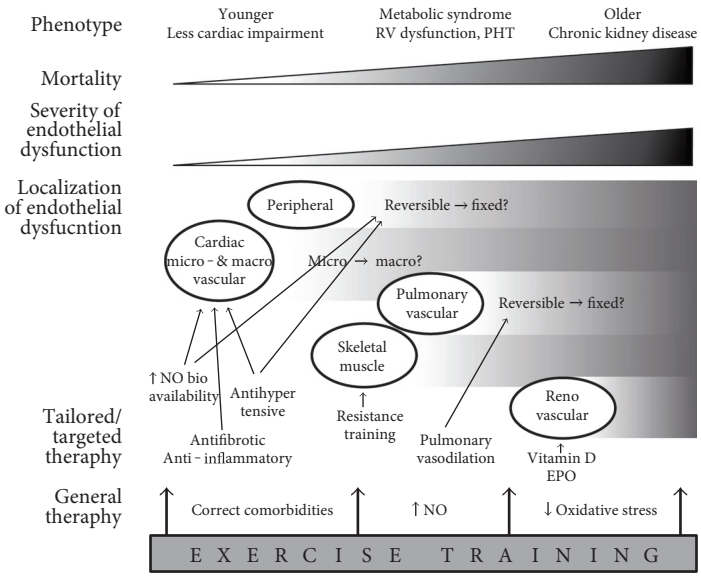

Figure 3: Possibilities for exercise training and targeted therapies depending on HFpEF phenotype. Cardiac ED is an early hallmark in all HFpEF patients. In older patients, pulmonary and renal vasculature are more frequently involved, and mortality is higher. HFpEF therapy could be tailored for each phenotype. Younger patients could still benefit from correction of comorbidities, preventing further systemic inflammation and ED. Increasing NO bioavailability, antifibrotic, or anti-inflammatory therapy could also be useful in early stages. Pulmonary vasodilation can only be effective when pulmonary vascular ED is manifested and still reversible. Exercise training has possible benefits at each stage, as it is able to correct comorbidities (weight loss, better glycemic control), increase NO bioavailability, and reduce systemic oxidative stress. EPO=erythropoietin, NO=nitric oxide, PHT=pulmonary hypertension, RV=right ventricle.

Unravelling the beneficial effects of exercise training in HFpEF could lead to patient-specific new therapies. Such a tailored approach can be useful in patients who are unable to exercise, or as add-on to a training program. Pharmacological or nonpharmacological correction of comorbidities, increase of NO bioavailability, and antioxidative therapy are possible targets, some of which are being explored in clinical trials already [119, 149]. These can be combined with exercise training to compose a truly personalized treatment for each patient (Figure 3).

9. Conclusions

HFpEF is a multisystem pathology. Cardiac dysfunction is not the sole causative factor, but interacts with a heterogeneous range of organ dysfunctions, including pulmonary, renal, peripheral vascular, and skeletal muscle dysfunctions. Endothelial dysfunction could be a central mechanism in this system-wide CV maladaptation, as such it forms an attractive target for future HFpEF therapies. Exercise training is thus far the only therapy with proven beneficial effects in HFpEF. While exercise training does not improve macrovascular ED in HFpEF, evidence does suggest peripheral vascular and/or skeletal muscle function is enhanced. This warrants a shift in both fundamental and clinical research towards endothelial-targeted therapies, including exercise training, in the search for an effective therapeutic strategy for HFpEF.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

High-Intensity Exercise Reduces Cardiac Fibrosis and Hypertrophy but Does Not Restore the Nitroso-Redox Imbalance in Diabetic Cardiomyopathy

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Diabetic cardiomyopathy refers to the manifestations in the heart as a result of altered glucose homeostasis, reflected as fibrosis, cellular hypertrophy, increased oxidative stress, and apoptosis, leading to ventricular dysfunction. Since physical exercise has been indicated as cardioprotective, we tested the hypothesis that high-intensity exercise training could reverse the cardiac maladaptations produced by diabetes. For this, diabetes was induced in rats by a single dose of alloxan. Diabetic rats were randomly assigned to a sedentary group or submitted to a program of exercise on a treadmill for 4 weeks at 80% of maximal performance. Another group of normoglycemic rats was used as control. Diabetic rat hearts presented cardiomyocyte hypertrophy and interstitial fibrosis. Chronic exercise reduced both parameters but increased apoptosis. Diabetes increased the myocardial levels of the mRNA and proteins of NADPH oxidases NOX2 and NOX4. These altered levels were not reduced by exercise. Diabetes also increased the level of uncoupled endothelial nitric oxide synthase (eNOS) that was not reversed by exercise. Finally, diabetic rats showed a lower degree of phosphorylated phospholamban and reduced levels of SERCA2 that were not restored by high-intensity exercise. These results suggest that high-intensity chronic exercise was able to reverse remodeling in the diabetic heart but was unable to restore the nitroso-redox imbalance imposed by diabetes.

1. Introduction

Diabetes mellitus is one of the most common chronic diseases all over the world, becoming an epidemic, triggered probably by reduced physical activity and increased obesity in the population [1, 2]. Diabetic cardiomyopathy is the deterioration of the myocardial function and morphology produced by the altered glucose homeostasis imposed in diabetes, independent of coronary disease [3]. Diabetic cardiomyopathy is characterized initially by diastolic dysfunction and cardiac hypertrophy, with preserved ejection fraction. At the cellular level, the diabetic myocardium presents myocyte hypertrophy, interstitial fibrosis, and apoptosis [4]. This process of cardiac deterioration involves the generation of reactive oxidative species (ROS) [5]. Oxidative stress exists when the production of ROS outweighs their degradation by antioxidant systems [6]. The resultant elevation of ROS has numerous harmful effects on the cardiovascular system via cellular damage by oxidation, disruption of vascular homeostasis through interference with NO, and detrimental intracellular signaling pathways [7]. In a variety of animal models of diabetes and humans with diabetic cardiomyopathy, there is excessive ROS production from both mitochondrial and extramitochondrial sources, and ROS have been implicated in all stages of the development of heart failure, from cardiac hypertrophy to fibrosis, contractile dysfunction, and failure [5, 8].

Several ROS sources contribute to the observed oxidative stress in the diabetic heart such as xanthine oxidoreductase (XOR) [9], nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) [10], mitochondria [9], and uncoupled nitric oxide synthases (NOS) [10–13].

A direct consequence of the increased production of reactive oxygen species (ROS) is NOS uncoupling. This is the aftermath of the oxidation of tetrahydrobiopterin (BH_4), an essential cofactor for NOS activity. When NOS is uncoupled, its activity is redirected towards the production of superoxide, instead of nitric oxide (NO), further contributing to the oxidative process.

Physical exercise is prescribed as part of the rehabilitation for patients with heart diseases since it reduces cardiac risk factors, protects against myocardial damage, and improves cardiac function [14]. In type 2 diabetic patients, exercise is also advised as part of the nonpharmacological treatment, since it exerts a number of benefits such as improved insulin sensitivity and reduction in body weight [15]. Nevertheless, there is less information regarding type 1 diabetic patients. A recent report indicates that intense exercise is associated to reduction in the risk of cardiovascular events in type 1 diabetes patients [16]. In animal models of type 1 diabetes, low-to-moderate exercise training has been found to improve cardiac glucose metabolism [17], reduce apoptosis [18], and improve ventricular function [19].

The intensity of exercise for diabetic patients is a matter of intense investigation, being suggested that in particular, high-intensity interval training may confer additional cardiometabolic protection [20]. For instance, while moderate-intensity exercise did not improved cardiac function in type 2 diabetic patients [21], a similar study but with a high-intensity interval training showed improved cardiac function and remodeling [22]. Another study showed no difference between moderate- and high-intensity exercises [23], although this later did not include the assessment of cardiac parameters.

As mentioned before, in animal models of type 1 diabetes, low- and moderate-intensity exercise have been reported to reverse or prevent some of the cardiac maladaptations of diabetic cardiomyopathy [18, 24]. Nevertheless, the information regarding high-intensity exercise in diabetic cardiomyopathy is less abundant.

The mechanisms by which exercise may produce it beneficial effects include an increase in nitric oxide production [25] and a reduction in oxidative stress [26]. In this work, we tested the hypothesis that a high-intensity exercise training program could reverse the cardiac maladaptations and oxidative stress that are produced by diabetes.

2. Methods

2.1. Animals and Training Protocol. Diabetes was induced in 3-month-old male Sprague–Dawley rats by a single dose of alloxan (Sigma-Aldrich, St. Louis, MO), 200 mg/kg, intraperitoneal. Six days day after alloxan injection, hyperglycemia was confirmed (plasma glucose levels >300 mg/dL). Diabetic rats were randomly assigned to a sedentary group ($n = 5$) or submitted to a program of exercise on a motor-driven treadmill, 5 days/week, for 4 weeks. The maximal intensity of the training for each rat was assessed by stepwise increases in the treadmill speed. Two days prior to the test, the animals were submitted to a period of acclimatization in the treadmill, walking at 0.6 km/h, twice a day, the first day. During the second and third days, the incremental velocity test was applied that consisted of increasing the treadmill velocity from 0.6 km/h to 0.2 km/h every 3 min, with no upper limit, until the animal reached exhaustion that defines the end of the test. The angle of the treadmill was kept constant at 0°. Once the animal reached its maximal velocity for at least 3 minutes, this value was assigned as the maximal performance (100%). Then, the animal was trained at the 80% of its capacity (velocity and time), once a day, five days a week, for four weeks. Every week, maximal capacity was reevaluated for each animal, to adjust its training load for the next week.

To determine the duration (in minutes) of the training, a tolerance test was applied that consisted in determining the maximal time that the rat was able to sustain the 80% of the velocity previously determined. Finally, the training consisted of the 80% of the maximal velocity for the 80% of maximal time. This finally determines the volume of training for each animal. The rats were maintained in the animal facility of the Universidad de Talca, with food and water ad libitum, at room temperature (22 ± 5°C) and with cycles of 12 hrs light/darkness. The diet used was obtained from Champion® (20.5% crude protein, 5% fiber, 4% fat). A group of normoglycemic rats was used as control ($n = 7$). Finally, the rats were euthanized after two days after the training protocol finished, to avoid potential confounding effects of acute exercise. To extract the heart, the animals were induced deep anesthesia with ketamine (75 mg/100 g body weight) and xylazine (15 mg/100 g body weight), checking for the complete absence of sensitive reflexes.

Plasma glucose determinations were performed using the glucose oxidase system (Valtek Diagnostics, Santiago, Chile), following the manufacturer instructions, and using a spectrophotometer (Rayleigh UV-9200).

All procedures were performed in conform to the *NIH Guide for the Care and Use of Laboratory Animals*. The protocol was approved by the Bioethical Committee of the Universidad de Talca.

2.2. Histological Staining. After excision, hearts were fixed in 4% paraformaldehyde. After fixation, the samples underwent a series of dehydrations and were embedded in paraffin blocks. After this, 5 μm sections were obtained with a Microm HM 325 microtome and then mounted on xylanized slides. Sections of hydrated and deparaffinized xylene tissue were stained with hematoxylin and eosin (H&E), used for TUNEL analysis or Sirius red staining.

2.3. Assessment of Cardiac Apoptosis. Apoptosis was evaluated by the TUNEL assay [27], which detects fragmented DNA in situ in the cell nucleus. The assay was performed using the TUNEL Apoptosis Detection Kit (EMD Millipore, Temecula, CA), according to the manufacturer's instructions with some modifications. Cardiac sections prepared as above indicated, then incubated with 50 μL of proteinase K for

30 min in a humid chamber and washed with PBS. Then, sections were incubated with the TdT end-labeling cocktail that contains TdT and biotinylated dUTP, for 5 min at room temperature. Each step was followed by PBS washes. Then, sections were incubated with blocking buffer for 20 min at room temperature. After this, FITC-labeled avidin was applied for 30 min at 37°C in a humid chamber. Sections were washed twice with PBS for 15 min at room temperature in the dark and then counterstained with propidium iodide (1 : 2000) for 15 min. Sections treated with DNAse I was used as positive controls. Sections in which treatment with proteinase K was replaced by PBS were used as negative control. After finishing the protocol, sections were observed with a Zeiss LSM-700 confocal microscope and TUNEL positive cells and total cells were counted. The apoptotic index represents the number of $TUNEL^+$ cells to total number of cells. This percentage was compared between groups.

2.4. Real-Time PCR. For quantitative polymerase chain reaction (qPCR), total RNA was extracted from rats hearts using TRIzol reagent and reversed transcribed using high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). qPCR was performed in triplicate for each heart, using a 20 μL mixture containing 1 ng cDNA, TaqMan Master Mix, and TaqMan® Gene Expression Assays (Applied Biosystems) for NOX2 (Rn00576710_m1) and NOX4 (Rn00585380_m1). As an internal control, glyceraldehyde 3-phosphate dehydrogenase GAPDH (Rn01775763_g1) was determined in each reaction. Reaction conditions were set according to the manufacturer: one cycle of 50° for 2 min, one cycle of 90° for 10 min, and 40 cycles of 15 s at 95° and 60° for 1 min using an Mx3000P qPCR system (Agilent Technologies, CA, USA). Relative fold change was calculated by the $2^{\Delta Ct}$ method and compared with baseline values as previously described [28].

2.5. Biopterin Measurements. Tetrahydrobiopterin (BH_4) and dihydrobiopterin (BH_2) were measured form cardiac homogenates, following the procedure described by Fukushima and Nixon [29], with modifications, by HPLC (Perkin Elmer series 200) separation and fluorescence detection at 350 nm (Shimadzu RF-20A). The procedure involves a differential oxidation BH_4 and BH_2 with iodine in acidic and basic conditions. In acidic conditions, both BH_4 and BH_2 are oxidized to biopterins, while under basic conditions, only BH_2 is oxidized to biopterin. The difference in the content of biopterin between both oxidations represents the amount of BH_4. Intracardiac BH_4 content was normalized to the total protein content of the sample.

2.6. Western Blotting. Cardiac tissue was homogenized in 3 mL of lysis buffer (Tris 50 mM, SDS 0.1%, NaCl 30 mM, EDTA 2 mM) supplemented with 30 μL proteases inhibitors cocktail (MP Biomedicals, Solon, OH) using an ultraturrax, as previously described [30]. Then, the homogenate was centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was removed and protein concentration was quantified using the BCA method (BCA Protein Assay Kit, Thermo Fisher Scientific, Rockford, IL). For this, 100 μg of protein were mixed with Laemmli buffer, separated by SDS-PAGE, and blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Antibodies used were the following: for NOX2 (anti-$gp91^{phox}$ 1 : 1000, catalog number: 611415, Lot: 15660), $p67^{phox}$ (1 : 1000, catalog number: 610913, Lot: 000079141) and eNOS (1 : 2000, catalog number: 610297, Lot: 35170) from BD Biosciences (San Jose, CA); for NOX4, from Thermo Scientific (1 : 1000, catalog number: PA1-46014), serine 16 phosphorylated phospholamban (1 : 1000, catalog number: A010-12, Lot: 0214-01) and total phospholamban (1 : 1000, catalog number: A101-14, Lot: 642016) were obtained from Badrilla (Leeds, UK); SERCA2 (1 : 2000, catalog number: SC 8094, Lot: C1115); GAPDH (1: 1000, catalog number: SC-365062, Lot: A2715); and secondary antibodies from Santa Cruz (Santa Cruz, CA). The protein bands were visualized using Supersignal West Femto Reagent (Pierce, Rockford, IL). Western blots were scanned and bands were quantified by densitometry analysis using ImageJ software.

2.7. Statistical Analysis. Results are expressed as mean ± standard error. Comparisons between groups were performed using one-way analysis of variance (ANOVA) with Newman-Keuls and Dunnett as post hoc analysis, for data with normal distribution. For nonparametric data, Kruskal-Wallis test was applied, with Dunn's multiple comparisons test as post hoc analysis. Analysis of exercise training was performed using ANOVA with repeated measures. Statistical significance was set at a value of $p < 0.05$. These analyses were performed using the SPSS statistical package and the GraphPad Prism 5 software (San Diego, CA).

3. Results

3.1. Alloxan-Induced Diabetes in Rats. Alloxan injection induced hyperglycemia in rats as expected for a type 1 model of diabetes (Table 1). Control animals increased body weight while both diabetic groups showed a slight weight reduction. All the animals in the training group completed the protocol and increased significantly their aerobic capacity (Figure 1). Diabetic rats submitted to the exercise protocol showed reduced plasma glucose levels compared to the sedentary diabetic rats ($p < 0.05$).

