PREDICTING INDIVIDUAL RESPONSES TO EXERCISE INTERVENTIONS

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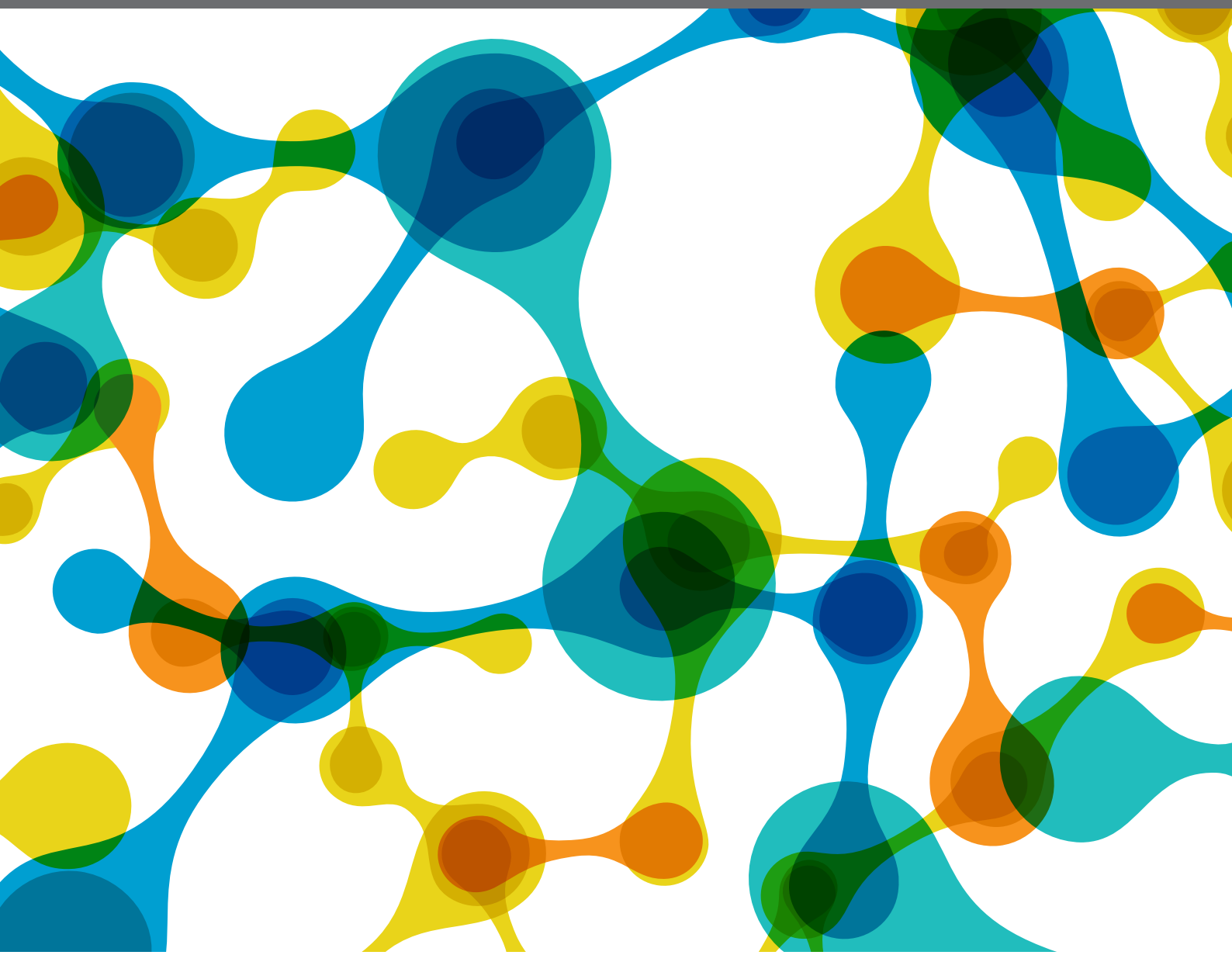

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PREDICTING INDIVIDUAL RESPONSES TO EXERCISE INTERVENTIONS

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Table of Contents

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EDITORIAL
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Editorial: Predicting Individual Responses to Exercise Interventions

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Editorial on the Research Topic

Predicting Individual Responses to Exercise Interventions

Despite the overwhelming evidence for the health benefits of regular exercise, there is limited knowledge regarding accurate prescription of exercise regimens on an individual basis, largely emanating from large interindividual differences in the biological responses to training. The present Research Topic has attempted to tackle this problem by gathering a sum of 14 papers attempting to discover predictors of the individual responses to exercise interventions.

Flück et al. report on potential association between gene polymorphism and performance in power and endurance elite athletes. In particular, by using an appropriate statistical approach, they tested whether gene polymorphism for angiotensin converting enzyme (ACE), tenascin-C (TNC), and actinin-3 correlates with diverse structural features of the skeletal muscle fibers known to contribute to the overall muscular function. Results indicated interaction effects between the athlete type and genotype for two gene polymorphisms of ACE (I/D) and TNC (A/T).

A similar approach was used by Ficek et al. to study the correlation between interleukin-15 (IL-15) polymorphism and training-induced changes in body composition in women subjected to a 12 week training programme. A significant interaction was found between genotype and changes in fat mass % (FM%) and fat-free mass (FFM). In particular, the genotype-phenotype interactions was robust for A allele and the [T;A] haplotype and FM% reduction and elevated FFM due to exercise.

The paper by Haun et al. deals with the identification of biomarkers in low and high previously well-trained responders to resistance training, clustered on the basis of changes in mean muscle fiber cross-sectional area (fCSA), muscle thickness, upper right leg lean soft tissue and mid-thigh circumference after 6 weeks of high-volume resistance training. Importantly, in this work regression analysis identified pre-exercise lower type II fiber percentage and fCSA as strong predictors of the hypertrophic response to resistance training.

The paper by Garai et al. deals with validity of the computer-based artificial neural network analysis (ANN) to predict individual responses to physical exercise. This approach is used to identify patterns of correlation between physiological and molecular datasets obtained in trained subjects in a concept-free manner. Using ANN, authors identified numerous molecular changes triggered by regular combined endurance-strength training in previously unaccustomed subjects and showed evidence for individual responsiveness at molecular level, identifying responders and not responders for each parameter.

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Blume and Wolfarth sought potential performance-related predictors in young competitive athletes by monitoring 146 young athletes through ∼10 months. The authors found that enhanced health senses and lower stress levels were related to performance progress. The authors propose that such subjective self-reported data should be added to the parameters routinely used to plan training, modify training intensity, and, ultimately, predict and ensure optimal performance development in young competitive athletes.