Exercise training tests at baseline (before diabetes) for maximal velocity and maximal time were performed. These tests were repeated after the induction of diabetes (week 0) and with these values was estimated the 80% of maximal performance that were used the next week of training for each animal. In the subsequent weeks, this procedure was repeated. In this way, diabetic rats increased their exercise capacity significantly (see Figure 1), specially the maximal velocity that increased each week, although they did not reach the baseline values (before diabetes). Nevertheless, at the end of the third and fourth week of training, the test maximal capacity (velocity and time) tests were not performed.

3.2. Cardiac Morphology. As it has been previously described, as part of the remodeling process that takes place in the diabetic heart, diabetes induced cellular hypertrophy of

Table 1: Body weight and plasma glucose.

Parameters	Control	Diabetic	Diabetic + exercise	p value
Baseline body weight, g	307.5 ± 7.5	333.0 ± 8.6	335.0 ± 10.6	0.1258
Final body weight, g	452.5 ± 19.3	318.0 ± 6.2†	298.0 ± 26.1†	0.0004
Baseline plasma glucose (mg/dL)	87.0 ± 8.7	78.0 ± 2.0	100.6 ± 4.5	0.0501
Plasma glucose at the beginning of training (mg/dL)	78.7 ± 3.5	573.5 ± 44.1†	516.6 ± 72.7†	<0.0001
Plasma glucose at the end of training (mg/dL)	108.4 ± 5.8	587.9 ± 15.9†	476.7 ± 42.6††	<0.0001

$^{\dagger}p < 0.005$ versus control; $^{\dagger\dagger}p < 0.05$ versus diabetic.

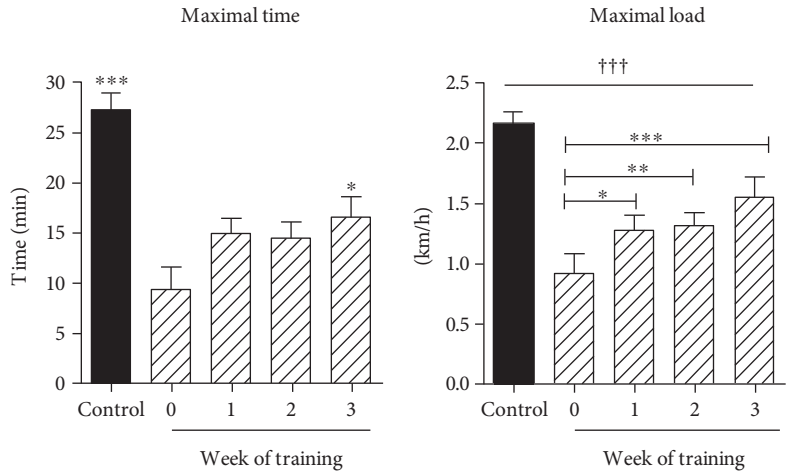

Figure 1: High-intensity training. Maximal time (min) and velocity (km/h) test. Rats were submitted to an exercise test before (control) and after induction of diabetes. Week 0 corresponds to the time point after the confirmation of diabetes. From week 0, the result of each week test was used to calculate the 80% of maximal capacity for the next week training. Then, every week, a maximal capacity test was performed. $^{\dagger\dagger\dagger}p < 0.005$ versus all the other groups; $^{***}p < 0.005$ versus week 0; $^{**}p < 0.001$ versus week 0; $^{*}p < 0.05$ versus week 0.

cardiomyocytes (Figure 2). Compared to the hearts from control normoglycemic rats, diabetic rat hearts presented increased perimeter of cardiomyocytes: 73 ± 7 μm in the control group and 89.5 ± 4.3 μm in the diabetic group ($p < 0.05$), and this value was reduced in diabetic rats that underwent exercise: 78.7 ± 2 μm. Consistent with this, myocyte area was increased in diabetic hearts compared to that in controls and reduced in animals that underwent physical training: 297 ± 17 μm^2 in control rats, 446 ± 26 m^2 in diabetic rats, and 363 ± 14 μm^2 in the diabetic + exercise group ($p < 0.05$).

As expected, diabetes induced an increase in fibrosis as collagen deposition in the heart as part of the cardiac damage (Figure 3). Nevertheless, chronic exercise reduced cardiac fibrosis: 4.43 ± 0.9% of fibrosis in the control group, 8.68 ± 0.7% in diabetic rats, and 5.72 ± 0.7% diabetic hearts from the rats that underwent high-intensity exercise.

3.3. Apoptosis. It has been observed that diabetes is associated with increased degree of apoptosis in the myocardium [31]. For this reason, we investigated this type of cell death in our experimental animals, using the TUNEL assay (Figure 4). In the control normoglycemic and diabetic rats, the level of apoptotic index was low: 1.9 ± 2.2 and 1.5 ± 2%, respectively. Unexpectedly, this level was increased substantially in the diabetic + exercise group: 6.5 ± 10.2% ($p < 0.05$). These data suggest that high-intensity exercise training induced an increase in cardiomyocytes apoptotic death.

3.4. NADPH Oxidases. Diabetes is associated with an increase in oxidative stress in the myocardium, particularly derived from NADPH oxidases as source of reactive oxygen species [32]. For this reason, we first looked into the expression at the level of NOX2 and NOX4 mRNA (Figure 5), the two main isoforms of NADPH oxidases that are expressed in the heart and, specifically, in the cardiac myocyte. Diabetes induced a twofold increase in the NOX2 mRNA level, compared to control normoglycemic animals ($p < 0.05$). Unexpectedly, exercise increased this level about eightfold. In the case of NOX4, diabetes also increased the mRNA levels ($p < 0.05$), and in the diabetes + exercise group, this increase was even more pronounced, although with substantial variability.

Next, we tested whether this increase in the NOX mRNA levels was followed by increased protein expression. For this purpose, we performed SDS-PAGE and Western blotting assays of cardiac samples (Figure 6). In the case of the protein levels of NOX2, both the diabetes and diabetes + exercise groups showed about a sevenfold increase. In the case of NOX4, there was a trend of increase but not statistically significant. On the contrary, the levels of $p67^{phox}$, one of the regulatory subunits of NOX2, also showed a significant increase in the diabetic + exercise group ($p < 0.05$). These data suggest that the components of NOX2 are upregulated, increasing the levels of myocardial oxidative stress, especially in the group submitted to high-intensity exercise training.

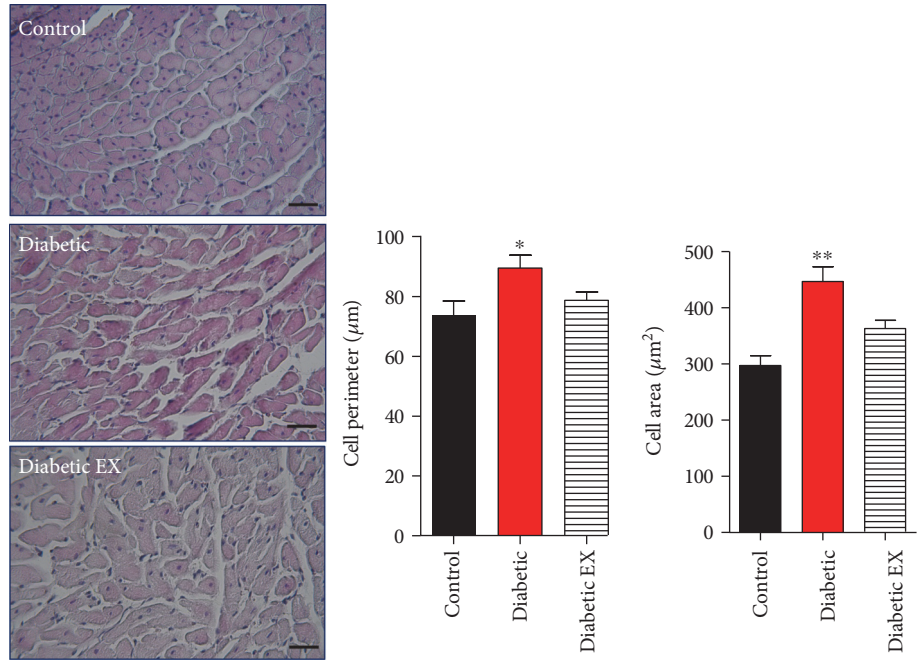

Figure 2: Cardiac hypertrophy induced by diabetes. Cardiac sections were stained with hematoxylin and eosin (see the photomicrographs). The bar graphs depict the statistical analysis for cellular hypertrophy (area and perimeter) in the control (nondiabetic), diabetic, and diabetic + exercise (diabetes EX) rats. The bar indicates 50 μm. $^{*}p < 0.05$ versus the other groups; $^{**}p < 0.01$ versus control. $N = 5$ hearts in each group.

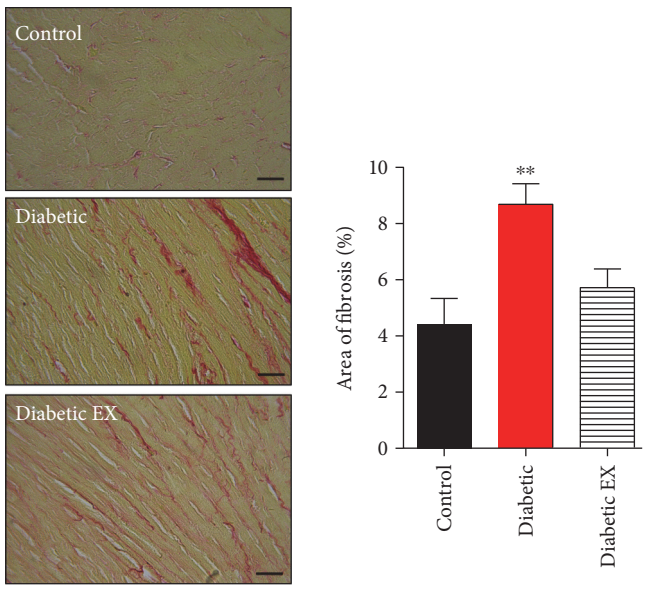

Figure 3: Cardiac fibrosis induced by diabetes. Cardiac sections were stained with Sirius red (see the photomicrographs). The bar graphs depict the statistical analysis for fibrosis in the control (nondiabetic), diabetic, and diabetic + exercise (diabetes EX) rats. $N = 5$ hearts in each group. The scale bar indicates 50 μm. $^{**}p < 0.01$ versus control.

3.5. Nitric Oxide Synthase. Next, we examined the nitric oxide pathway, which has been shown to be cardioprotective. First, we evaluated the cardiac levels of the endothelial nitric oxide synthase, by Western blotting (Figure 7). Neither diabetes nor exercise induced changes in the levels of eNOS ($p = 0.4139$).

A direct consequence of the increased production of reactive oxygen species (ROS) is the uncoupling of nitric oxide synthase. The oxidation of BH_4, an essential cofactor for NOS activity, leads to the dissociation of NOS as a dimer into monomers. We quantified the levels of eNOS as a dimer and a monomer by Western blot under nonreducing conditions (Figure 7). This analysis revealed that diabetes induced strong eNOS uncoupling, increasing the formation of its monomer. High-intensity exercise was unable to restore eNOS as dimer. The eNOS dimer/monomer ratio was 1.3 ± 0.4 in the control group, 0.38 ± 0.04 in the diabetic group, and 0.26 ± 0.03 in the diabetic + exercise group ($p < 0.05$). Furthermore, exercise was unable to restore the intracardiac levels of BH_4, an essential cofactor for NOS activity, that were reduced in diabetic rats: 111.1 ± 49.2 pmol/g protein in the control group, 17.9 ± 2.6 in the diabetic group, and 17.4 ± 3.7 in the diabetic + exercise group ($p < 0.05$). This result explains that both diabetic groups showed increased eNOS as monomer, in the uncoupled state. In most samples, BH_2 levels were undetectable, for this reason, results are expressed as BH_4 normalized to protein concentration, instead of a ratio BH_4/BH_2.

3.6. Calcium Handling Proteins. Finally, we evaluated the level of Ca^{2+} handling proteins, to gain information regarding the status of the excitation contraction coupling elements in the diabetic hearts. Phospholamban (PLB) is a negative regulator of the sarcoplasmic calcium pump SERCA2 [33]. When PLB phosphorylated at serine 16 (by protein kinase A), it unleashes SERCA2 from its inhibition, being able to increase the Ca^{2+} reuptake to the sarcoplasmic reticulum.

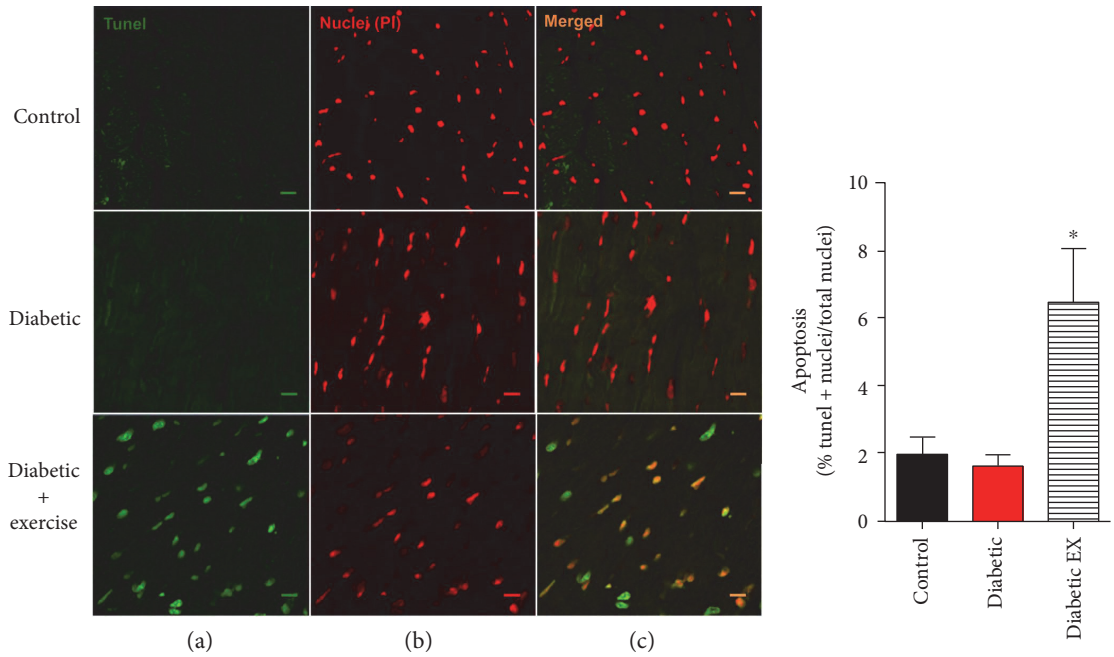

Figure 4: Cardiac apoptosis. Assessment of apoptosis by TUNEL. (a) Photomicrographs (green channel) show the positive nuclei for the TUNEL reaction of the representative cardiac sections from the control (nondiabetic), diabetic, and diabetic + exercise rats, $n = 5$ hearts in each group. (b) Photomicrographs (red channel) show total nuclei of the same sections stained with propidium iodide. (c) Photomicrographs show the merged signal for both channels. The scale bar indicates 10 μm. $^*p < 0.05$ versus the other groups.

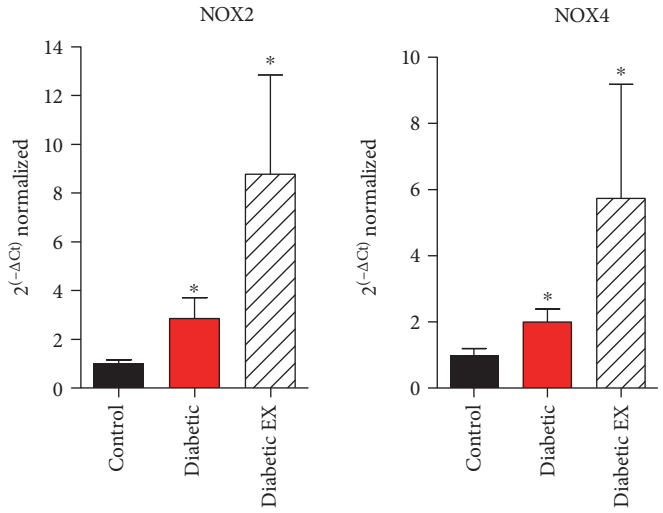

Figure 5: NOX2 and NOX4 mRNA expression in diabetic hearts. Quantification for NOX2 and NOX4 mRNA, normalized to the levels of GAPDH, by real-time PCR, from the control (nondiabetic), diabetic, and diabetic + exercise (diabetic EX) rat hearts; $n = 3$ hearts in each group. $^*p < 0.05$ versus control.

While the total levels of PLB were not altered by diabetes (Figure 8), the degree of PLB phosphorylation at serine 16 was reduced in diabetic animals and was not recovered by exercise ($p < 0.05$), suggesting impaired intracellular Ca^{2+} handling. We also assessed the levels of SERCA2. Both diabetic groups presented decreased levels of SERCA2 compared to nondiabetic rats ($p < 0.05$), which is associated with cardiac dysfunction, as the capacity to store Ca^{2+} in the sarcoplasmic reticulum is reduced (systolic function), and the velocity of relaxation is reduced (diastolic function).

4. Discussion

Exercise appears as an effective strategy for diabetic patients and has been recommended in moderate and high intensity [15]. In this study, we evaluated the effects of a high-intensity physical exercise training protocol on cardiac remodeling and myocardial proteins involved in the nitroso-redox balance in an animal model of type 1 diabetes. We found that this training program had a positive impact on cardiac remodeling, evidenced as reduction in myocyte

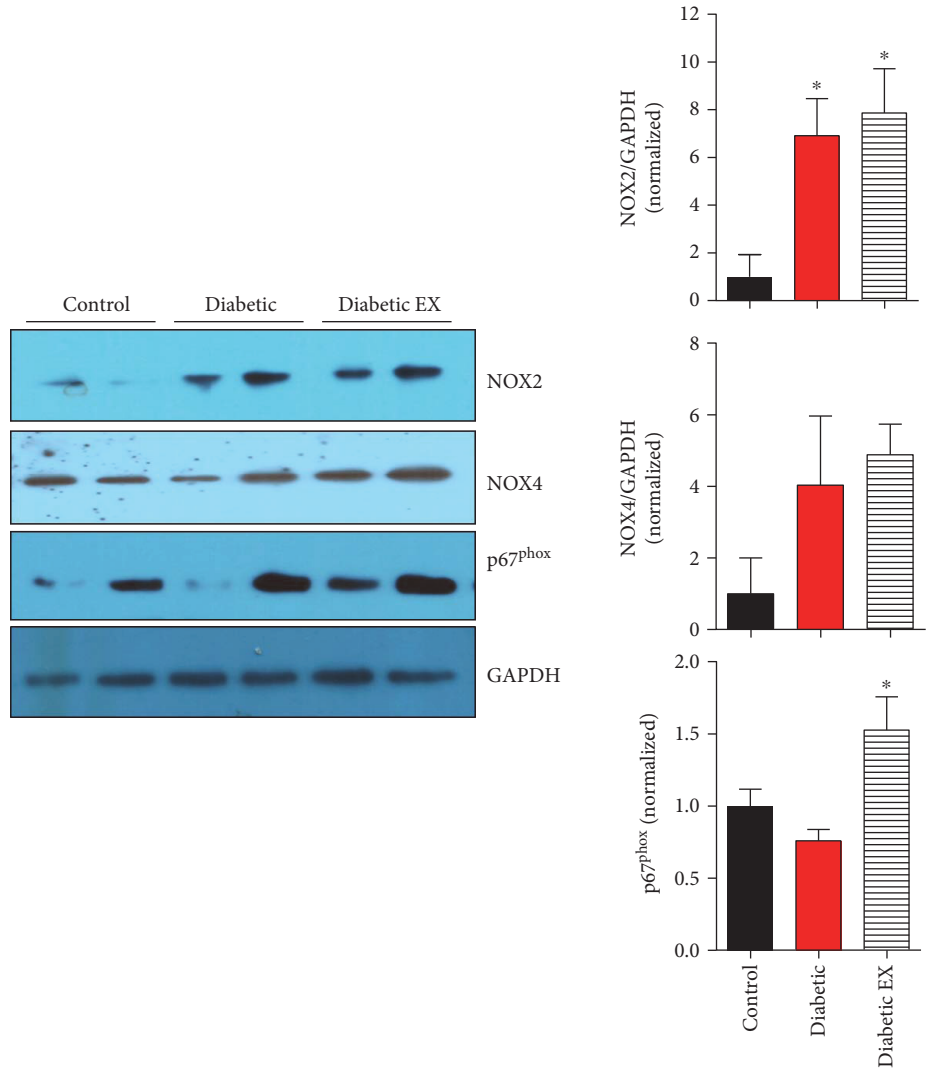

Figure 6: NOX proteins expression in diabetic cardiomyopathy. Representative Western blots for NOX2, NOX4, and $p67^{phox}$ in the control (nondiabetic), diabetic, and diabetic + exercise (diabetic EX) rat hearts. The bar graphs indicate the densitometry analysis for 5 hearts of each group, normalized by GAPDH levels. $^{*}p < 0.05$ versus control.

hypertrophy and reduced collagen deposition (fibrosis), but was unable to restore the nitroso-redox environment. NADPH oxidase 2 system (NOX2) was upregulated; there were no changes in eNOS levels, but the degree of eNOS uncoupling was increased, being a potential additional source of superoxide. Furthermore, this probably increase in oxidative stress was associated with increased degree of cardiac apoptosis in diabetic animals submitted to the exercise program and reduced phospholamban phosphorylation. These data also suggest that the reverse remodeling process may be independent of increased oxidative stress. For example, it has been suggested that moderate-intensity training decreases myocyte cross-sectional area [24]. Interestingly, the high-intensity training increased the degree of apoptosis. This is contrary to what has been observed in rats under low-intensity exercise [18], which reduced the apoptotic index associated with a recovery in the level of antioxidant enzymes glutathione peroxidase, catalase, and superoxide dismutase. Nevertheless, in that study, plasma levels of glucose reached about 300 mg/dL in the diabetic animals, much lower compared to those in our study (around 600 mg/dL in the diabetic rats). In another recent study, Gimenes et al. in a similar low-intensity exercise protocol also observed a recovery in the cardiac levels of catalase and glutathione peroxidase, with glucose plasma levels around 500 mg/dL (similar to this study) [34].

Regarding the NADPH oxidase system, diabetes induced an increase in both NOX2 and NOX4 mRNA that was not reversed by exercise. Furthermore, NOX2 protein and $p67^{phox}$, one of its regulatory subunits, were also increased in diabetes and not reversed by exercise, strongly suggesting an increased ROS production.

In a recent report, Sharma et al. described that in a protocol of exercise similar to ours, they observed a reduction in the levels of the mRNA (although not measured by quantitative PCR) and protein of $p47^{phox}$ and $p67^{phox}$, the regulatory subunits of NOX2, in the left ventricle of diabetic rats submitted to exercise training [35]. But again,

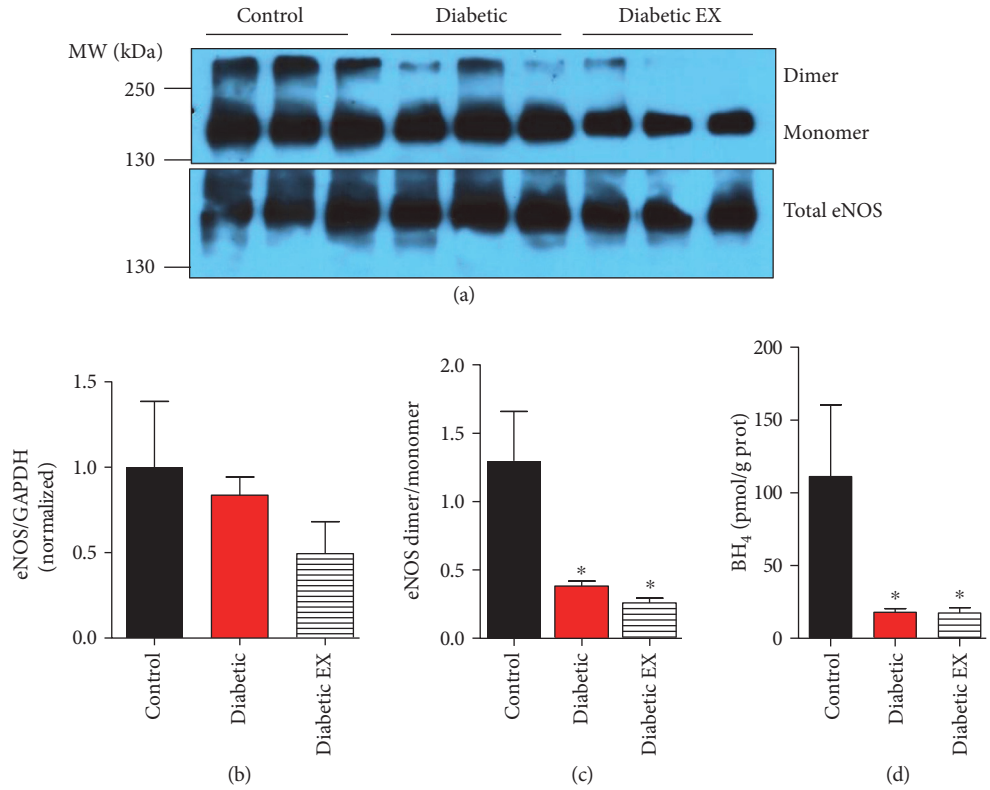

Figure 7: eNOS uncoupling in diabetes. (a) Representative Western blot for eNOS as dimer and monomer, and total cardiac eNOS from control (non-diabetic), diabetic and diabetic + exercise rats hearts (diabetic EX), $n = 5$ hearts each group. (b) Quantification for cardiac levels eNOS. (c) Ratio of dimer to monomer eNOS. (d) Intracardiac levels of tetrahydrobiopterin (BH_4). $^*p < 0.05$ versus control.

the plasma glucose levels in diabetic animals attained about 300 mg/dL, which appears to be in important factor on the observed results.

It is possible that the reverse remodeling observed in the exercise-trained rats may be independent of oxidative stress but dependent, for example, on reverse of metabolic pathways such as nonoxidative glucose pathways [26]. In our study, exercise reduced the plasma levels of the advanced glycation end-products (AGEs, data not shown) in the diabetic animals. On the other hand, NOX-derived ROS may have induced signaling pathways converging, for instance, in increased autophagy [36, 37]. Autophagy is a homeostatic process important during stress conditions, aimed to obtain energy and amino acids from the degradation of proteins and organelles [38]. ROS produced by NOX2 and 4 may increase the process of autophagy in the diabetic heart. This increased autophagic flux may be beneficial for the diabetic heart [39].

Interestingly, our study showed an increase in the degree of uncoupling of the enzyme, associated with a reduction of the NOS cofactor BH_4. It is possible that the increased oxidative stress derived from NOX2 and other sources may have been responsible for the reduced levels of BH_4, since this molecule has been shown to be redox sensitive [40, 41].

Strategies to recover cardiac BH_4 in diabetic animals have shown promising results reducing remodeling and improving ventricular function [11, 13]. Farah et al. showed that a high-intensity exercise increased the degree of eNOS dimerization [42]. Nevertheless, this increase was lost upon ischemia and even reduced upon reperfusion. This reinforces the concept that eNOS uncoupling is induced by oxidative environments, as those observed in cardiovascular diseases and diabetes [43–45].

In addition, it has been shown that NOS activity is related to phospholamban phosphorylation [46], a key protein in the regulation of the excitation-contraction coupling, as a regulator of SERCA2. On the other hand, it has been reported that diabetes reduces phospholamban phosphorylation [33, 46], in agreement with our observations. Exercise was unable to restore this phosphorylation, probably reducing the capacity of the sarcoplasmic reticulum for Ca^{2+} reuptake, along with the reduced levels of SERCA2. Nevertheless, in the diabetic heart, other abnormalities in the excitation-contraction coupling machinery are also present, like the ryanodine receptor and the Na/Ca exchanger [47–49].

Another possibility is that exercise training may have reduced inflammatory mediators in the diabetic myocardium. da Silva et al. found in rats treated with streptozotocin that a low-intensity swimming training reduced intramyocardial levels of TNF-alpha associated with a reverse remodeling of the diabetic hearts [50].

5. Conclusions

In conclusion, the present results suggest that high-intensity exercise is able to reverse cardiac remodeling in the diabetic

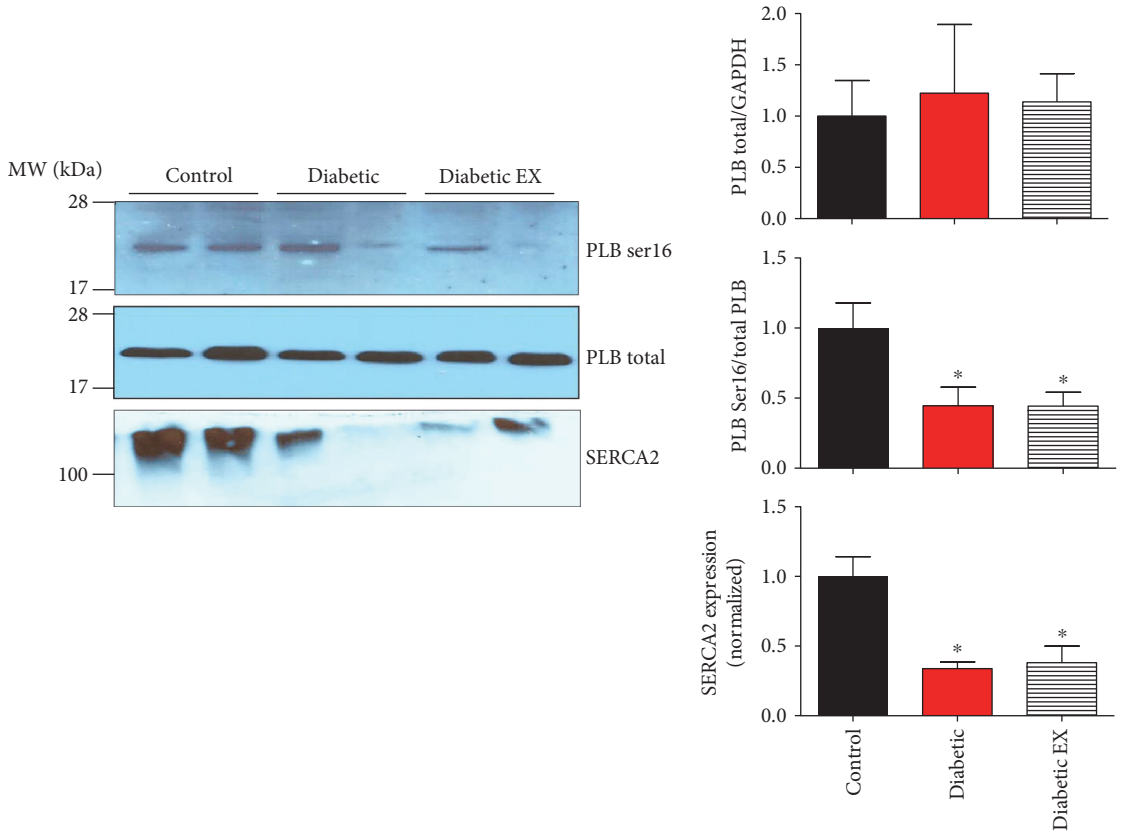

Figure 8: Phospholamban phosphorylation and SERCA2 expression. Levels of phospholamban (PLB) phosphorylation in control (non-diabetic), diabetic, and diabetic rats submitted to exercise training (diabetic EX). Representative Western blots for the phosphorylated form of phospholamban at serine 16 (PLB Ser16), total phospholamban (PLB), and SERCA2 from cardiac homogenates. The bar graphs correspond to the densitometry analysis for 5 hearts in each group. $^{*}p < 0.05$ versus control.

heart but is unable to restore the nitroso-redox imbalance imposed and observed in this condition. This later could by restored by pharmacological manipulations that may include antioxidants and tetrahydrobiopterin.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Neutrophil and Monocyte Bactericidal Responses to 10 Weeks of Low-Volume High-Intensity Interval or Moderate-Intensity Continuous Training in Sedentary Adults

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Neutrophils and monocytes are key components of the innate immune system that undergo age-associated declines in function. This study compared the impact of high-intensity interval training (HIIT) and moderate-intensity continuous training (MICT) on immune function in sedentary adults. Twenty-seven (43 ± 11 years) healthy sedentary adults were randomized into ten weeks of either a HIIT (>90% maximum heart rate) or MICT (70% maximum heart rate) group training program. Aerobic capacity (VO_{2peak}), neutrophil and monocyte bacterial phagocytosis and oxidative burst, cell surface receptor expression, and systemic inflammation were measured before and after the training. Total exercise time commitment was 57% less for HIIT compared to that for MICT while both significantly improved VO_{2peak} similarly. Neutrophil phagocytosis and oxidative burst and monocyte phagocytosis and percentage of monocytes producing an oxidative burst were improved by training similarly in both groups. Expression of monocyte but not neutrophil CD16, TLR2, and TLR4 was reduced by training similarly in both groups. No differences in systemic inflammation were observed for training; however, leptin was reduced in the MICT group only. With similar immune-enhancing effects for HIIT compared to those for MICT at 50% of the time commitment, our results support HIIT as a time efficient exercise option to improve neutrophil and monocyte function.