Ross et al. evaluated responses to exercise for health outcomes in overweight or obese adults at an individual level. By assigning 382 previously sedentary, overweight or obese individuals to either an inactive control group or to different exercise training programs, they found no association between changes in aerobic capacity and changes in visceral fat, the lipidemic profile or fasting plasma insulin. These findings underline the need for additional research on the biochemical, physiological, and genetics factors influencing individual responsiveness to exercise training regimens.

Tung et al. and Tung et al. explored physiological and biochemical differences between mice of intrinsically high and low exercise capacities (in terms of endurance and strength) through multiomics approaches. They report that, compared to the latter, the former displayed, on one hand, lower fatigue and injury biomarkers and, on the other hand, higher levels of muscle microRNAs associated with performance-related functions and key proteins related to muscle function and carbohydrate metabolism. Groups also differed in the gut microbiome.

O'Donoghue et al. examined whether phenotypic characteristics at baseline or phenotypic responses to a 12-week lifestyle intervention can explain inter-individual variability in change in glucose tolerance in 285 individuals at high risk of type 2 diabetes. Despite finding an overall improvement and despite performing a meticulous analysis, the authors found no sufficient phenotypic characteristics, among standard clinical and physiological parameters, that could explain the inter-individual variability in the response of glucose tolerance to the intervention.

The review by Petrigna et al. examined the reliability and feasibility of available procedures testing countermovement and squat jump in the context of public health examination in adolescence. By analyzing 117 relevant studies in the literature, the authors noted a lack of method standardization for both jumps. Based on the literature, they proposed standard operating procedures, which may facilitate the comparison of data between studies in testing lower limb muscle strength and power in adolescents.

Loro et al. examined differences in the activation of metabolic pathways within exercised skeletal muscle between control mice and mice lacking IL-15 receptor alpha (IL15RA). Mice lacking IL15RA demonstrated higher fatigue resistance, recovery capacity, and increased habitual activity compared to control mice. Skeletal muscle metabolomic analysis revealed 8 resting and 11 post-exercise differences in muscle metabolite content. These results provide some mechanistic insight into how IL15RA ablation improves exercise performance and highlight the potential impact of genetic variability on exercise response.

El Abed et al. studied changes in markers of oxidative stress in blood following sprint interval, endurance, and combined training in trained judokas. Although the comparison between exercise protocols yielded mixed results, the data do support the potential for differential oxidative stress responses following different intensities of exercise. These results provide a mechanistic bases for the importance of considering exercise intensity as we move toward personalized exercise prescription.

Sierra et al. examined differences in hemtological and iron metabolism responses between participants with/without the ACTN3 R577X polymorphism following a marathon race. Between genotype differences were not observed for fitness or for mean changes in hematological and iron metabolism responses. The proportion of participants with large changes in iron levels and hematological parameters was smaller following the marathon in athletes with the ACTH3 XX genotype. The results presented by Sierra et al. support the existence of a small genetic component in the determination of exercise response, however, future studies with larger samples and more rigorous statistical comparisons are needed.

Müllers et al. review the impact of physical activity on the development of dementia and explore the potential for personalized exercise prescription in the context of individual variability in response to exercise. The authors discuss the factors underlying inter-individual variability in response to exercise, the potential for individual patterns of response, and methods for ensuring benefit in most individuals. They conclude that more research is needed to expand our understanding of the potential for personalized exercise prescription. It seems particularly important for future work to determine if personalized exercise prescription can enhance cognitive responses to exercise and help to reduce/delay the onset of dementia.

He et al. examine the time course of myokine response to different exercise intensities. Higher intensity exercise increased peak post-exercise FGF-21 and follistatin but had no effect on IL-15, myostatin, irisin, resistin, or omentin. These data suggest that changes in submaximal intensity have inconsistent impact on the post-exercise myokine response. Whether individual variability exists in the acute myokine response to exercise is an interesting avenue for future research.

In conclusion, the articles in this Research Topic highlight the complexity of the factors determining individual responses to exercise interventions. We believe that, through both their positive and negative findings, they contribute to our understanding of the problem. Key improvements have been made by the discovery of new potential genotype-phenotype connections, as well as molecular, morphological and even psychological predictors of the responses to training. Future research on the topic should involve larger (and, possibly, multicenter) studies with sufficient statistical power to detect predictors of the responses to exercise interventions, along with an expansion of the spectrum of potential predictors in the areas of physiology, biochemistry, molecular biology, biomechanics, and psychology through interdisciplinary collaboration.

AUTHOR'S NOTE

We hope that the readers will enjoy, and benefit from, reading this collection. We welcome your feedback.

AUTHOR CONTRIBUTIONS

All authors have written and reviewed the editorial.

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ORIGINAL RESEARCH
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Pre-training Skeletal Muscle Fiber Size and Predominant Fiber Type Best Predict Hypertrophic Responses to 6 Weeks of Resistance Training in Previously Trained Young Men

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Limited evidence exists regarding differentially expressed biomarkers between previously-trained low versus high hypertrophic responders in response to resistance training. Herein, 30 college-aged males (training age 5 ± 3 years; mean ± SD) partook in 6 weeks of high-volume resistance training. Body composition, right leg vastus lateralis (VL) biopsies, and blood were obtained prior to training (PRE) and at the 3-week (W3) and 6-week time points (W6). The 10 lowest (LOW) and 10 highest (HIGH) hypertrophic responders were clustered based upon a composite hypertrophy score of PRE-to-W6 changes in right leg VL mean muscle fiber cross-sectional area (fCSA), VL thickness assessed via ultrasound, upper right leg lean soft tissue mass assessed via dual x-ray absorptiometry (DXA), and mid-thigh circumference. Two-way ANOVAs were used to compare biomarker differences between the LOW and HIGH clusters over time, and stepwise linear regression was performed to elucidate biomarkers that explained significant variation in the composite hypertrophy score from PRE to W3, W3 to W6, and PRE to W6 in all 30 participants. PRE-to-W6 HIGH and LOW responders exhibited a composite hypertrophy change of +10.7 ± 3.2 and −2.1 ± 1.6%, respectively ($p < 0.001$). Compared to HIGH responders, LOW responders exhibited greater PRE type II fCSA (+18%, $p = 0.022$). Time effects ($p < 0.05$) existed for total RNA/mg muscle (W6 > W3 > PRE), phospho (p)-4EBP1 (PRE > W3&W6), pan-mTOR (PRE > W3 < W6), p-mTOR (PRE > W3 < W6), pan-AMPKα (PRE > W3 < W6), pan-p70s6k (PRE > W3), muscle ubiquitin-labeled proteins (PRE > W6), mechano growth factor mRNA (W6 > W3&PRE), 45S rRNA (PRE > W6), and muscle citrate synthase activity (PRE > W3&W6). No interactions existed for the aforementioned biomarkers and/or other assayed targets (muscle 20S proteasome activity, serum total testosterone, muscle androgen receptor protein levels, muscle glycogen, or serum creatine kinase).