1. Introduction

Neutrophils and monocytes are key components of the innate immune system and comprise the first line of defence against foreign pathogens [1, 2]. With falling birth rates and increasing longevity, we are an aging society with current demographic trends suggesting that 1 in 4 adults will be aged over 65 by 2050. Importantly, risk of infection is increased by an age-associated decline in neutrophil and monocyte function, which occurs as early as middle age [3, 4]. Key aspects of functional decline in neutrophils include reduced chemotaxis, phagocytosis, reactive oxygen species (ROS), and neutrophil extracellular trap (NET) production [3, 5, 6]. Similarly, dysfunctional monocytes are characterized by an altered phenotype including increased surface CD16 and impaired toll-like receptor (TLR) expression and function [7–9]. Furthermore, monocyte differentiation into macrophages is altered with a skewing towards a proinflammatory phenotype (M1) and reduced phagocytic capacity and antigen presentation [4]. Critically, dysfunctional immune

responses are also associated with an elevated proinflammatory phenotype and likely contribute to age-related systemic inflammation, termed inflammaging, which increases the risk of several age-related chronic diseases [10, 11]. Identifying mechanisms to prevent or reverse neutrophil and monocyte dysfunction and reduce inflammaging is critical to improving immunity and reducing risk of infection and chronic disease as our population ages.

A growing body of evidence now suggests that exercise has systemic effects on immune function and inflammation. Although some of the anti-inflammatory effects of exercise can be attributed to the changes in adipose tissue, cellular immune function appears to be directly impacted also [12–14]. Our recent work suggests that neutrophil chemotaxis is better preserved in older adults who performed twice as many steps daily as age-matched controls [15]. Others have shown enhanced neutrophil phagocytosis and improved total numbers in relation to exercise training [16–18]. Exercise training has also been reported to influence monocyte function: CD16 expression and TLR expression were reduced; proinflammatory cytokine production was reduced; expression of the costimulatory molecule CD80 was increased [19–21]. Despite the evidence of an immune-modifying effect of exercise training, involvement in physical activity is low in the general population and is known to decline dramatically with age [22]; therefore, there is an urgent need to determine the optimal exercise exposure to benefit immune function.

As time constraints are considered a major barrier to exercise participation, novel exercise approaches in nonathletic sedentary populations have gained increasing attention [23]. High-intensity interval training (HIIT) offers an attractive approach by reducing the time commitment for exercise while providing cardiorespiratory fitness benefits similar to or greater than those of traditional moderate-intensity continuous training (MICT). Our group and others have shown significant improvements, comparable to MICT, in physiological, cardiometabolic, and psychological health with HIIT [24–27]. Although there is growing evidence that HIIT improves cardiometabolic health, there is less evidence to suggest that longitudinal HIIT has an effect on systemic inflammation and cellular immune function. Recently, Robinson and colleagues provided the first direct evidence that HIIT alters cellular immunity [20]. In this seminal paper, the authors demonstrated that 10 days of HIIT reduces monocyte, but not neutrophil, expression of TLR4, and lymphocyte expression of TLR2. The relevance of these changes to primary cellular functions is unclear. Furthermore, no studies have investigated the impact of a longer duration HIIT intervention on primary cellular immune function.

The purpose of our study was to determine the impact of ten weeks of group-based HIIT and MICT on neutrophil and monocyte function and systemic inflammation in sedentary healthy individuals. Specifically, we aimed to determine whether a shorter amount of exercise training time (HIIT) was comparable to a longer amount of exercise training (MICT). We examined neutrophil and monocyte phagocytosis and oxidative killing of *E. coli*; expression of CD16, TLR2, and TLR4; serum cytokine and specific hormone levels at baseline and following each intervention. We hypothesized that HIIT would be comparable to MICT in improving cellular immune function.

2. Methods

2.1. Participants and Experimental Procedures. Twenty-seven (43 ± 11 years) healthy (free of any known metabolic or cardiovascular disease and not taking any medication), inactive individuals were recruited for this substudy of a larger investigation examining the effects of HIIT on a range of cardiovascular, metabolic, psychological, and physical activity outcomes [24]. For experimental procedures including fitness testing, training procedures, body composition, and randomization, please refer to our previous publication [24]. Ethical approval was given by the University of Birmingham Research Ethics Committee, and all participants gave their written informed consent.

Briefly, aerobic capacity (VO_{2peak}) was determined by a progressive exercise test to volitional exhaustion on an electronically braked cycle ergometer (Lode BV, Groningen, The Netherlands). Body composition was assessed using a single frequency bioimpedance device (Tanita BC 418 MA Segmental Body Composition Analyzer, Tanita, Japan). Participants were stratified into subgroups by age, gender, and body mass index (BMI) and randomly assigned to either HIIT or MICT.

All exercise sessions were conducted in a dedicated cycle suite at the University of Birmingham sports centre in groups of 10–15 participants. Training was carried out on commercial spinning bikes (Star Trac UK Ltd., Buckinghamshire, UK) and was led by a spin class instructor. Both groups were asked to attend three supervised sessions per week, with the MICT group prescribed two additional self-administered sessions for 10 weeks and participants instructed to achieve heart rate targets determined from the VO_{2peak} assessment. For both groups, each session was designed to be a group exercise-class design, which would differ slightly in duration or style depending on the day or week. As such, each person within groups completed similar programs and none were prescribed only one exercise duration per intervention. High-intensity interval training (HIIT) began with a 5-minute warm-up of low-intensity cycling before repeated high intensity sprints of between 15 and 60 seconds, interspersed with periods of active rest (45–120 seconds). Participants self-adjusted the resistance of the bikes to elicit a heart rate of >90% HR_{max} during the sprint intervals. Each HIIT session lasted 18–25 minutes. Moderate-intensity continuous training (MICT) began with a 5-minute warm-up of low intensity cycling before participants adjusted the resistance to elicit a heart rate of ~70% HR_{max}. Each MICT session lasted 30–45 minutes; during the training period, participants were instructed to maintain their habitual dietary and physical activity patterns. As such, cumulative exercise exposure for each group amounted to 55 ± 10 minutes/week (HIIT) and 128 ± 44 minutes/week (MICT).

2.2. Blood Sampling. Participants arrived at the laboratory, having abstained from exercise in the previous 24 hours

and at least 48 hours after their final exercise training. These times gave us confidence that any effects observed were for a training effect and not for an acute effect of the last session. Participants were seated for 15 minutes before peripheral venous blood samples were collected into vacutainers containing either heparin, EDTA, or a clotting agent for serum. Samples were then processed immediately for plasma and immune cell isolation while serum was left at room temperature for 30 minutes to clot. Serum and plasma were separated from blood by centrifugation (3000 ×g for 10 minutes), snap frozen in liquid nitrogen, and stored at −80°C until analysed.

2.3. Whole Blood Counts. Complete blood differentials, including leukocyte counts, were completed using EDTA-treated whole blood immediately after sampling on a fully automated Coulter™ ACT^{diff} haematology analyser (Beckman-Coulter, High Wycombe, UK). All samples were analysed in triplicate.

2.4. Mononuclear Cell Isolation. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised blood using density centrifugation. Briefly, blood was diluted 1 : 1 with phosphate-buffered saline (PBS) and layered over Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden) at a blood Ficoll ratio of 4 : 3 mL. This was centrifuged at 400 ×g for 30 minutes at room temperature with no brake. Following centrifugation, mononuclear cells suspended at the interface of the Ficoll and plasma were removed and washed twice, 400 ×g for 10 minutes, in PBS. Cells were counted and viability assessed by Trypan Blue exclusion before being resuspended in PBS+ 1% bovine serum albumin (BSA, Sigma-Aldrich, Poole, UK) at 1×10^6 cells·mL^{-1} and prepared for immunofluorescence staining.

2.5. Cell Surface Receptor Expression. Freshly isolated PBMCs were stained with combinations of anti-CD14-PcB (BD Bioscience, Oxford, UK, clone M5E2), anti-CD16-FITC (BD Bioscience, clone 3G8), anti-TLR2-APC (BD Bioscience, clone TL2.1), anti-TLR4-APC (Affymetrix eBioscience, Hatfield, UK, clone HTA-125), or their relevant concentration-matched isotype control for 30 minutes on ice in the dark. Following incubation, cells were washed in PBS/1% BSA and resuspended in PBS/1% BSA for analysis by flow cytometry. Mononuclear cells were identified by their typical forward versus side scatter, and 7000–10,000 CD14$^+$ monocytes were acquired for analysis on a CyAn ADP™ 430 flow cytometer (Beckman-Coulter, High Wycombe, UK) and data were analysed using Summit v4.3 software (Dako, Cambridgeshire, UK).

For neutrophil surface phenotype, staining with the above antibodies, CD16, TLR2, and TLR4, was assessed in 100 μL of whole blood. Blood was aliquoted into FACS tubes and placed on ice. Combinations of antibodies or isotype controls were added to blood and were incubated for 1 hour on ice in the dark. Following incubation blood was washed twice in PBS before adding 2 mL of 1x Fix/Lyse Buffer (Affymetrix eBioscience). Blood was placed at room temperature in the dark for 15 minutes to allow complete RBC lysis and fixation of WBCs. Following this, cells were washed twice in PBS and resuspended in PBS for analysis by flow cytometry. Granulocytes were gated by their typical forward versus side scatter, and 10,000 CD16$^+$ neutrophils were acquired for analysis on a CyAn ADP 430 flow cytometer (Beckman-Coulter, High Wycombe, UK) and data were analysed using Summit v4.3 software (Dako, Cambridgeshire, UK).

2.6. Neutrophil and Monocyte Phagocytosis and Oxidative Burst. Phagocytosis and oxidative burst were assessed in whole blood using commercially available kits and manufacturers' guidelines (Phagotest and Phagoburst, BD Biosciences). Briefly, phagocytosis was assessed in heparin-treated whole blood and incubated at 4°C (control) or 37°C (test) with opsonised FITC-labelled *E. coli*. Phagocytosis was halted by the addition of cold phosphate-buffered saline (PBS) while cell surface-bound FITC was quenched by addition of Trypan Blue solution. Unbound-free bacteria were removed by washing in PBS and erythrocytes lysed and leukocytes fixed using 1% Fix/Lyse solution provided in the kit. Cell DNA was counterstained by an addition of propidium iodide (PI) before flow cytometry analysis was performed.

Oxidative burst was assessed in heparin-treated blood that was incubated at 37°C with opsonised *E. coli* (test) or PBS (control) for 10 minutes. Solution-containing dihyrdorodamine-123, which is converted to fluorescent rodamine-123 in the presence of reactive oxidants, was included for 10 minutes at 37°C. Oxidative burst was halted by the addition of erythrocyte lysis/leukocyte fixation buffer before leukocyte DNA was stained and flow cytometry analysis performed.

Phagocytosis and oxidative burst quantitation was performed on a CyAn ADP 430 flow cytometer equipped with three solid-state lasers. FITC and R-123 were detected in FL1 while PI was detected in FL2 using the Argon (405 nm) laser. 10,000 neutrophils and 5000–10,000 monocytes were acquired for analysis. Following compensation of FL1 versus FL2 phagocytic and oxidative burst was determined by the relative increase in median fluorescence intensity (MFI) in FL1 compared to negative controls. Data were analysed using Summit v4.3.

2.7. Serological Analyses. All measurements were made in duplicate using commercially available kits and manufacturer's guidelines. Serum interleukin- (IL-) 1β, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17, granulocyte/macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor (TNF)α were simultaneously measured by multiplex luminometry (Bio-Rad, Hemel Hempstead, UK). Samples we0re analysed using a Bio-Plex Luminex^{200} platform equipped with a 635 nm red and 532 nm green laser using Bio-Plex Manager™ software. Detection of C-reactive protein (CRP) was by high-sensitivity enzyme-linked immunosorbent assay (ELISA) using a commercial kit (IBL International, Hamburg, Germany). Plasma cortisol and DHEAs were assessed separately by ELISA (IBL International, Hamburg, Germany) and plasma adiponectin and leptin were assessed separately by solid phase sandwich ELISA (R&D Systems, Abingdon, UK).

Table 1: Group baseline and final values for participant body composition and fitness measures.

	HIIT		MICT	
	Baseline	Final	Baseline	Final
n (males/females)	14 (4/10)		13 (5/8)	
Age (years)	42 (12)		45 (10)	
Body composition				
Height (cm)	167 (7)		166 (9)	
Body mass (kg)	78.1 (19.9)	77.7 (19.4)	79.0 (15.2)	78.4 (16.2)
BMI (kg/m^2)	28.1 (6.1)	27.9 (6.0)	28.1 (4.7)	27.9 (5.0)
Fat mass (%)	32.9 (8.2)	31.8 (8.2)	32.2 (8.6)	30.9 (8.7)*
Physical fitness				
VO_{2max} ($L\,min^{-1}$)	2.4 (0.6)	2.7 (0.7)***	2.6 (0.6)	2.9 (0.7)***
VO_{2max} ($mL/kg\,min^{-1}$)	31.5 (6.4)	35.0 (8.4)***	32.6 (5.8)	35.8 (7.1)***

BMI: body mass index. Data are mean (SD) unless otherwise stated. $^{*}p < 0.05$, $^{***}p < 0.001$ are significant within group change scores.

Table 2: Group baseline and final values for neutrophil and monocyte phagocytosis of *E. coli* and oxidative burst towards *E. coli*.

	HIT		MICT	
	Baseline	Final	Baseline	Final
Neutrophil				
Phagocytosis (MFI)	130.6 (16.6)	152 (17.2)*	126.2 (12.5)	145.5 (14.1)*
Oxidative burst (MFI)	69.0 (24.5)	74.6 (22.1)*	77.6 (18.2)	104.8 (16.5)**
Monocyte				
Phagocytosis (MFI)	99.3 (10.4)	113.3 (11.6)**	92.9 (12.2)	110.4 (13.8)**
Oxidative burst (MFI)	27.5 (8.4)	22.7 (9.1)	30.9 (11.7)	27.8 (13.5)
Oxidative burst (%)	74.4 (10.0)	81.6 (11.1)*	74.4 (13.7)	85.6 (12.1)**

MFI: median fluorescence intensity. Data are mean (SD). $^{*}p < 0.05$ and $^{**}p < 0.01$ are significant within group change scores.

2.8. Statistical Analysis. Statistical analysis was conducted using PASW version 18.0 (Chicago, IL, USA), and data are presented as mean ± SD unless otherwise stated. Normality was assessed using Kolmogrov-Smirnov analysis; natural log transformation of distributed variables violating normality was completed. Data were analysed using repeated measures ANOVA to assess the effect of training on immune function and interactions with training × exercise group. Age and body fat percentage were included as covariates due to the influence of age on immune function and inflammation, and the small changes in body fat were observed in the MICT group. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Participant Characteristics, Exercise Capacity, and Body Composition. Body composition and aerobic capacity measures are presented in Table 1. No differences were detected between the groups at baseline ($p > 0.05$). As with the larger study, the HIIT group completed on average 57% less total training time commitment compared to the MICT group ($p < 0.001$). There were significant main effects of training for VO_{2peak} [$F(1,25) = 49.6$; $p < 0.001$; $\eta_p^2 = .67$] with increases postexercise of 9% for both HIIT and MICT (both $p < 0.001$). Neither body mass nor BMI (both $p > 0.05$) was reduced by training in this cohort. However, there were significant main effects of training for body fat percentage [$F(1,25) = 7.9$; $p = 0.01$; $\eta_p^2 = .27$], with reductions observed for MICT ($p = 0.04$) but not HIIT ($p = 0.08$). No differences between groups for effects of training were observed.

3.2. Immune Responses. A primary mechanism of bacterial clearance by neutrophils and monocytes is an ingestion of microbes through phagocytosis and subsequent killing in the phagolysosome as a result of exposure to reactive oxygen species. Table 2 shows the neutrophil and monocyte bactericidal capacity following HIIT or MICT.

All neutrophils (100%) from subjects in both groups ingested opsonized *E. coli* and produced an oxidative burst. There were significant main effects of training for the amount (MFI) of *E. coli* ingested by neutrophils [$F(1,25) = 7.5$; $p = 0.011$; $\eta_p^2 = .24$], with increases observed for HIIT ($p = 0.023$) and MICT ($p = 0.049$). There were also significant main effects of training for the amount (MFI) of ROS produced against *E. coli* by neutrophils [$F(1,25) = 12.2$; $p = 0.002$; $\eta_p^2 = .36$], with increases observed for HIIT ($p = 0.03$) and MICT ($p = 0.004$).

Similarly, there were significant main effects of training for the amount (MFI) of *E. coli* ingested by monocytes [$F(1,25) = 18.7$; $p < 0.001$; $\eta_p^2 = .46$], with increases observed

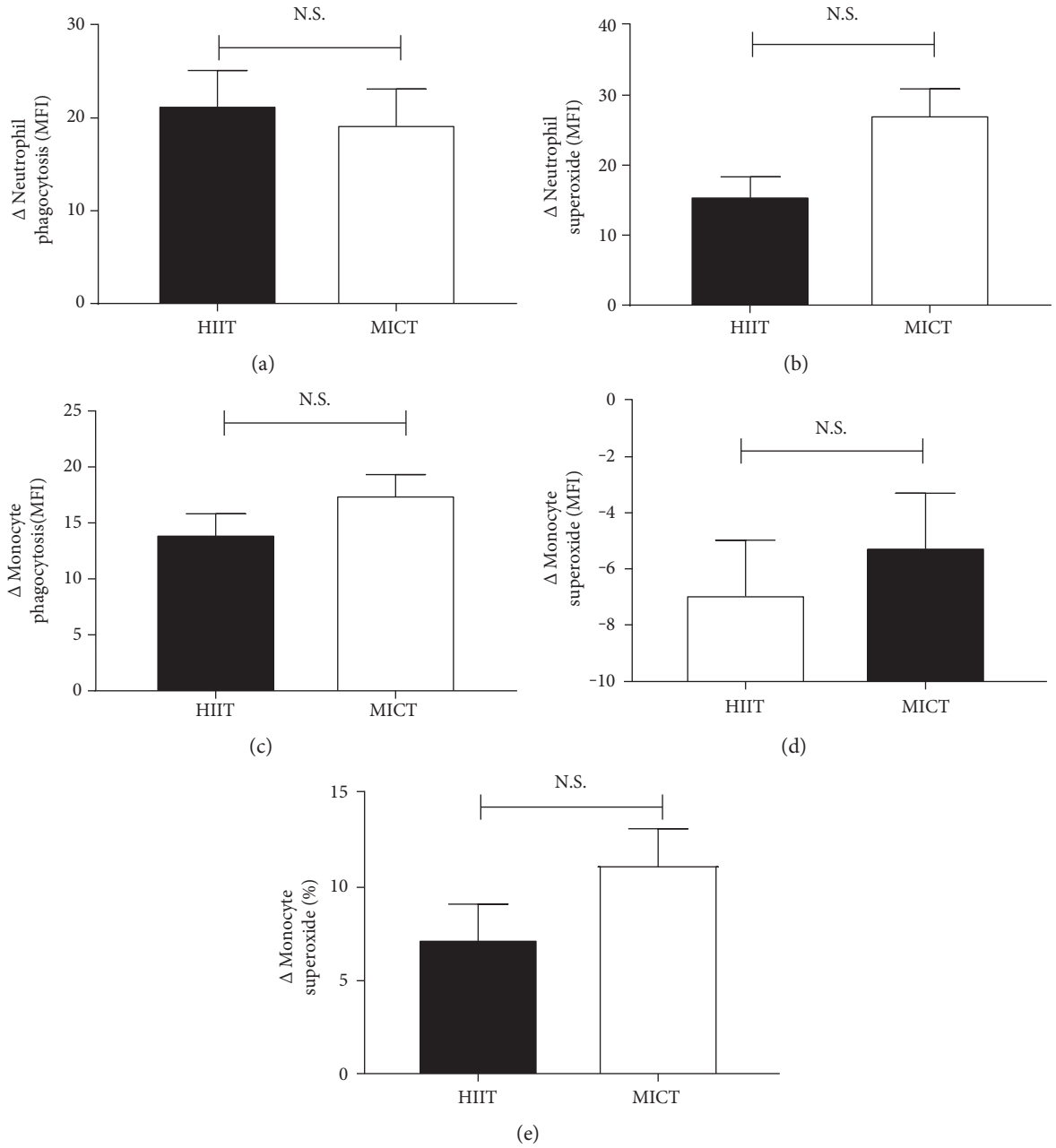

Figure 1: Final minus baseline change scores for group immune function. HIIT was comparable to MICT for changes in neutrophil phagocytosis (a), neutrophil superoxide (b), monocyte phagocytosis (c), monocyte superoxide (d), and percentage of monocytes-producing superoxide (e). N.S.—nonsignificant differences between groups.

for HIIT ($p = 0.005$) and MICT ($p = 0.002$). Although there were no increases in monocyte oxidative burst ($p > 0.05$), there were significant main effects of training for the percentage of monocytes producing an oxidative burst [$F(1,25) = 11.1$; $p = 0.003$; $\eta_p^2 = .33$], with increases observed for HIIT ($p = 0.03$) and MICT ($p = 0.006$).

There were no differences between groups for any of the functional measures assessed suggesting both routines are equally effective (Figure 1), though there was a trend for MICT to have greater improvements in neutrophil superoxide production (Figure 1(b), $p = 0.094$).

In order to determine whether changes in cell function reflect changes in total cell numbers, Table 3 shows complete blood differentials before and after training. There were no effects of training on total white blood cell (WBC), lymphocyte, neutrophil, or monocyte counts (all $p > 0.05$). To better understand the changes in cellular function observed, we assessed expression of CD16, TLR2, and TLR4 on monocytes and neutrophils. No effect of exercise was observed for TLR2, TLR4, or CD16 on neutrophils (data not shown; $p > 0.05$ for all). There were significant main effects of training on the percentages of monocyte subsets. Training increased the

Table 3: Group baseline and final values for total numbers of white blood cells, percentages of monocyte subsets, and expression of TLR4 and TLR2.

	HIIT		MICT	
	Baseline	Final	Baseline	Final
Total WBC ($\times 10^9\,L^{-1}$)	7.4 (2.7)	8.2 (2.1)	8.2 (1.1)	7.7 (2.4)
Lymphocytes ($\times 10^9\,L^{-1}$)	2.4 (0.9)	2.5 (0.7)	2.5 (0.4)	2.7 (0.5)
Neutrophils ($\times 10^9\,L^{-1}$)	4.6 (1.8)	5.2 (1.7)	5.2 (1.2)	4.4 (2.0)
Monocytes ($\times 10^9\,L^{-1}$)	0.3 (0.2)	0.4 (0.2)	0.4 (0.2)	0.5 (1.1)
$CD14^+/CD16^-$ (%)	87.2 (4.7)	91.1 (4.5)**	86.9 (5.8)	89.8 (6.1)*
TLR4 (MFI)	3.9 (1.2)	3.8 (1.4)	3.9 (1.2)	3.8 (1.3)
TLR2 (MFI)	119 (11.2)	120 (10.1)	121 (13.4)	123 (14.1)
$CD14^+/CD16^{int}$ (%)	4.4 (2.8)	3.1 (2.2)*	4.7 (2.4)	3.6 (2.2)*
TLR4 (MFI)	5.0 (2.1)	4.9 (2.8)	5.1 (2.0)	4.9 (2.1)
TLR2 (MFI)	123 (15.3)	116 (16.5)*	124 (18.9)	119 (19.9)*
$CD14^+/CD16^{bright}$ (%)	8.1 (4.4)	5.8 (3.9)*	8.4 (3.1)	6.9 (4.2)
TLR4 (MFI)	4.6 (3.2)	4.0 (2.7)**	4.5 (3.0)	3.9 (2.7)**
TLR2 (MFI)	86 (10.9)	85 (12.6)	91 (13.2)	93 (14.0)

WBC: white blood cell; CD: cluster of differentiation; TLR: toll-like receptor; MFI: median fluorescence intensity. Data are mean (SD). $^*p < 0.05$ and $^{**}p < 0.01$ are significant within group change scores.

percentage of $CD14^+/CD16^-$ [$F(1,25) = 11.3$; $p = 0.004$; $\eta_p^2 = .31$] with increases observed for HIIT ($p = 0.008$) and MICT ($p = 0.024$). Reductions were observed for the percentage of $CD14^+/CD16^{int}$ monocytes in both groups ($p < 0.05$). Reductions in the percentage of $CD14^+/CD16^{bright}$ monocytes were observed in the HIIT ($p = 0.031$) group but not in the MICT ($p = 0.071$). There were significant main effects of training for expression of TLR4 on $CD14^+/CD16^{bright}$ monocytes [$F(1,25) = 16.54$; $p < 0.001$, $\eta_p^2 = .26$] with reduced expression observed for HIIT ($p = 0.001$) and MICT ($p = 0.001$). TLR2 expression was higher at all times on $CD14^+/CD16^-$ and $CD14^+/CD16^{int}$ compared to $CD14^+/CD16^{bright}$ populations ($p < 0.05$). There were significant main effects of training for expression of TLR2 on $CD14^+/CD16^{int}$ monocytes [$F(1,25) = 19.42$; $p < 0.001$, $\eta_p^2 = .20$] with reduced expression observed for HIIT ($p < 0.001$) and MICT ($p = 0.001$). No differences for training between groups were observed. There were no differences within or between groups for surface expression of TLR2 or TLR4 on $CD16^+$ neutrophils (data not shown).

3.3. Serological Analysis. Exposure to systemic inflammation, neuroendocrine hormones, and metabolic hormones can influence immune cell function. However, no differences were observed for any inflammatory cytokines or acute phase proteins or endocrine hormones at baseline or in response to exercise training, Table 4 (all $p > 0.05$). There were significant main effects of training for the metabolic hormone leptin [$F(1,25) = 6.9$; $p = 0.014$; $\eta_p^2 = .22$] with reductions in the MICT group ($p = 0.043$) but not in the HIIT group ($p = 0.127$).

4. Discussion

This study shows that, in sedentary men and women, ten weeks of low-volume high-intensity interval training was comparable to moderate-intensity continuous training at improving neutrophil and monocyte bactericidal capacity while reducing CD16, TLR2, and TLR4 on $CD14^+$ monocytes but not neutrophils. Systemic inflammation and endocrine responses were unaffected by either of the training interventions, although leptin was lower following MICT which was associated with a reduced body fat percentage. As previously reported in our primary study, VO_{2peak} was significantly improved and body fat percentage was marginally reduced in MICT with no difference observed between groups [24]. Critically, no differences between groups were observed for our immunological analyses suggesting that for half the prescribed exercise time, HIIT can improve immune function to a similar extent as MICT. Therefore, we suggest that HIIT might be an effective means to improve fitness and immune function in populations who find typically prescribed continuous exercise difficult.

4.1. Changes in Immune Cell Function. Although changes in primary immune cell function are associated with risk of infectious episodes, surprisingly, little is known about exercise training and neutrophil and monocyte bactericidal functions. Both cells are central to the early resolution of infection, primarily by phagocytosis of the pathogen and oxidative killing of the pathogen.

When compared to sedentary healthy matched controls, physically active individuals aged between 20 and 60 years have increased neutrophil phagocytosis and ROS production [16, 18]. Neutrophil phagocytosis was also improved following 2 months of moderate-intensity exercise training in middle-aged healthy men [28]. Our data adds to this literature in suggesting that HIIT and MICT are equally capable of improving neutrophil bactericidal capacity and likely reducing risk of infectious episodes.

It is still unclear how these functional improvements occur. We found no effect of training on neutrophil

Table 4: Group baseline and final values for inflammatory cytokines and acute phase proteins, endocrine, and metabolic hormones.

	HIIT		MICT	
	Baseline	Final	Baseline	Final
Proinflammatory				
IL-1β (pg mL^{-1})[a]	0.1 (0.08)	0.1 (0.1)	0.5 (1.2)	0.4 (0.9)
IL-6 (pg mL^{-1})[a]	1.4 (1.6)	1.0 (1.0)	1.2 (1.0)	0.8 (0.8)
IL-8 (pg mL^{-1})	5.7 (2.1)	4.4 (1.5)	5.7 (2.5)	5.4 (2.3)
IL-17 (pg mL^{-1})[a]	5.5 (17.9)	5.2 (15.7)	13.8 (23.4)	5.5 (14.7)
TNFα (pg mL^{-1})[a]	0.8 (2.1)	0.1 (0.4)	ND	0.2 (0.5)
CRP (mg L^{-1})[a]	3.0 (4.3)	2.1 (2.2)	1.2 (1.4)	1.6 (1.8)
GM-CSF (pg mL $^{-1}$)[a]	0.02 (0.06)	ND	0.02 (0.07)	0.02 (0.07)
Anti-inflammatory				
IL-4 (pg mL^{-1})[a]	0.04 (0.1)	0.06 (0.1)	0.02 (0.05)	0.05 (0.07)
IL-10 (pg mL^{-1})[a]	0.3 (1.1)	ND	ND	ND
IL-13 (pg mL^{-1})[a]	0.7 (1.2)	0.6 (0.9)	1.1 (1.8)	0.7 (1.1)
Endocrine				
Cortisol (nmol L^{-1})[a]	200 (61.3)	190 (62.1)	197 (98.6)	183 (56.0)
DHEAs (nmol L^{-1})	3563 (1965)	3841 (2087)	4464 (2335)	4144 (1739)
Cortisol: DHEAs	0.07 (0.03)	0.06 (0.04)	0.05 (0.03)	0.05 (0.02)
Metabolic				
Adiponectin (μg mL^{-1})	3.8 (1.9)	3.6 (1.8)	3.3 (1.2)	3.1 (1.3)
Leptin (ng mL^{-1})	19.3 (11.3)	18.0 (10.8)	16.4 (9.6)	14.6 (10.2)*
Leptin: adiponectin	6.0 (4.4)	6.1 (4.3)	6.0 (4.6)	5.8 (5.3)

IL: interleukin; TNF: tumour necrosis factor; ND: nondetectable; CRP: high sensitivity C-reactive protein; GM-CSF: granulocyte/macrophage colony-stimulating factor; DHEAs: dehydroepiandrosterone sulphate. Data are mean (SD). [a]Not normally distributed and were log transformed. *$p < 0.05$ significant within group change scores.

expression of TLR4, the primary TLR responsible for recognition of the lipopolysaccharide (LPS) membrane component of *E. coli*. Although Robinson and colleagues did not assess neutrophil bactericidal capacity, expression of TLR4 was reduced following 10 days of MICT but not HIIT [20]. It is unclear why we did not observe a similar effect or whether reduced TLR4 would in fact reduce bactericidal function. Our study and theirs assessed immune function greater than 48 hours after the last exercise session, and so, we are confident it is not due to blood draw timing. One potential explanation is that acute bouts of exercise are capable of selective clearance of dysfunctional immune cells [29]. If exercise clears dysfunctional neutrophils from the system acutely, improving the functionality of the remaining pool in the longer term, then the 10-week intervention used here could benefit from this change and explain why we saw improved neutrophil function.

We have previously shown that habitual physical activity (~10,000 versus ~5000 steps/day) is associated with enhanced neutrophil functions in the absence of surface receptor differences [15]. Our results in this cohort also suggest that neutrophil CD16 expression is not influenced by exercise training. Our lack of change in TLR and CD16 expression suggests that there are instead intrinsic cell signalling alterations associated with improved neutrophil function. Neutrophil bactericidal functions are regulated by several signalling pathways, downregulation of which can compromise mechanism such as phagocytosis and oxidative burst. Such effects are seen with aging, with reduced MAP kinase amongst the differences seen in neutrophils from older donors [30]. To our knowledge, there are no reports of the effects of exercise on MAP kinase signalling in immune cells, though this has been reported in skeletal muscle [31]. Future research should aim to determine neutrophil signalling pathways influenced by exercise training in order to understand exercise-mediated mechanisms.

With a progressive age-associated decline in neutrophil function, there is an enhanced risk of infection. As neutrophils are the first line of defence against pathogenic invasion, they are integral to the effective resolution of infection. Although our participants were aged on average 43 years old (23–60 years), our results indicate a potential route to improving neutrophil function in at risk groups. To date, no study has assessed the impact of HIIT on neutrophil function in over 60-year olds. Future studies should aim to assess immune functional responses to HIIT in populations showing clear signs of immunosenescence in order to determine whether it is possible to reverse or delay immunosenescence. Critically, our data highlights that neutrophil bactericidal capacity can be improved by higher intensity exercise with significantly less time commitment.