Regression analysis indicated PRE type II fiber percentage (R^2 = 0.152, β = 0.390, p = 0.033) and PRE type II fCSA (R^2 = 0.207, β = −0.455, p = 0.019) best predicted the PRE-to-W6 change in the composite hypertrophy score. While our sample size is limited, these data suggest: (a) HIGH responders may exhibit more growth potential given that they possessed lower PRE type II fCSA values and (b) possessing a greater type II fiber percentage as a trained individual may be advantageous for hypertrophy in response to resistance training.

Keywords: high responder, resistance training, mTOR, ribosome biogenesis, proteolysis

INTRODUCTION

Low and high hypertrophic responders exist following weeks to months of structured resistance training. This interest was initially spurred by Bamman's laboratory who reported that different hypertrophic response clusters existed following 16 weeks of resistance training (Bamman et al., 2007). Bamman's laboratory (Kim et al., 2007; Petrella et al., 2008; Thalacker-Mercer et al., 2013; Stec et al., 2016), our laboratory (Mobley et al., 2018; Roberts et al., 2018b), and others (Davidsen et al., 2011; Ogasawara et al., 2016; Morton et al., 2018) have subsequently examined low and high responders following weeks to months of resistance training with the intent of deciphering biomarkers that exist between each cluster. Determining biomarkers or mechanisms which may explain the variance in the hypertrophic response to resistance training is important for a variety of reasons. First, follow-up studies can be performed in low responders in order to determine if increasing Caloric intake or various nutritional supplements (e.g., creatine or protein) improves the training response. Additionally, if low responders demonstrate an enhanced inflammatory or muscle damage profile to training, then reducing training stress or providing adjuvant therapies (e.g., anti-inflammatory strategies) may be a viable strategy to optimize training adaptations.

Various research groups have suggested that ribosome biogenesis is greater in high versus low responders following weeks to months of structured resistance training (Figueiredo et al., 2015; Stec et al., 2016; Mobley et al., 2018). There is also evidence to suggest that a greater degree of satellite cell proliferation and subsequent myonuclear accretion occurs in high versus low responders during training (Petrella et al., 2008), although contrary evidence exists when only examining college-aged males (Mobley et al., 2018). High responders may also possess an altered microRNA profile in skeletal muscle which acts to enhance insulin-like growth factor-1 (IGF-1) mRNA expression (Davidsen et al., 2011). Recent evidence also suggests that, in previously-trained college-aged men, skeletal muscle androgen receptor content is greater in high responders (Morton et al., 2018). Likewise, other studies have suggested that an up-regulation in muscle androgen receptor content is associated with skeletal muscle hypertrophy (Ahtiainen et al., 2011; Mitchell et al., 2013).

We recently published a study which subjected previously-trained college-aged males to 6 weeks of a very high-volume resistance training protocol (Haun et al., 2018b). Herein, we re-purposed said dataset to identify biomarkers related to myonuclear accretion, ribosome biogenesis, mTORc1 signaling, proteolysis, muscle damage, androgen signaling, and muscle metabolism which may have delineated the hypertrophic response to the 6-week training protocol. Unlike past reports which have examined biomarker differences between low and high hypertrophic responders that were previously untrained (Bamman et al., 2007; Davidsen et al., 2011; Ogasawara et al., 2016; Stec et al., 2016; Mobley et al., 2018), the current study is only one of two studies to make these observations in previously trained subjects (Morton et al., 2018). We hypothesized that HIGH responders would experience greater increases in myonuclear accretion as well as biomarkers related to ribosome biogenesis, androgen signaling, mTORc1 signaling, and mitochondrial biogenesis relative to LOW responders.

MATERIALS AND METHODS

Ethics Statement and Study Design

Prior to engaging in data collection, this study was approved by the Institutional Review Board at Auburn University (Protocol #17-425 MR 1710). All subjects provided verbal and written consent, and this study conformed to the standards set by the latest revision of the Declaration of Helsinki. The testing procedures, resistance training protocol, molecular analyses, and histology methods employed herein are outlined below. Readers are referred to Haun et al. (2018b) for more in-depth descriptions of supplementation and nutritional recommendations.

PRE, W3, and W6 Testing Sessions

During the prior to training (PRE), W3, and W6 testing sessions participants were instructed to arrive for testing batteries in an overnight fasted condition. At W3 and W6, testing sessions occurred 24 h following Friday workouts. Testing procedures pertinent to this dataset are described below, although other assessments were performed and described in greater detail elsewhere (Haun et al., 2018b). It should be noted that urine specific gravity assessments occurred first followed by body composition measures (DXA and ultrasound described below), and blood draws and muscle biopsies occurred last. Additionally, lower body strength testing occurred approximately 1 week prior to the PRE testing session procedures described below.

DXA and Ultrasound Assessments

At the beginning of each testing session participants submitted a urine sample (∼5 mL) to assess normal hydration specific gravity levels (1.005–1.020 ppm) using a handheld refractometer (ATAGO; Bellevue, WA, United States). Participants with a urine specific gravity ≥1.020 were asked to consume 400 ml tap water and were re-tested ∼20 min thereafter. Following hydration testing, height and body mass were assessed using a digital column scale (Seca 769; Hanover, MD, United States) with body masses and heights collected to the nearest 0.1 kg and 0.5 cm, respectively. Participants were then subjected to a full-body DXA scan (Lunar Prodigy; General Electric, Fairfield, CT, United States) while wearing general sports attire (i.e., athletic shorts or compression shorts and an athletic shirt). According to previous data published by our laboratory, the same-day reliability of the DXA during a test-calibrate-retest on 10 participants produced an intra-class correlation coefficient (ICC) of 0.998 for total body lean soft tissue mass (Kephart et al., 2016). After all DXA scans were complete, a technician (K.C.Y.) used a region of interest tool in the software to segment the upper right leg using standardized landmarks (enCORE version 15.00), and bone-free lean tissue mass (referred to as upper right leg lean soft tissue mass throughout) was generated through the Lunar software. Following DXA scans, participants were subjected to an ultrasound assessment to determine right leg mid-thigh muscle thickness with a 3 to 12 MHz multi-frequency linear phase array transducer (Logiq S7 R2 Expert; General Electric). Measurements were taken from the midway point between the iliac crest and patella of the right femur whereby participants were in a standing position and weight was placed on the left leg. Reliability for muscle thickness during a test–retest at PRE on 33 participants produced an ICC of 0.994 (Haun et al., 2018b). Notably, all DXA scans and ultrasound assessments were completed by the same investigators (M.A.R., and P.W.M., respectively) as suggested by previous research interventions (Lohman et al., 2009; Lockwood et al., 2017) in order to minimize variability in testing procedures.