Monocytes make up a relatively small proportion of circulating leukocytes (2–12%). However, due to their diverse role in immune function and inflammation, they have received more interest than neutrophils in the exercise literature. The majority of literature is focused on monocyte phenotype and proinflammatory cytokine production and less on bactericidal activity [32–34]. We show for the first time

that monocyte bactericidal function is improved by 10 weeks of HIIT. We observed a significant improvement in phagocytic capacity in both exercise groups. Although we did not see more ROS production on a per cell basis, the percentage of ROS-producing monocytes was significantly increased. It is unclear how these improvements were achieved. The percentage of cells producing ROS suggests that not all monocytes were equally phagocytically active, and our results may be influenced by the training-induced increase in CD16-negative monocytes (see below) which could influence ROS production. Schaun and colleagues observed no difference in monocyte phagocytosis following 12 weeks of aerobic exercise training [35], though they used a TLR2 agonist (zymosan) rather than bacteria. Of interest is that basal and stimulated monocyte ROS production and phagocytosis is suggested to be higher in obese individuals [36]. However, we saw no association with weight and ROS production or phagocytosis and as there was minimal weight loss, we can assume that increased phagocytosis was not influenced by changes in body fat.

Research on monocyte function and phenotype has focused on acute single session or short-term (days) bouts of exercise. The Nieman group have pioneered monocyte function research over the last twenty-years and have shown interesting acute effects of exercise [37–39]. Acute exercise is associated with an intensity-dependent increase in numbers of circulating monocytes that are predominated by a proinflammatory phenotype [40–42]. Monocyte phagocytosis is transiently increased by acute exercise and is associated with the degree of inflammatory response to exercise [43]. However, Nieman and colleagues also recently showed that monocyte bactericidal capacity is diminished in response to overtraining, muscle damage and elevated inflammation [37]. Although we did not assess acute exercise-mediated immune responses, it is likely that each exercise session resulted in transient changes in function. We can only assume that each exercise bout has a small but significant impact on basal function, progressively improving it over time.

We observed significant changes in monocyte phenotype following exercise training, suggesting an altered inflammatory potential. Specifically, there were reductions in the cell surface expression of CD16. CD16 is typically associated with a proinflammatory subtype with higher basal and stimulated production of cytokines such as TNFα [44]. Furthermore, we observed significant reductions of TLR4 expression on intermediate and TLR2 on nonclassical $CD16^+$ proinflammatory monocytes. Our work is in agreement with Robinson and colleagues who showed that 2 weeks of HIIT was sufficient to reduce monocyte expression of TLR4. Although their study did not show reduced TLR2 on monocytes, they did find an effect on lymphocytes suggesting that TLR2 is influenced by training and monocyte expression may require more or less training time [20].

As with our neutrophil data, we did not observe functional monocyte improvements associated with relevant changes in cell surface receptor expression (i.e., more phagocytosis with more TLR4). Therefore, functional improvements are likely associated with intrinsic cell signalling changes similar to what we suggest in neutrophils. Recent gene expression analysis of acute interval exercise bouts suggests that monocytes may be directed towards an anti-inflammatory profile with downregulation of TNF, TLR4, and CD36 genes [41]. Additionally, metabolic disorders such as type 2 diabetes and obesity are associated with increased TLR expression and activation [45]. Although our participants were metabolically healthy, glucose tolerance and insulin sensitivity were improved following training suggesting a link with metabolic control and TLR expression [24]. Taken together, HIIT has the potential to modify monocyte proinflammatory phenotype and contributes to improved bactericidal capacity. Future research should attempt to determine the acute and chronic mechanisms by which exercise influences inflammatory monocyte functions.

4.2. Impact of HIIT on Serological Measures. Although we observed altered cellular responses to training which can be associated with an anti-inflammatory effect, there were no reductions in serological markers of inflammation. The anti-inflammatory effects of exercise have been extensively researched and reviewed [12, 46]. However, it remains unclear what the inflammatory response is in the absence of weight loss and what the consequences of this might be [47]. Acute exercise is associated with both an immune cell redistribution and an inflammatory response which following exercise cessation returns to normal levels between 0.5 and 24 hours [48–50].

These effects have led many to conclude that exercise training has a role in controlling chronic low-grade inflammation. However, it is becoming clear that low-grade inflammation may be influenced less by exercise training and more by weight loss [20, 32]. When accounting for weight change in the analysis of exercise-mediated effects on inflammation markers such as CRP, effects of exercise are often lost [32, 51]. In agreement with these reports, we observed no changes in concentrations of a number of basal inflammatory cytokines or acute phase proteins. Similarly, Robinson and colleagues also found no significant effects of HIIT or MICT on systemic inflammation [20]. A number of recent studies have highlighted the acute inflammatory response to HIIT and its similarity to MICT; however, these snapshots do not inform of longitudinal responses [50, 52]. As inflammatory biomarkers are used more in prediction of disease outcomes, future research should aim to determine the discrete and analogous effects of intervention-specific weight and fitness responses on inflammation.

We did find that MICT but not HIIT was associated with a small but significant 1.4% reduction in body fat. This was aligned with a significant 10% reduction in the adipose tissue-derived adipokine leptin. Although leptin inhibits hunger, it can have proinflammatory effects on immune cells [53]. Whether the reduction in leptin was directly associated with immune function remains unclear; however, it is unlikely to be influential on systemic inflammatory responses.

With a growing body of evidence suggesting links between the endocrine system, systemic inflammation, and physical activity, we measured cortisol and DHEAs [11, 54–56]. Although others have shown exercise effects for cortisol and DHEAs with reductions in cortisol [57],

we saw no effect in the present study. These findings are not surprising in light of the lack of changes observed for inflammatory cytokine measures. Taken together, our results are in agreement with other exercise studies showing that exercise training with minimal weight change is not associated with reduced systemic inflammation.

4.3. Limitations. Our study is not without limitations. We have previously described the major study limitations and, here, will describe the substudy-specific limitations [24]. The high proportion of women may have influenced our findings. We were unable to control for menstrual cycle during our measurements and as such, women were likely at different stages of menses during the study measurements. It is unclear the interactions of the menstrual cycle, exercise training, and immune function in our study. Others have suggested that the menstrual cycle during acute exercise may be associated with altered mucosal immunity and may transiently alter subsets of lymphocytes [58, 59]. Although we are unaware of previous studies assessing menstrual cycle interactions with exercise training and neutrophil or monocyte bactericidal function, we cannot discount a possible influence. Future studies should aim to assess the role of the menstrual cycle in relation to exercise and immune function. Although previous studies have shown gender differences in response to HIIT, these are often focused on metabolic control including glycogen breakdown and insulin sensitivity [60]. Although we did not have statistical power to analyse gender differences, the results of female participants were the same as when combined with men.

Our study was designed to determine whether a shorter amount of exercise training time, at a higher intensity, was comparable to a longer amount of exercise time, at a lower intensity for key outcome measures. The groups differed on total exercise time per session, with HIIT performing 18–25 minutes/session while MICT performed 30–45 minutes/session. HIIT participants performed on average 2.6 sessions/week, while MICT participants performed on average 3.4 sessions/week. Cumulative exercise time per week was 55 ± 10 minutes (HIIT) versus 128 ± 44 minutes (MICT). As such, these differences for time present a technical limitation, which prevents us from determining a mechanistic role for exercise intensity. Although this was not our aim, had we controlled for exercise time, the results such as VO_2 would likely have been different. Given that HIIT was 57% less time-consuming than MICT and provided similar benefits, it could be surmised that longer durations of HIIT would provide greater benefits than MICT. However, the feasibility of these remains unknown and it might be that adherence would drop from 82%.

In addition to time limitations, we also did not control for and were unable to assess energy expenditure between the groups. Although HIIT might have had higher energy expenditure per session, it is likely that the MICT group had greater overall energy expenditure as evident by the small but significant reduction in body fat percentage in the MICT group. The HIIT group, although not significant, had a trend towards reduced body fat percent. Both groups were advised not to alter their diet or current physical activity levels. We did not specifically control for diet and as such, we are uncertain whether participants changed their diet that resulted in a reduced body fat percentage in the MICT group. Because of this small change, we cannot discount that the effects seen in MICT were driven by changes in body fat. However if this is the case, then the effects of HIIT and MICT are through different pathways, one adipose and the other fitness mediated. It is likely that small changes in fitness and small changes in adipose tissue synergize to result in larger changes in other organs. Until exercise studies can discriminate between fat and fitness, we will be unable to give specific cause and effects. However, HIIT might be a useful model to start teasing out these differences in at risk populations.

5. Conclusion

In summary, this study is the first to demonstrate in sedentary adults an immune bactericidal enhancing effect of HIIT. As these adaptations were comparable to those following MICT despite less than 57% of the total exercise time, these results support HIIT as a time-efficient exercise option to improve neutrophil and monocyte function. Furthermore, as previously reported, HIIT had better adherence than MICT ($82 \pm 14\%$ versus $62 \pm 13\%$, resp.) [24]. Although time commitment was reduced, it is unclear what total workload performed was for each group. Because both groups improved physiological fitness and immunological parameters similarly, it is likely that total work performed was similar between the groups. As such, for those considering HIIT as an exercise program, reduced time does not equate to less work. The same amount of work must be performed, just in less amount of time. Therefore, reduced time commitment and higher adherence combined with the social aspect of group HIIT training could provide an effective means to engaging older adults, most in need of immune function improvements, in exercise training.

Our findings support the proposal that improved function of innate immune cells is a potential anti-immunosenescence response to exercise training of high- or moderate-intensity exercise training. Both HIIT and MICT altered monocyte, but not neutrophil, expression of key surface receptors suggesting that functional improvements are related to intrinsic cellular signalling pathway changes. Neutrophils and monocytes are key intermediaries in the resolution of infection, tissue repair, and control of chronic systemic inflammation. Their age-associated functional decline is central to the development of many age-related diseases including cardiovascular disease and metabolic disease. Future research should aim to determine if the cellular immune responses to exercise training are associated with altered intrinsic cellular signalling and whether this translates directly to improved cardiometabolic health, reduced disease risk, and reduced risk of infection in at risk populations.

Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

All authors declare they have no conflicts of interest.

Authors' Contributions

David B. Bartlett and Janet M. Lord conceived and designed the immune and serological experiments. David B. Bartlett performed the experiments. Sam O. Shepherd, Christopher S. Shaw, and Anton J. M. Wagenmakers conceived and designed the exercise training. Sam O. Shepherd, Oliver J. Wilson, and Ahmed M. Adlan performed the exercise-related experiments. David B. Bartlett wrote the manuscript, and all authors contributed to the final manuscript.

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Research Article

Association of the Composite Inflammatory Biomarker GlycA, with Exercise-Induced Changes in Body Habitus in Men and Women with Prediabetes

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GlycA is a new composite measure of systemic inflammation and a predictor of many inflammatory diseases. GlycA is the nuclear magnetic resonance spectroscopy-derived signal arising from glucosamine residues on acute-phase proteins. This study aimed to evaluate how exercise-based lifestyle interventions modulate GlycA in persons at risk for type 2 diabetes. GlycA, fitness, and body habitus were measured in 169 sedentary adults (45–75 years) with prediabetes randomly assigned to one of four six-month exercise-based lifestyle interventions. Interventions included exercise prescription based on the amount (energy expenditure (kcal/kg weight/week (KKW)) and intensity (%VO_{2peak}). The groups were (1) low-amount/moderate-intensity (10KKW/50%) exercise; (2) high-amount/moderate-intensity (16KKW/50%) exercise; (3) high-amount/vigorous-intensity (16KKW/75%) exercise; and (4) a Clinical Lifestyle (combined diet plus low-amount/moderate-intensity exercise) intervention. Six months of exercise training and/or diet-reduced GlycA (mean Δ: $-6.8 \pm 29.2\,\mu$mol/L; $p = 0.006$) and increased VO_{2peak} (mean Δ: 1.98 ± 2.6 mL/kg/min; $p < 0.001$). Further, visceral (mean Δ: -21.1 ± 36.6 cm^2) and subcutaneous fat (mean Δ: -24.3 ± 41.0 cm^2) were reduced, while liver density (mean Δ: $+2.3 \pm 6.5$HU) increased, all $p < 0.001$. When including individuals in all four interventions, GlycA reductions were associated with reductions in visceral adiposity ($p < 0.03$). Exercise-based lifestyle interventions reduced GlycA concentrations through mechanisms related to exercise-induced modulations of visceral adiposity. This trial is registered with Clinical Trial Registration Number NCT00962962.

1. Introduction

When combined with a dietary intervention, physical activity can be an effective means of slowing the progression to type 2 diabetes mellitus (T2DM) [1–3]. Combined lifestyle changes make it difficult to distinguish differences between the dietary and the exercise components. Although Pan et al. [4] suggested that exercise alone can reduce the risk of diabetes and is comparable to diet alone or diet+exercise, most interventions assess changes in risk factors, including glucose, lipids, and inflammation [5–9]. As such, even with minimal weight loss, exercise in participants who are overweight and sedentary has beneficial effects on cardiometabolic risk [5–8]. Often, responses to exercise interventions are assessed with combined measures of body composition, lipoproteins, and proinflammatory biomarkers; such markers include interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), and C-reactive protein (CRP) [10, 11]. Although the use of

these inflammatory risk factors as defining measures of clinical health status is well documented, their responses to lifestyle and exercise interventions are not clear [12–18].

GlycA is a marker of systemic inflammation, measured via nuclear magnetic resonance spectroscopy (NMR), whose signal arises from the N-acetyl glucosamine residues on the glycans of acute-phase proteins (APPs) [19, 20]. Although the GlycA signal is associated with concentrations of IL-6, TNF-α, fibrinogen, and CRP, these proteins contribute negligibly to concentrations of GlycA [20, 21]. GlycA levels are raised in acute febrile illnesses [22]. In addition, GlycA is positively correlated with body mass index (BMI), insulin resistance, markers of metabolic syndrome, and the ratio of leptin to adiponectin, suggesting that it is related to adipose tissue-associated low-grade inflammation [23, 24]. As such, GlycA is a biomarker for cardiometabolic disease risk [25]. In support of this claim are data that show that GlycA is associated with CVD in a secondary prevention cardiovascular cohort (CATHGEN) [26] and with incident CVD events in the Women's Health Study (WHS) [27], the Prevention of Renal and Vascular End-Stage Disease (PREVEND) study [28], the Multi-Ethnic Study of Atherosclerosis (MESA) [29], and the JUPITER trial [30], independent of traditional CVD risk factors. Notably, following adjustment for traditional risk factors (e.g., BMI, LDL, and age), these associations were often only slightly attenuated by CRP, implying that GlycA measures CVD risk independently of CRP and may be a marker of a different inflammatory process. GlycA was also associated with incident T2DM [31, 32] even after adjusting for clinical T2DM risk factors. GlycA concentrations are higher in patients with inflammatory autoimmune diseases such as rheumatoid arthritis (RA) [33, 34], systemic lupus erythematosus (SLE) [35, 36], and psoriasis [37]. Moreover, GlycA is associated with disease activity [33, 34] and coronary atherosclerosis in RA [33] as well as active vascular inflammation in psoriasis [37]. Taken together, these data support the concept that GlycA is a marker of systemic inflammation and a risk factor for cardiometabolic disease. To date, no study has assessed the effects of exercise-based interventions on GlycA concentrations in an at-risk population in an attempt to understand how exercise might modulate this unique risk factor.

The purpose of Studies of Targeted Risk Reduction Interventions through Defined Exercise-Prediabetes (STRRIDE-PD) was to compare the effects of different amounts and intensities of exercise without diet to a Clinical Lifestyle (exercise plus diet) program modeled after the first six months of the Diabetes Prevention Program (DPP) [1]. The outcomes were focused on cardiometabolic risk factors in individuals at-risk for diabetes. Because little is known about the effects of diet and exercise on the GlycA biomarker, we sought to evaluate GlycA levels in the STRRIDE-PD study, determine whether exercise alone or exercise plus diet would favorably modify circulating GlycA concentrations, and try to understand how exercise effects might be mediated.

2. Methods

2.1. Study Design. Study design and experimental procedures have been described previously [9]. Briefly, 195 sedentary 45- to 75-year-old adults were recruited with a BMI between 25 and 35 kg/m^2 and identified as having prediabetes by two consecutive fasting plasma glucose measurements between 95 and 125 mg/dL taken one week apart. GlycA measures were not available for all 195 recruits due to either insufficient plasma sample volume for NMR analysis or samples that did not produce an NMR spectrum that was good enough for GlycA quantification. Specifically, interferents can arise in the NMR spectrum that do not allow quantification of various analytes. Interferents can arise from such things as medication use or hemolysis of the sample. Therefore, of the 195 recruited into the study, only 169 participants had GlycA measured at both time points and these participants are reported here. Exclusion criteria included an inability to exercise, smoking, diabetes, uncontrolled hypertension, musculoskeletal disorders, and/or cardiovascular disease. The protocol was approved by the Duke Institutional Review Board and informed written consent was obtained.