Muscle Tissue and Blood Collection

Muscle biopsies were collected using a 5-gauge needle under local anesthesia as previously described (Haun et al., 2018b). Immediately following tissue procurement, ∼20–40 mg of tissue was embedded in cryomolds containing optimal cutting temperature (OCT) media (Tissue-Tek®, Sakura Finetek, Inc., Torrance, CA, United States). Embedding was performed whereby tissue was placed in cryomolds for cross-sectional slicing in a non-stretched state prior to rapid freezing. A desktop light microscope was used with a 4x objective to ensure tissue was situated appropriately within OCT cryomolds prior to freezing, and fine needles were used to make adjustments if needed. Cryomolds were then frozen using liquid nitrogen-cooled isopentane and subsequently stored at −80°C until histological analyses occurred. The remaining tissue was teased of blood and connective tissue, wrapped in pre-labeled foils, flash frozen in liquid nitrogen, and subsequently stored at −80°C until molecular analyses occurred. Venous blood samples were also collected into a 5 mL serum separator tube (BD Vacutainer, Franklin Lakes, NJ, United States) during the waiting period for local anesthesia to take effect. Creatine kinase (CK) activity, cortisol, and total testosterone were elected as serum targets of interest and more detailed methods of these assays are described below.

Resistance Training Protocol

Participants were familiarized with the design of training and technical parameters during testing of 3RMs which occurred 3–7 days prior to PRE testing and training initiation. Strict technical parameters were employed for testing to ensure accurate reflections of strength under direct supervision of research staff holding the Certified Strength and Conditioning Specialist Certification from the National Strength and Conditioning Association.

Following 3RM testing and the PRE testing battery, resistance training occurred 3 days per week. Loads corresponding to 60% 1RM, based on three repetition maximum (3RM) testing, were programmed for each set of each exercise. Sets of 10 repetitions were programmed for each set of each exercise throughout the study. Exercises were completed one set at a time, in the following order during each training session: days 1 and 3 each week – barbell (BB) back squat, BB bench press, BB stiff-legged deadlift (SLDL), and an underhand grip cable machine pulldown exercise designed to target the elbow flexors and latissimus dorsi muscles (lat pulldown); day 2 of each week – BB back squat, BB overhead press, BB SLDL, and lat pulldown. The 3 day per week protocol involved a progressive increase from 10 sets per week to 32 sets per week for each exercise. Thus, on the last week of training participants performed 32 sets of 10 repetitions of BB back squats, 32 sets of 10 repetitions of BB bench press and OH press combined, 32 sets of 10 repetitions of BB SLDL, and 32 sets of 10 repetitions of lat pulldowns. Readers are referred to Haun et al. (2018b) for more in-depth descriptions of training.

Muscle Tissue Processing

For protein and RNA analyses tissue foils were removed from −80°C and crushed using a liquid nitrogen-cooled mortar and pestle. For protein analysis, ∼30 mg of powdered tissue was placed in 1.7 mL microcentrifuge tubes containing 500 μL of ice-cold cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton; Cell Signaling, Danvers, MA, United States] pre-stocked with protease and Tyr/Ser/Thr phosphatase inhibitors (2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin). Samples were then homogenized by hand using tight micropestles, insoluble proteins were removed with centrifugation at 500 *g* for 5 min, and obtained sample lysates were stored at −80°C prior to Western blotting and other biochemical assays (described below).

For total RNA analysis, ∼15–30 mg of powdered tissue was weighed using an analytical scale with a sensitivity of 0.001 g (Mettler-Toledo; Columbus, OH, United States). Tissue was then homogenized in 1.7 mL microcentrifuge tubes containing 500 μL of Ribozol (Ameresco; Solon, OH, United States) via micropestle manipulation and RNA isolation was performed per manufacturer recommendations. Total RNA concentrations were then determined in duplicate using a NanoDrop Lite

spectrophotometer (Thermo Fisher Scientific; Waltham, MA, United States), and total RNA per unit muscle weight was used as a surrogate for ribosome density as in past publications (Nader et al., 2005; Mobley et al., 2016).

Immunohistochemistry for fCSA and Myonuclear Number Determination

Methods for immunohistochemistry have been employed previously in our laboratory and described elsewhere (Hyatt et al., 2015; Martin et al., 2016; Mobley et al., 2017a). Briefly, sections from OCT−preserved samples were cut at a thickness of 8 μm using a cryotome (Leica Biosystems; Buffalo Grove, IL, United States) and were adhered to positively-charged histology slides. Once all samples were sectioned, batch processing occurred for immunohistochemistry. During batch processing sections were air-dried at room temperature for up to 10 min, permeabilized in a phosphate-buffered saline (PBS) solution containing 0.5% Triton X-100, and blocked with 100% Pierce Super Blocker (Thermo Fisher Scientific) for 25 min. Sections were then incubated for 20 min with a pre-diluted commercially-available rabbit anti-dystrophin IgG antibody solution (catalog #: GTX15277; Genetex, Inc., Irvine, CA, United States) and spiked in mouse anti-myosin I IgG (catalog #: A4.951 supernatant; Hybridoma Bank, Iowa City, IA, United States; 40 μL added per 1 mL of dystrophin antibody solution). Sections were then washed for 2 min in PBS and incubated in the dark for 20 min with a secondary antibody solution containing Texas Red-conjugated anti-rabbit IgG (catalog #: TI-1000; Vector Laboratories, Burlingame, CA, United States), and Alexa Fluor 488-conjugated anti-mouse IgG (catalog #: A-11001; Thermo Fisher Scientific) (∼6.6 μL of all secondary antibodies per 1 mL of blocking solution). Sections were washed for 5 min in PBS, air-dried, and mounted with fluorescent media containing 4,6-diamidino-2-phenylindole (DAPI; catalog #: GTX16206; Genetex, Inc.). Following mounting, slides were stored in the dark at 4°C until digital immunofluorescent images were obtained.

After staining was performed on all sections, digital images were captured using a fluorescent microscope (Nikon Instruments, Melville, NY, United States) at a 10x objective. Approximate exposure times were 600 ms for TRITC and FITC imaging and 80 ms for DAPI imaging. This staining method allowed the identification of cell membranes (detected by the Texas Red filter), type I fiber green cell bodies (detected by the FITC filter), type II fiber black cell bodies (unlabeled), and myonuclei (detected by the DAPI filter). Standardized measurements of types I and II fiber cross-sectional areas (fCSAs) were performed using the open-sourced software CellProfiler^{TM} (Carpenter et al., 2006) per modified methods previously described whereby the number of pixels counted within the border of each muscle fiber were converted to a total area (μm^2) (Mobley et al., 2017a). A calibrator slide containing a 250,000 μm^2 square image was also captured, and pixels per fiber from imaged sections were converted to area using this calibrator image. On average, 113 ± 26 fibers per cross-section were identified for analysis at each sampling time. A *post hoc* experiment performed in our laboratory to examine potential differences in fCSA measurements between sections on the same slide (n = 27 slides) revealed strong reliability using this method (ICC = 0.929). Measurements of fiber type-specific myonuclear number were also performed using a custom script in CellProfiler^{TM} which discriminates the fiber border that corresponded to each myonucleus (Mobley et al., 2017a).

Western Blotting

Whole-tissue sample lysates obtained through cell lysis buffer processing (described above) were batch process-assayed for total protein content using a BCA Protein Assay Kit (Thermo Fisher Scientific). Lysates were then prepared for Western blotting using 4x Laemmli buffer at 1 μg/μL. Following sample preparation, 18 μL samples were loaded onto 4–15% SDS-polyacrylamide gels (Bio-Rad; Hercules, CA, United States) and subjected to electrophoresis (180 V for 45–60 min) using pre-made 1x SDS-PAGE running buffer (Ameresco; Framingham, MA, United States). Proteins were then transferred (200 mA for 2 h) to polyvinylidene difluoride membranes (Bio-Rad), Ponceau stained and imaged to ensure equal protein loading between lanes. Membranes were then blocked for 1 h at room temperature with 5% non-fat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST; Ameresco). Rabbit anti-human phospho-p70s6k (Thr389) (1:1,000; catalog #: 9234; Cell Signaling), rabbit anti-human pan p70s6k (1:1,000; catalog #: 2708; Cell Signaling), rabbit anti-human phospho-4EBP1 (Thr37/46) (1:1,000; catalog #: 2855; Cell Signaling), rabbit anti-human pan 4EBP1 (1:1,000; catalog #: 9644; Cell Signaling), rabbit anti-human phospho-mTOR (Ser2448) (1:1,000; catalog #: 2971; Cell Signaling), rabbit anti-human pan mTOR (1:1,000; catalog #: 2972; Cell Signaling), rabbit anti-human phospho-AMPKα (Thr172) (1:1,000; catalog #: 2535; Cell Signaling), rabbit anti-human pan AMPKα (1:1,000; catalog #: 2532; Cell Signaling), rabbit anti-human androgen receptor (1:1,000; catalog #: 5153; Cell Signaling) and rabbit anti-human ubiquitin (1:1,000; catalog #: 3933; Cell Signaling) were incubated with membranes overnight at 4°C in TBST with 5% bovine serum albumin (BSA). The following day, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit (catalog #: 7074; Cell Signaling) in TBST with 5% BSA at room temperature for 1 h (1:2,000). Membrane development was performed using an enhanced chemiluminescent reagent (Luminata Forte HRP substrate; EMD Millipore, Billerica, MA, United States), and band densitometry was performed using a gel documentation system and associated densitometry software (UVP, Upland, CA, United States). Densitometry values for all targets were divided by whole-lane Ponceau densities. All Western blotting data are expressed as relative expression units (REUs).

Real-Time PCR

Two μg of RNA was reverse transcribed into cDNA for RT-PCR analysis with cDNA synthesis reagents (Quanta Biosciences, Gaithersburg, MD, United States) per the manufacturer's recommendations. RT-PCR was performed using gene-specific primers and SYBR green chemistry (Quanta Biosciences). Primer sequences used were as follows: 45S pre-rRNA forward

primer 5′-GAACGGTGGTGTGTCGTT-3′, reverse primer 5′-GCGTCTCGTCTCGTCTCACT-3′; mechano growth factor (MGF): forward primer 5′-CGAAGTCTCAGAGAAGGAAA GG-3′, reverse primer 5′-ACA GGTAACTCGTGCAGAGC-3′; myostatin (MSTN): forward primer 5′-GACCAGGAGAAG ATGGGCTGAATCCGTT-3′, reverse primer 5′-CTCATCACAG TCAAGACCAAAATCCCTT-3′; beta-2-microglobulin (B2M, housekeeping gene 1): forward primer 5′-ATGAGTATGC CTGCCGTGTGA-3′ reverse primer 5′-GGCATCTTCAAACC TCCATG-3′; cyclophilin (PPIA, housekeeping gene 2): forward primer 5′-CGATGTCTCAGAGCACGAAA-3′, reverse primer 5′-CCCACCTGTTTCTTCGACAT-3′. PCR calculations were performed as previously described by our laboratory (Haun et al., 2018a). Briefly, $2^{-\Delta Cq}$ values for each gene of interest at each time point were calculated whereby ΔCq = gene of interest Cq – geometric mean housekeeping gene Cq values. All W3 and W6 values for a given mRNA target were then normalized to PRE values within subject, and PCR data were expressed as fold-change scores. Prior melt curve analyses from our laboratory confirmed that only one RT-PCR product was obtained with the primer sets being used.

Citrate Synthase Activity Assay

Whole-tissue sample lysates obtained through cell lysis buffer processing (described above) were batch processed for citrate synthase activity as previously described (Roberts et al., 2018b), and this metric was used as a surrogate for mitochondrial content per the findings of Larsen et al. (2012) suggesting citrate synthase activity exhibits a strong correlation with electron micrograph images of mitochondrial content (r = 0.84, $p < 0.001$). The assay utilized is based on the reduction of 5,50-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 nm (extinction coefficient: 13.6 mmol/L/cm) coupled to the reduction of acetyl-CoA by the citrate synthase reaction in the presence of oxaloacetate. Briefly, 12.5 μg of skeletal muscle protein were added to a mixture composed of 0.125 mol/L Tris-HCl (pH 8.0), 0.03 mmol/L acetyl-CoA, and 0.1 mmol/L DTNB. The reaction was initiated by the addition of 5 μL of 50 mmol/L oxaloacetate and the absorbance change was recorded for 1 min. The average coefficient of variation for all duplicates was ∼8%.