2.2. Exercise and Dietary Interventions. Participants were randomized by gender and race (white women, white men, nonwhite women, and nonwhite men) into one of the four six-month intervention groups; exercise mode was predominantly using treadmills and also included elliptical trainers, rowing, and bicycle ergometers. VO_2 reserve was chosen for exercise prescription and calculated as previously described [38]. The Clinical Lifestyle program, a combined diet and exercise program, is based on the Diabetes Prevention Program which resulted in a better risk reduction for diabetes than metformin [1]. In effect, this is the gold standard lifestyle intervention for reducing the risk of diabetes.

(1) Low-amount/moderate-intensity exercise (Low-Mod)—exercise energy expenditure of 10 kcal per kg per week (KKW) at 50% VO_2 reserve

(2) High-amount/moderate-intensity exercise (High-Mod)—16 KKW at 50% VO_2 reserve

(3) High-amount/vigorous-intensity exercise (High-Vig)—16 KKW at 75% VO_2 reserve

(4) Clinical Lifestyle intervention (Clinical Lifestyle)—10 KKW at 50% VO_2 reserve plus a calorically restricted diet designed to reduce body weight by 7% over six months.

2.3. Fitness and Body Composition. Fitness and body composition were evaluated at baseline and at the end of six months of prescribed exercise training. Exercise treadmill testing was used to determine cardiorespiratory fitness as previously described [39]. Briefly, aerobic capacity (VO_{2peak}) was determined by a graded maximal treadmill test which started at 3 mph/0% grade and then increased speed and/or grade such that the metabolic demand increased at approximately 3.5 mL/kg/min until volitional exhaustion. Height was measured to the nearest 0.1 cm using a stadiometer (Seca-220, Hamburg, Germany). Body composition was assessed according to Siri's three-compartmental model [40]. Body weight was first assessed using a calibrated scale before

assessment of body fat and lean tissue mass with air-displacement plethysmography (BodPod System; Life Measurement Corporation, Concord, CA) [41, 42]. Waist circumference was measured around the minimal waist and blood pressure was taken following 15 minutes sitting quietly.

2.4. Abdominal Computerized Tomography (CT). Single 10 mm thick axial sections of the abdomen were scanned before and after the intervention using a General Electric CT/I scanner (GE Medical Systems, Milwaukee, WI). Liver density and abdominal cross-sectional areas for visceral adipose tissue (VAT) and subcutaneous adipose depots were assessed using OsiriX© Software (Pixmeo, Geneva, Switzerland). The determination of adipose tissue depots and liver density by CT are very accurate, precise, and reliable [43, 44].

2.5. Plasma Measures. Peripheral venous blood was drawn into vacutainers containing EDTA and placed in ice. Blood was centrifuged at 1600 ×*g* at 4°C for 10 minutes and plasma flash frozen in liquid nitrogen and stored at −80°C until analyses was conducted. Plasma samples were acquired following an overnight fast and at between 16 and 24 hours following their final exercise session. Plasma GlycA, glucose, and insulin were quantified as previously described [9, 33]. Briefly, glucose was measured by a clinical analyzer (Beckman Coulter Dx C600, Brea, CA, USA) and insulin by electrochemiluminescent plate assay (Meso Scale Discovery, Gaithersburg, MD, USA). GlycA was assessed by NMR spectroscopy, where NMR spectra were acquired from plasma samples as previously described for the NMR Lipoprofile (lipoprotein particle distribution) at LipoScience, Laboratory Corporation of America Holdings (Raleigh, NC, USA) [45]. The NMR Profiler platform is comprised of a 9.4 T (400 MHz ^{1}H frequency) spectrometer (Bruker Biospin, Billerica, MA, USA) with an integrated fluidics sample delivery system. The intra-assay and interassay variability for GlycA measurement is 1.9% and 2.6%, respectively [20].

2.6. Statistical Analyses. All analyses were conducted using PASW v21.0 (Chicago, IL, USA) and all data are presented as mean (SD) unless otherwise stated. Normality was assessed using Kolmogorov-Smirnov analysis and natural log transformation of variables violating normality was completed. Repeated measures ANOVA were used to assess changes in outcomes. Models included group, time, and group-by-time interactions. Sex differences were assessed at baseline and for change scores with a univariate ANOVA. There were no differences for change scores between men and women and so, data was not split by sex for each group. Even though we have low power to detect significant interactions in four training groups, we assessed GlycA changes within groups with pair-wise post hoc comparisons correcting for multiple comparisons using the method of Bonferroni. Linear regression analysis of log-normalized data was used to test significant associations between changes in outcome measures and changes in GlycA. Following our analyses of GlycA, a sample size estimation was performed with change in GlycA as an outcome (GPower v3.1.9.2, Universitat Dusseldorf, Germany) [46]. Using our correlation between baseline and follow-up values of 0.804, calculated partial η^2 of 0.035 which gave an effect size of 0.190, it would have taken 52 participants in each of the four groups to observe significant differences between groups. Statistical significance was accepted at $p \leq 0.05$.

3. Results

We previously reported [9] metabolic and cardiorespiratory fitness indices in the whole cohort ($n = 195$). Here, we report similar findings for the 169 participants who had GlycA reliably assessed at both time points.

3.1. Baseline Analyses. Baseline characteristics for the entire group and by intervention are shown in Table 1. There were no differences between groups for any of the data presented (all $p > 0.05$). As expected, men and women differed on baseline cardiorespiratory fitness, body fat percentage, and subcutaneous and visceral adiposity (all $p < 0.001$), data not shown. As previously reported, when compared to men, women had greater concentrations of GlycA ($p < 0.001$), data not shown [33]. Although we have previously reported exercise prescription for the study [9], Table 2 highlights the prescription and adherence values for the 169 participants who had GlycA measured at both time points.

3.2. Effect of Intervention

3.2.1. Cardiorespiratory Fitness. As expected, there was a significant main effect for time ($F(1, 168) = 95.3$, $p < 0.001$, $\eta^2 = 0.390$) with increases in absolute VO_{2peak} for each group following the interventions (all $p < 0.01$), Figure 1(a). There were significant group × time interactions ($F(3, 165) = 4.4$, $p < 0.01$, $\eta^2 = 0.082$); following post hoc analyses, this was because the High-Vig group increased VO_{2peak} to a greater extent than the Low-Mod group ($p = 0.011$) and a trend towards greater than the High-Mod group ($p = 0.08$).

3.2.2. Body Composition. For liver density, a surrogate measure of liver adiposity, there was a significant main effect for time ($F(1, 168) = 18.6$, $p < 0.001$, $\eta^2 = 0.105$) with liver density increased by 5% in the High-Vig group ($p = 0.002$) and 8% in the Clinical Lifestyle group ($p < 0.001$), Figure 1(b). There were significant group × time interactions ($F(3, 165) = 3.3$, $p = 0.021$, $\eta^2 = 0.06$); following post hoc analyses, this was because liver density increases were greater in the Clinical Lifestyle group compared to that in the High-Mod ($p = 0.048$) and Low-Mod ($p = 0.025$) but not that in the High-Vig ($p = 0.163$) group. For subcutaneous adiposity, there was a significant main effect for time ($F(1, 168) = 68.6$, $p < 0.01$, $\eta^2 = 0.303$) with reductions observed in each group (Figure 1(c); all $p < 0.01$). For visceral adiposity, there were was a significant main effect for time ($F(1, 168) = 69.0$, $p < 0.001$, $\eta^2 = 0.304$) with reductions observed in each group (Figure 1(d); all $p < 0.05$). There were significant group × time interactions for subcutaneous ($F(3, 165) = 7.7$, $p < 0.001$, $\eta^2 = 0.127$) and visceral fat reductions ($F(3, 165) = 7.0$, $p < 0.001$, $\eta^2 = 0.118$); which was because of greater reductions in subcutaneous and visceral fat in the Clinical Lifestyle intervention compared to that in every other group (all $p < 0.01$).

Table 1: Baseline demographics, fitness, body composition, inflammation, glucose, and insulin for each intervention group.

	Total group ($n = 169$)	Low-Mod ($n = 41$)	High-Mod ($n = 45$)	High-Vig ($n = 40$)	Clinical Lifestyle ($n = 43$)
Age (years)	59 (7.5)	57 (7.9)	61 (6.8)	61 (7.0)	58 (7.9)
Men/women	67/102	17/24	18/27	15/25	17/26
Race					
Caucasian	132	32	36	31	33
African American	29	9	8	7	5
Others	8	0	1	2	5
Fitness					
VO_{2peak} (mL/kg/min)	24.5 (5.1)	25.0 (5.8)	24.2 (5.0)	23.9 (5.2)	24.9 (4.5)
Body composition					
Body mass index (kg/m^2)	30.5 (2.7)	30.6 (2.6)	30.0 (2.4)	30.4 (2.7)	31.0 (3.2)
Minimal waist (cm)	98.7 (8.8)	98.3 (8.0)	98.2 (8.2)	99.8 (8.6)	98.6 (10.2)
Body fat (%)	40.8 (7.8)	39.7 (8.0)	40.9 (6.7)	41.5 (8.2)	41.0 (8.4)
Total abdominal adiposity (cm^2)	523 (110)	517 (89)	522 (102)	523 (137)	528 (112)
Subcutaneous (cm^2)	330 (97)	327 (81)	324 (89)	335 (127)	335 (92)
Visceral (cm^2)	192 (73)	190 (83)	198 (68)	188 (72)	193 (71)
Liver density (HU)	59 (10)	60 (8)	60 (7)	56 (13)	59 (10)
Blood pressure					
Systolic BP (mmHg)	126 (14)	126 (14)	127 (15)	128 (13)	124 (14)
Diastolic BP (mmHg)	75 (10)	77 (9)	74 (7)	75 (11)	75 (11)
Inflammation					
GlycA (μmol/L)	348 (47)	348 (51)	342 (47)	351 (44)	352 (47)
Glucose and insulin					
Fasting Glucose (mg/dL)	106 (9)	106 (11)	106 (8)	104 (8.5)	106 (10.9)
Fasting insulin (μU/mL)	7.4 (5.0)	7.3 (3.6)	6.7 (4.6)	7.7 (6.7)	7.9 (4.8)

Data are mean (SD). No significant differences observed between groups.

Table 2: Exercise prescription and actual completed exercise for each group.

	Total group	Low-Mod	High-Mod	High-Vig	Clinical Lifestyle
Exercise prescription (Rx)					
Intensity (% peak VO_2)		50	50	75	50
Amount (KKW)		10	16	16	10
Time (min/week)	212 (63)	179 (35)	290 (55)***	194 (37)	179 (35)
Exercise completed					
Adherence (% of Rx time)	85 (17)	89 (16)	82 (16)	85 (17)	85 (19)
Amount (KKW)	11 (3.2)	9 (1.9)	13 (2.6)	14 (2.7)	9 (1.9)
Frequency (sessions/week)	3.3 (0.8)	3.1 (0.7)	3.9 (0.9)***	3.2 (0.7)	3.0 (0.7)

KKW (kcal per kg per week). Data are mean (SD). $^{***}p < 0.001$ different from all other groups

3.2.3. Metabolic Measures. For fasting glucose, there was a significant main effect for time ($F(1, 168) = 5.3$, $p = 0.022$, $\eta^2 = 0.035$) with glucose reduced by 5% in only the Clinical Lifestyle group ($p < 0.001$). There was a significant group × time interaction ($F(3, 165) = 7.6$, $p < 0.001$, $\eta^2 = 0.136$) because of greater reductions in the Clinical Lifestyle group compared to that in every other group (all $p < 0.001$), indicating that diet, rather than exercise, was responsible for reduced fasting glucose, Figure 1(e). For fasting insulin, there was also a significant main effect for time ($F(1, 168) = 36.4$, $p < 0.001$, $\eta^2 = 0.201$) with reductions in the Low-Mod ($p = 0.015$), the High-Vig ($p = 0.005$), and the Clinical Lifestyle ($p < 0.001$) groups, Figure 1(f). There was a significant group × time interaction ($F(3, 165) = 4.4$, $p = 0.005$, $\eta^2 = 0.083$) because Clinical Lifestyle was better at reducing fasting insulin than any of the other groups (all

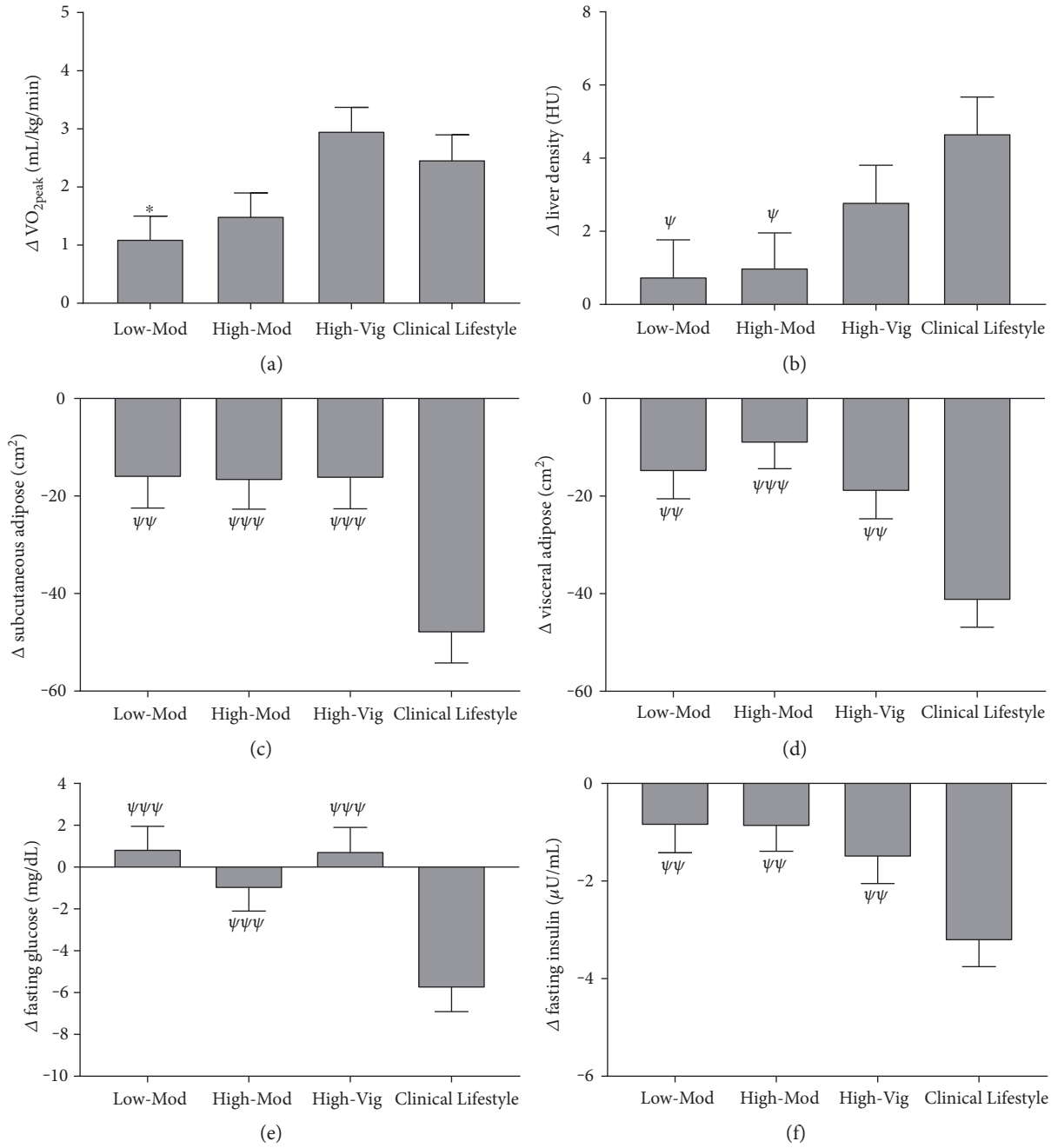

Figure 1: Mean (SEM) change scores for VO_{2peak} (a), liver density (b), subcutaneous (c) and visceral adiposity (d), fasting glucose (e), and insulin (f) for each intervention. $^{**}p < 0.01$ different from the High-Vig group; $^{\psi}p < 0.05$; $^{\psi\psi}p < 0.01$; $^{\psi\psi\psi}p < 0.001$ different from Clinical Lifestyle.

$p < 0.01$), indicating that exercise alone can reduce insulin but exercise and diet combined are superior at reducing fasting insulin.