20S Proteasome Activity Assay

Forty μg of skeletal muscle protein from whole-tissue sample lysates obtained through cell lysis buffer processing (described above) were batch processed for 20S proteasome activity as previously described using commercially available fluorometric kits (catalog #: APT280; Millipore Sigma; Burlington, MA, United States) per the manufacturer's instructions which are similar to methods previously published by our laboratory (Mobley et al., 2017b). Assay readings are presented as relative fluorometric units (RFUs). The average coefficient of variation for all duplicates was 8.7%.

Glycogen Assay

Whole-tissue sample lysates were batch processed for glycogen determination using commercially-available fluorometric kits (catalog #: MAK016; Millipore Sigma) per the manufacturer's instructions. This assay was piloted with whole tissue lysates, frozen wet tissue, and lyophilized tissue, and all three methods yielded similar results. Thus, given that whole tissue lysates were available for most participants, we opted to assay this tissue fraction. A standard curve was used to determine glycogen content of whole-tissue sample lysates, this value was multiplied by the starting cell lysis buffer volume (500 μL) to derive total glycogen content per sample, and the resultant value was divided by input wet muscle weights to obtain nmol glycogen/mg wet muscle weight. The average coefficient of variation for all duplicates was 9.8%.

Serum Assays

Upon blood collection, serum tubes were centrifuged at 3,500 *g* for 5 min at room temperature. Aliquots were then placed in 1.7 mL polypropylene tubes and stored at −80°C until batch-processing. An activity assay was used to determine serum levels of CK (Bioo Scientific; Austin, TX, United States). In cases where samples were missing or where the standard curve indicated that blood levels were negative, values were not considered in the analysis. Commercially-available ELISA kits (ALPCO Diagnostics; Salem, NH, United States) were used to assay serum total testosterone and cortisol. All kits were performed according to manufacturer's instructions and plates were read using a 96-well spectrophotometer (BioTek, Winooski, VT, United States). The average coefficient of variation of duplicate values for each target were as follows: serum testosterone = 6.9%, serum cortisol = 2.8%, and serum CK activity = 1.4%.

Statistical Analysis and Responder Clustering

Statistical tests were performed in RStudio (Version 1.0.143) and SPSS (Version 25). Regarding HIGH and LOW hypertrophic cluster analysis we adopted methods similar to Davidsen et al. (2011), as well a recent publication from our laboratory in untrained participants (Roberts et al., 2018b), wherein the 10 lowest (LOW) and 10 highest (HIGH) hypertrophic responders were clustered based upon a composite hypertrophy score of PRE-to-W6 percent changes in:

(a) right leg VL muscle thickness assessed via ultrasound
(b) upper right leg lean soft tissue assessed via DXA
(c) right leg mid-thigh circumference
(d) right leg VL mean (type I and type II) muscle fCSA.

Our rationale for using multiple indices to delineate LOW and HIGH responders is due to previous data from our laboratory demonstrating that defining clusters based upon VL thickness alone did not yield between-cluster differences in 3RM back squat strength changes following 12 weeks of resistance training (Mobley et al., 2018). However, using a combination of DXA-based, histology-based, and ultrasound-based hypertrophic indices to cluster LOW and HIGH responders in this same subject pool yielded between-cluster differences in this strength metric [HIGH pre-to-post delta (Δ)3RM squat = 42 ± 3 kg, LOW Δ3RM squat = 31 ± 9 kg, respectively; p = 0.005]

(Roberts et al., 2018b). Moreover, we sought to cluster in an unbiased manner toward any hypertrophic assessment and felt equally weighting each variable in forming the composite score by using percent change was appropriate. Further, since many biomarkers were derived from the biopsy sample of the VL, and fCSA typically produces greater percent changes than other levels of hypertrophic assessment, allowing fCSA percent changes to leverage the composite score served as additional rationale. **Figure 1** below demonstrates the percent change score for the composite hypertrophy variable as well as absolute values for a–d above between clusters.

All dependent variable comparisons over time were analyzed between LOW and HIGH clusters using 2 × 3 (cluster [LOW, HIGH] × time [PRE, W3, W6]) mixed factorial repeated measures ANOVAs with the exception of histology data and self-reported food log data which were analyzed using 2 × 2 (cluster [LOW, HIGH] × time [PRE, W6]) models. If a significant cluster × time interaction was observed, LSD *post hoc* tests were performed within each cluster and between clusters at each time point. Significance was established at $p < 0.05$.

Aside from comparing the 10 HIGH and 10 LOW responders, stepwise linear regression was also performed to predict the PRE to week 3 (W3), W3 to W6, and PRE to W6 composite hypertrophy score responses in all 30 participants using PRE biomarker data as well as percent changes in biomarkers from PRE to W3, W3 to W6 and PRE to W6. For regression analysis, the following variables at PRE were measured and considered for inclusion in the overall analysis as predictors: (a) PRE type I fiber percentage, (b) PRE type II fiber percentage, (c) PRE type I fCSA, (d) PRE type II fCSA, (e) PRE type I fiber myonuclear number, (f) PRE type II fiber myonuclear number, (g) PRE muscle 20S proteasome activity, (h) PRE muscle glycogen, (i) PRE citrate synthase activity, (j) PRE serum creatine kinase activity, (k) PRE serum testosterone, (l) PRE serum cortisol, (m) PRE muscle pan 4EBP1, (n) PRE muscle p-4EBP1, (o) PRE muscle pan mTOR, (p) PRE muscle p-mTOR, (q) PRE muscle pan AMPK, (r) PRE muscle p-AMPK, (s) PRE muscle pan p70s6k, (t) PRE muscle p-p70s6k, (u) PRE

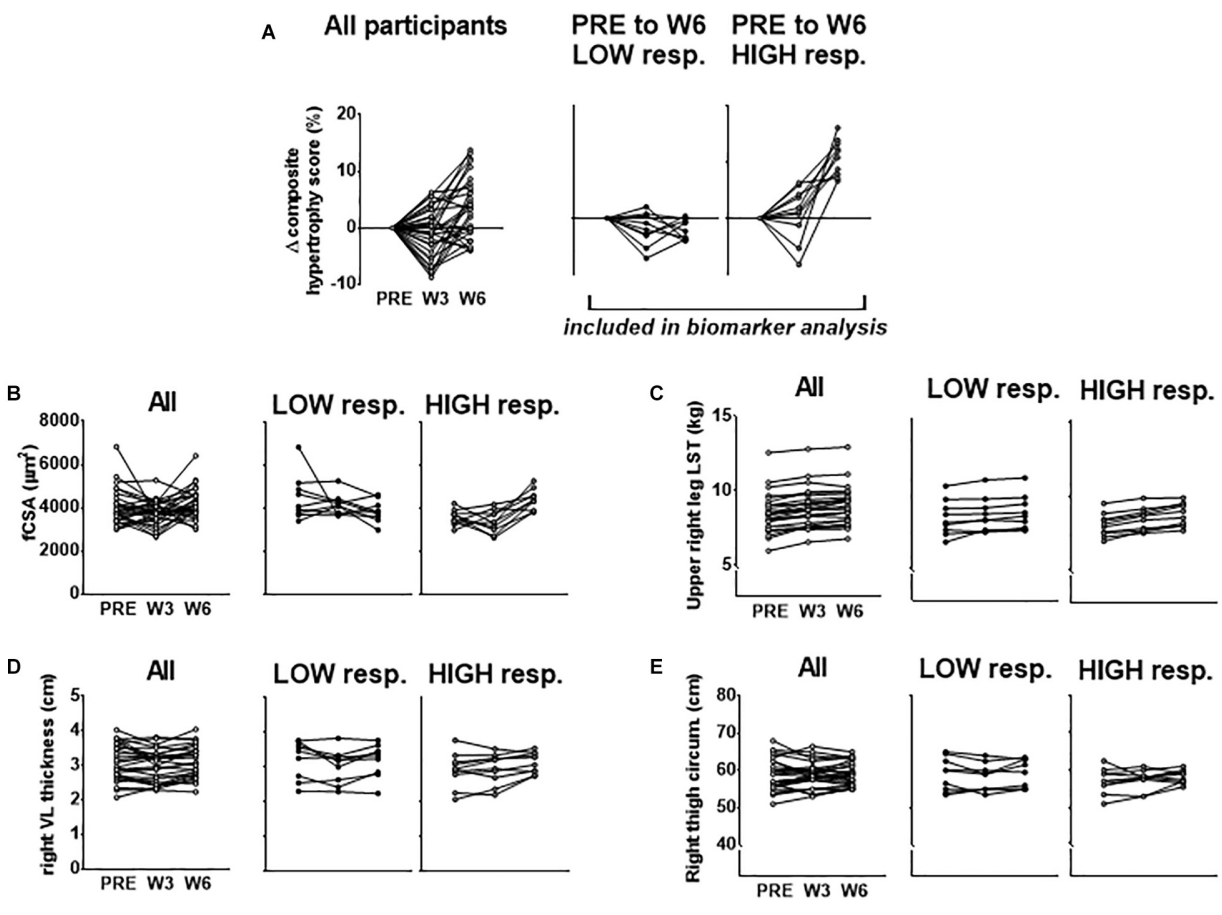

FIGURE 1 | PRE to W6 percent changes in hypertrophic indices used to cluster LOW and HIGH responders. **(A)** PRE to W3 and PRE to W6 changes in the composite hypertrophy score in all participants as well as LOW (n = 10) and HIGH (n = 10) responders. Response clusters were ranked according to the PRE to W6 composite score which was made up of percent changes in mean fiber cross sectional area (fCSA), upper right leg DXA lean soft tissue (LST), right leg vastus lateralis (VL) thickness, and right thigh circumference. Raw values for each of these composite score variables for all subjects as well as response clusters are presented in **(B–E)**.

muscle pan poly Ub, (v) PRE muscle RNA, (w) PRE muscle MGF mRNA, (x) PRE muscle 45S rRNA, and (y) PRE muscle MSTN mRNA. Percent change scores were also calculated for the predictors above, except for fiber percentages and fCSA to avoid multicollinearity, and were considered for inclusion in models explaining variation in the hypertrophic response from PRE to W3 (PRE-W3), W3 to W6 (W3–W6), and PRE to W6 (PRE-W6). Percent change variables are named below as time point to time point percent change (e.g., PRE-W3 muscle p-mTOR percent change). Critically, although baseline predictors were based on raw values, percent change values were calculated for all biomarker predictors for consistent, standardized inference (e.g., percent change in x relative to percent change in y). In order to construct more meaningful models and due to statistical power, bivariate correlations were completed prior to stepwise regression for each predictor and the dependent variable of the model to eliminate predictors weakly correlated with the dependent variable at each level of time ($r < 0.3$). Upon identification of predictors correlating with the dependent variable beyond $r = 0.3$ and satisfaction of assumptions tests, analysis proceeded. Measurement of each predictor variable occurred for each subject at each time point (PRE, W3, and W6), except for AR protein content where only HIGH and LOW clusters were examined as a *post hoc* analysis (further described below) or in the case of a lack of sample. Power analyses in RStudio using general linear model parameters in the "pwr" package (Version 1.2–1) revealed $\geq$ 80% power (power = $1 - \beta$) for the discovery of large effects when models included 30 subjects and up to 3 predictor variables. However, analyses were underpowered to identify small and medium effects (<80%). Therefore, we intended to construct parsimonious models with fewer predictors to identify large effects and avoid commission of type 1 or 2 errors. Additionally, effort was made to include all datum in the analysis that were biologically plausible and outlier detection was only considered if datum exceeded 3 standard deviations from the mean of each individual predictor. However, if datum exceeded 3 standard deviations from the mean but were considered biologically possible, analysis proceeded. To assist in the identification of potential outliers and to further protect against erroneous conclusions, assumptions tests and regression diagnostics were also performed. These included: (a) Shapiro–Wilk tests for normality of residual distributions, (b) Levene's tests of homogeneity of variance between levels of time for each predictor, (c) homogeneity of regression slopes, and (d) multicollinearity assessments. If Levene's test was violated, a Greenhouse–Geisser adjustment to degrees of freedom was made for more conservative *p*-value inference. Homogeneity of regression slopes were evaluated by examining predictor variable's individual relationship with the dependent variable in order to build more meaningful models. This assessment was to ensure predictors in models had consistently positive or negative relationships with the composite hypertrophy score which was verified through bivariate correlations. Multicollinearity was assessed through examining which predictors were correlated and models were also inspected via variance inflation factor scores (VIF < 10). If predictor variables were strongly correlated ($r > 0.5$) or VIF scores exceeded 10, predictors were eliminated, or separate models were constructed to examine the explained variation by individual predictors. For clarity, only the strongest predictors from the overall stepwise regression analysis are discussed in the results section below.

The sample size for the overall stepwise regression analysis was 30 subjects, while specific sample sizes for each analysis and cluster analysis are reported in figures or text where appropriate. All data for this study are provided in **Supplementary File S1**.