3.2.4. GlycA. For GlycA, there was a significant main effect for time ($F(1, 168) = 7.9$, $p = 0.006$, $\eta^2 = 0.05$), with an average GlycA reduction of 2% among the whole cohort, Figure 2. There were no group × time interactions and no differences between groups for absolute changes in GlycA ($F(3, 165) = 2.0$, $p = 0.123$, $\eta^2 = 0.035$). As this study is assessing responses for a novel biomarker of chronic inflammation and is hypothesis generating, we felt obliged to perform Bonferroni-corrected pair-wise analyses on the within-groups change for GlycA. This would give some indication of how individual groups were contributing to the 2% reduction in GlycA and allows the readers a chance to draw their own conclusions. Our analyses revealed that GlycA was reduced on average by 3% in the High-Vig group ($p = 0.033$) and on average by 4% in the Clinical Lifestyle intervention ($p = 0.007$). The Low-Mod group reduced GlycA on average by 1% ($p = 0.305$) while the High-Mod group increased GlycA on average by 1% ($p = 0.705$).

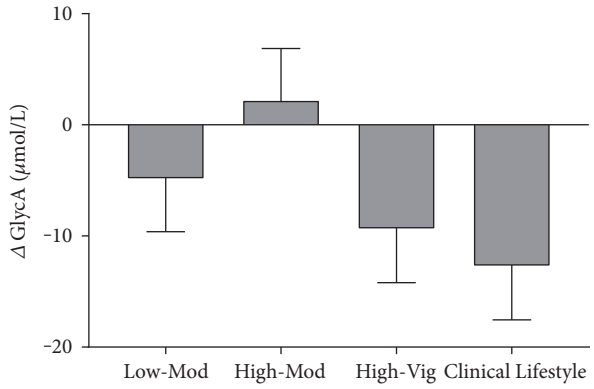

Figure 2: Mean (SEM) change scores for GlycA. Although there was a significant effect for time ($p = 0.006$) with an overall 2% reduction in GlycA, no differences were observed between groups ($p > 0.05$).

Table 3: Associations of changes in fitness, body composition, liver density, fasting glucose, and insulin on changes in GlycA concentrations.

	Change in log GlycA		
	R^2 adj	B (95% C.I.) × 10^3	p
Fitness			
VO_{2peak} (mL/kg/min)	0.627	−2.0 (−4.0, 0.0)	0.06
Body composition			
BMI (kg/m^2)	**0.645**	**4.0 (0.0, 8.0)**	**0.03**
Body fat (%)	**0.616**	**2.0 (0.0, 3.0)**	**0.03**
Total abdominal adiposity (cm^2)[a]	**0.642**	**120 (23.0, 217)**	**0.02**
Subcutaneous (cm^2)[a]	0.631	88 (−11.0, 18.8)	0.08
Visceral (cm^2)[a]	**0.637**	**79 (15.0, 14.2)**	**0.02**
Liver density (HU)[a]	0.629	−28.0 (−121, 66.0)	0.56
Glucose and insulin			
Fasting glucose (mg/dl)[a]	0.621	129 (−84, 342)	0.23
Fasting insulin (μU/mL)[a]	**0.63**	**44 (11, 77)**	**0.01**

[a]Log transformed

3.2.5. Relationships. To investigate the contributions of various physiological improvements to GlycA responses, we evaluated relations between GlycA and changes in fitness, liver density, and various measures of body composition (Table 3). Reductions in GlycA were associated with reductions in BMI ($p = 0.03$), body fat percentage ($p = 0.03$), total abdominal adiposity ($p = 0.02$), and visceral adiposity ($p = 0.02$). Furthermore, reductions in GlycA were associated with reduced fasting insulin concentrations ($p = 0.01$) but not fasting glucose ($p = 0.23$). As BMI, body fat percentage, and visceral adiposity are interrelated, we conducted multivariable analyses with changes in fasting insulin and visceral adiposity. Multivariable regression analysis indicated that changes in GlycA were somewhat associated with changes in reductions in visceral adiposity ($p = 0.06$) but not with fasting insulin ($p = 0.124$). Taken together, these data suggest an anti-inflammatory response in our interventions—effects likely mediated by reductions in ectopic fat more than by improved insulin sensitivity.

4. Discussion

We describe, for the first time, the effects of exercise-based lifestyle interventions on a newly described composite measure of systemic inflammation, GlycA. Further, this is associated, at least in part, with exercise-induced modifications of ectopic fat stored in the visceral compartment. We were marginally underpowered to detect differences between groups for change in GlycA. However, as this is the first study to examine responses of GlycA to exercise-based lifestyle interventions, we are compelled to present as complete a story as we can and give our best interpretation. We realize that others may offer alternative interpretations and future research should aim to determine with an adequately powered randomized control trial the influence of exercise and/or diet on GlycA. In overweight individuals with prediabetes, a six-month lifestyle intervention of either exercise alone or exercise combined with diet resulted in a small (2%) significant reduction in circulating GlycA. Without a control (no intervention) group, it is unclear from our analyses which groups were responsible for the changes or whether simply any intervention would result in a change in GlycA. Our interpretation suggests that a Clinical Lifestyle (combined exercise and diet) program and a similar period of high-amount vigorous-intensity exercise significantly contributed to reductions in plasma GlycA concentrations. However, we cannot discount that with more participants or longer periods of training, low-amount moderate-intensity or high-amount moderate-intensity exercise might also reduce GlycA concentrations. In Table 3, all intervention groups are combined to assess relations between physiologic responses to exercise-containing regimens and GlycA. These analyses imply that lifestyle interventions that modify GlycA do so primarily in relation to their ability to modify ectopic energy stores—primarily those in the visceral compartments. Although the strengths of the associations were small, with baseline GlycA the largest predictor of follow-up concentrations, when controlling for this these small associations remain significant. This would explain why adding a diet regimen to reduce body fat can augment the effects of exercise interventions on changes in systemic inflammation as measured by GlycA.

Also of interest is the observation that when matched for energy expenditure (High-Vig and High-Mod), the effects of vigorous intensity exercise outperform those of moderate intensity exercise. This establishes a contribution of exercise intensity on the responses and incorporates the near significant association between exercise-induced changes in peak VO_2 ($p = 0.06$; Table 3). It is well established and confirmed in this study that when matched for energy expenditure, vigorous intensity exercise induced a greater increase in cardiorespiratory fitness (Figure 1).

4.1. Acute-Phase Protein Responses to Exercise and Diet. The GlycA signal originates from specific glycan residues found on APPs, primarily α1-acid glycoprotein (AGP), haptoglobin, α1-antitrypsin, α1-antichymotrypsin, and transferrin

[20]. CRP, IL-6, and fibrinogen contribute negligibly to the GlycA signal, and any changes in these APPs and cytokines are unlikely to impact our findings [20]. APPs serve as regulators of inflammation through various functions such as collating iron and reducing oxidative damage, reduction of aberrant tissue damage by inhibiting proteases, and providing efficient pharmacokinetics [47–49]. Most circulating APPs are N-linked glycoproteins whose glycan structures are modified during both acute and chronic inflammatory conditions [48, 50, 51]. In support of APP glycosylation as a risk factor for disease, synthesis and glycosylation of AGP, haptoglobin, and transferrin are associated with a number of chronic diseases such as pancreatitis, rheumatoid arthritis, diabetes, and inflammatory lung conditions [52–54]. Additionally, in a large population-based study, a recently created NMR-based risk score, which includes AGP, was found to predict all-cause and CVD mortality [55]. Given that GlycA concentrations reflect a composite measure of systemic inflammation rather than concentrations of individual APPs, it may be a more consistent and accurate method to determine overall inflammatory and cardiometabolic disease risk than traditional markers [33].

To date, no studies have assessed the response of GlycA to exercise training. However, the responses of specific APPs—such as AGP and haptoglobin—to exercise training and/or diet have been studied. Prescott and colleagues showed no change in AGP following 8 weeks of exercise in elderly heart failure patients [56]. However, they also reported no change in body composition or aerobic fitness, supporting the premise that changes in body composition, particularly visceral adiposity, mediate the observed exercise-induced changes in APPs. In a recent study by Lavoie and colleagues, the combination of high physical activity levels and superior dietary quality, assessed by the Canadian Healthy Eating Index (C-HEI), was associated with reductions in the cardiometabolic risk factors CRP and apolipoprotein B in postmenopausal women [57]. This study also reported reductions in APPs known to contribute to the GlycA signal. Specifically, high physical activity levels with good C-HEI scores were associated with reduced concentrations of haptoglobin while lower AGP was associated with reduced fat mass.

Together, the studies by Lavoie, Prescott, and ours suggest that APP reductions are associated with fat loss and exercise-induced modification of cardiorespiratory fitness. In support, GlycA is associated with the leptin/adiponectin ratio [23]. The leptin/adiponectin ratio is a surrogate marker of adipose tissue function, and higher ratios are associated with a dysfunctional phenotype that contributes to inflammation and insulin resistance in nondiabetic individuals [58]. Visceral adipose tissue is a component of the metabolic syndrome and is a major producer of IL-6, TNF-α, and MCP-1 [59, 60]. Therefore, it is possible that our interventions reduced visceral adiposity enough to impact the secretion and enhanced glycosylation of circulating APPs.

4.2. GlycA Changes in Relation to Health and Disease. We report here a small but significant reduction in plasma GlycA concentration. Whether this reduction is relevant to disease risk remains unknown; however, a number of cross-sectional studies suggest that small changes in GlycA are associated with risk of disease and mortality. Most recently, hazard ratio (HR) risk of mortality from colorectal cancer was 1.24 per SD increment in GlycA [61]. Interestingly, GlycA ranges of 327–369 μmol/L was associated with an HR of 0.92, 370–416 μmol/L was associated with an HR of 1.26, and over 416 μmol/L was associated with an HR of 1.46, suggesting that small increments in GlycA concentrations are physiologically relevant for risk of mortality [61]. Furthermore, the difference in GlycA between patients with rheumatoid arthritis, a highly inflammatory disease, and matched controls was 20–30 μmol/L (353 ± 67 versus 329 ± 54) suggesting that small differences have significant disease associations [34]. GlycA was measured in the PREVEND study, and the highest GlycA quartile (>384 μmol/L) was at a significantly increased risk of developing type 2 diabetes compared to the lowest quartile (<306 μmol/L) [32]. Taken together, we suggest that small GlycA changes may be physiologically relevant and even small reductions may reflect reduced risk of inflammatory-mediated damage that is observed in many chronic diseases.

4.3. GlycA Responses and Vigorous Intensity Exercise. One observation points to the contribution of exercise intensity to the exercise-training response: the High-Vig group did not reduce visceral adiposity more than the moderate intensity groups, even when matched for energy expenditure. This may be due to the lack of adequate statistical power to detect these differences. A previous work with larger numbers of participants has shown both greater fat depot improvements with larger amounts of exercise than our study [62, 63]. Therefore, it is possible that reductions in GlycA in the High-Vig group could be influenced by exercise intensity-induced visceral adipose loss and increased cardiorespiratory fitness. In young individuals, exercise-induced APP responses are specified by exercise intensity, duration, mode, and timing of APP measurements following the last exercise exposure [64–66]. Liesen and colleagues observed exercise intensity-specific responses to an acute bout of exercise in many of the APPs contributing to GlycA [66]. Of interest, acute bouts of exercise induced smaller increases in AGP and haptoglobin following nine weeks of higher intensity endurance exercise training with no significant weight loss. These findings suggest that these APPs can be regulated by the intensity of exercise, independent of body weight changes.

4.4. GlycA and Hepatic Fat. The primary source of APPs is hepatic synthesis and steatosis can be accompanied by steatohepatitis. Assessing liver fat content is difficult; gold standard needle biopsies are invasive and present a risk of mortality [67]. Over the last 20 years, the use of computed tomography to determine liver fat content has become a safer and an easily accessible alternative to biopsies [68, 69]. With a good correlation between CT scans and needle biopsies [70], the lower the attenuation of the liver, the lower the tissue density and thus, the greater the fat content. As such, liver density is an established surrogate measure for liver fat content [71].

Our data suggests that liver fat was reduced in both the High-Vig and Clinical Lifestyle groups. There are clear links between liver fat accumulation and insulin sensitivity, glucose control, inflammation, and the risk of T2DM [72–74]. Therefore, as the High-Vig and Clinical Lifestyle groups reduced fasting insulin and subcutaneous and visceral fat, and as the Clinical Lifestyle also reduced fasting glucose, it would seem likely that ectopic liver fat was reduced in both groups. These findings are similar to our previous study showing, as compared to a control group of no exercise, that aerobic exercise training was associated with increased liver density (lower fat) and reduced plasma alanine aminotransferase (ALT), which is associated with reduced hepatic insulin sensitivity and risk for T2DM [75, 76]. However, a consequence of each of our four lifestyle interventions is an exercise and/or dietary modification of metabolism. With this change in metabolism comes the potential to generate confounding assumptions from CT data. Changes in water content, depending on the analyses technique and dispersion of measured molecules, could result in assumptions of either increased or decreased fat content [77, 78]. We are unaware of exercise studies assessing CT-derived liver density in response to extreme changes in hepatic water only content, such as dehydration. Acute exercise with no carbohydrate intake and dieting are associated with temporal changes in liver metabolite (i.e., glycogen) content [79, 80]. As glycogen is stored with water, these changes could potentially influence liver density. In their very elegant review paper discussing liver glycogen metabolism responses to exercise, Gonzalez and colleagues extracted data from three studies and suggest that unlike muscle glycogen content, there are no differences in basal liver glycogen content between those with T2DM, healthy controls, or endurance-trained athletes [80–82]. Together, these data suggest that it is unlikely that our interventions resulted in noticeable increased (or decreased) glycogen storage, which would confound our interpretation of fat content. Instead, glycogen utilization during exercise would be improved following a period of training, which would contribute to an increased energy reserve during exercise [79]. Therefore, although it is possible that changes in liver density are a reflection of changes in metabolite content, potentially explaining the lack of association with density and GlycA, we do not believe this to be the case. Instead, from our previous data and studies of correlations between liver density and liver adiposity, plus our observation of subcutaneous and visceral fat reductions, we believe that the majority of changes can be attributed to reduction in liver fat content. The role that liver fat and other fat depots play on glycosylation of APPs remains unclear; future work should determine the relationship of ectopic fat and GlycA in order to understand better the role of glycosylation in health and disease.

4.5. Limitations. We recognize this study has several limitations. We have already discussed the limitations of the GlycA analyses and the lack of a control group. Although we have speculated about the possible mechanisms whereby exercise modifies the APP components of GlycA, we are unable to determine the molecular and cellular physiology whereby exercise-based interventions actually modify GlycA concentrations. Future work should aim to determine which organs and biological processes underlie these adaptations. For instance, one might ask whether the reductions in GlycA plasma concentrations are evident because of reductions in concentrations of particular APPs or through the reduction in glycosylation of specific acute-phase proteins—or both. This will be important when designing lifestyle interventions for individuals at risk of chronic disease.

5. Conclusion

GlycA is a composite and accurate measure of systemic inflammation and is reduced in individuals at risk for the development of T2DM following 6 months of an exercise-based lifestyle intervention. These adaptations appear to be mediated by intervention-induced reductions in ectopic fat stores—particularly in the visceral compartment—coupled in part with favorable modifications of cardiorespiratory fitness. The implications of these studies for disease prevention are significant. Increased GlycA is associated with incidence of T2DM and CVD and with vascular disease in patients with RA and psoriasis [27, 31–33, 37]. Thus, diet and/or exercise-induced GlycA reductions may reflect a reduced risk of developing T2DM and diabetes-associated cardiometabolic complications for a number of inflammatory conditions. Which type (mode, volume, and intensity) of exercise and/or diet that modifies GlycA remains unclear and critically does a better job than another. The current study suggests that a 6-month exercise-based lifestyle intervention which improves cardiorespiratory fitness and reduces body fat content can reduce GlycA concentrations. Our study is relevant to the future of personalized prescription of exercise and diet in clinical populations who find such interventions difficult to maintain.

Abbreviations

AGP: α1-acid glycoprotein
ALT: Alanine aminotransferase
APP: Acute-phase-protein
C-HEI: Canadian Healthy Eating Index
CRP: C-reactive protein
CT: Computerized tomography
CVD: Cardiovascular disease
DPP: Diabetes Prevention Program
IL: Interleukin
KKW: kcal per kg per week
MCP: Monocyte chemoattractant protein
PREVEND: Prevention of Renal and Vascular End-Stage Disease
STRRIDE-PD: Studies of Targeted Risk Reduction Interventions through Defined Exercise-Prediabetes
T2DM: Type 2 diabetes mellitus
TNF: Tumor necrosis factor
WHS: Women's Health Study.

Disclosure

The present affiliation of author Lori Bateman is Center for Health Promotion and Disease Prevention, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA.

Conflicts of Interest

MAC is an employee of LabCorp and all other authors declare no conflict of interest.

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