RESULTS

Baseline Characteristics and Training Volume Differences Between Clusters

Pre-training cluster differences in age, self-reported resistance training age, body mass, body composition, fiber type, pre-training three repetition back squat strength, and back squat training volume differences throughout the intervention are presented in **Table 1**. Notably, there were no significant differences between clusters regarding these variables ($p > 0.05$).

Self-Reported Macronutrient Intakes Between Clusters

PRE and W6 energy and absolute as well as relative (per kg) macronutrient intake differences between clusters are presented

TABLE 1 | Baseline characteristics at PRE and back squat training volume between clusters.

Variable	LOW (*n* = 10)	HIGH (*n* = 10)	*p*-value
Age (years)	22 ± 1	21 ± 2	0.376
Training age (years)	6 ± 2	6 ± 2	1.000
Body mass (kg)	83.1 ± 12.8	78.8 ± 8.0	0.381
DXA LST (kg)	65.1 ± 9.7	62.2 ± 5.9	0.430
DXA FM (kg)	14.5 ± 4.9	13.5 ± 4.9	0.649
Type II fiber (%)	50 ± 14	59 ± 17	0.189
3RM back squat (kg)	135 ± 14	127 ± 23	0.342
Total back squat training volume (kg) from weeks 1 to 6	111,821 ± 12,962	106,610 ± 18,679	0.478
Number of participants in different nutritional groups from Haun et al. (2018a)			
GWP	5	3	Chi-square *p*-value
MALTO	1	3	
WP	4	4	0.199

These data demonstrate that pre-training age, training age, body composition, muscle fiber type, and three repetition maximum (3RM) back squat strength, and back squat training volume during the 6-week intervention did not differ between LOW and HIGH responders. Moreover, the LOW and HIGH responders were not influenced by different nutritional interventions reported in the study by Haun et al. (2018b). All data are presented as mean ± standard deviation values. DXA LST, lean soft tissue assessed using dual x-ray absorptiometry; DXA FM, fat mass assessed using dual x-ray absorptiometry. MALTO, participants that supplemented once daily with 30 g of maltodextrin; WP, participants that supplemented once daily with 25 g of whey protein concentrate; GWP, participants that supplemented 25 g/d WP during week 1, 50 g/d during week 2, 75 g/d during week 3, 100 g/d during week 4, 125 g/d during week 5, and 150 g/d during week 6.

in **Table 2**. There were no significant cluster effects, time effects, or cluster × time interactions for these variables.

Cluster Differences in Types I and II fCSA and Myonuclear Number

Significant cluster × time interactions were observed for type I fCSA ($p < 0.001$; **Figure 2A**) and type II fCSA ($p = 0.001$; **Figure 2D**). No PRE differences existed between clusters for type I fCSA values, although type II fCSA values were greater at PRE and W3 in LOW versus HIGH responders ($p < 0.05$). Percent changes in types I and II fCSA from PRE to W6 were significantly different between clusters ($p < 0.001$). Type I fCSA decreased by −15.62 ± 13.66% and type II fCSA by −7.44 ± 11.47% in LOW responders whereas type I fCSA increased by 20.25 ± 18.27% and type II fCSA by 21.96 ± 20.17% in HIGH responders. No significant time effects, cluster effects, or cluster × time interactions existed for type I or type II fiber myonuclear number (**Figures 2B,E**). A significant time effect existed for type I myonuclear domain size (PRE > W6, $p = 0.045$; **Figure 2C**), but no cluster effect or interaction existed. No significant time effect, cluster effect, or interaction existed for type II fiber myonuclear domain size (**Figure 2F**). Representative images from a LOW and HIGH responder are presented in **Figure 2G**.

TABLE 2 | Self-reported macronutrient intakes between clusters at PRE and week 6.

Variable Group	PRE mean ± SD	W6 mean ± SD	Statistics
Energy intake (kcal/d)			
LOW	2,874 ± 499	2,459 ± 1,015	Cluster $p = 0.454$
HIGH	3,067 ± 186	2,699 ± 524	Time $p = 0.096$
			C × T $p = 0.918$
Energy intake (kcal/kg/d)			
LOW	34.1 ± 6.0	28.7 ± 11.2	Cluster $p = 0.122$
HIGH	39.2 ± 3.0	33.5 ± 6.2	Time $p = 0.064$
			C × T $p = 0.963$
Protein intake (g/d)			
LOW	177 ± 29	186 ± 100	Cluster $p = 0.511$
HIGH	194 ± 38	204 ± 41	Time $p = 0.641$
			C × T $p = 0.972$
Protein intake (g/kg/d)			
LOW	2.1 ± 0.2	2.2 ± 1.1	Cluster $p = 0.187$
HIGH	2.5 ± 0.8	2.6 ± 0.6	Time $p = 0.824$
			C × T $p = 0.975$
Carbohydrate intake (g/d)			
LOW	263 ± 95	223 ± 93	Cluster $p = 0.347$
			Time $p = 0.230$
HIGH	290 ± 30	259 ± 70	C × T $p = 0.870$
Carbohydrate intake (g/kg/d)			
LOW	3.2 ± 1.2	2.6 ± 1.0	Cluster $p = 0.130$
HIGH	3.7 ± 0.3	3.2 ± 0.8	Time $p = 0.172$
			C × T $p = 0.913$
Fat intake (g/d)			
LOW	126 ± 27	98 ± 39	Cluster $p = 0.976$
HIGH	124 ± 30	101 ± 25	Time $p = 0.013$ (W1 > W6)
			C × T $p = 0.756$
Fat intake (g/kg/d)			
LOW	1.5 ± 0.3	1.2 ± 0.5	Cluster $p = 0.576$
HIGH	1.3 ± 0.6	1.1 ± 0.5	Time $p = 0.012$ (W1 > W6)
			C × T $p = 0.704$

These data demonstrate that PRE and week 6 (W6) self-reported energy and macronutrient intakes were similar between clusters. Notably, only n = 6 HIGH and n = 9 LOW were included in the analysis given that the other participants per cluster did not regularly self-report food intakes; refer to Haun et al. (2018a) for a more in-depth description of dietary analysis and subject removal due to non-compliant reporting. All data are presented as mean ± standard deviation values. Additionally, W6 data contains added supplemental macronutrients. C × T, cluster by time interaction.

Cluster Differences in Ribosome Biogenesis Markers

A significant time effect existed for increases in total RNA density (i.e., ribosome density) (W3&6 > PRE, $p < 0.001$), but no significant cluster effect or cluster × time interaction existed (**Figure 3A**). A significant time effect also existed for decreases in 45S rRNA fold-change scores (W6 < PRE, $p = 0.008$), but no significant cluster effect or cluster × time interaction existed (**Figure 3B**).

Cluster Differences in mTORc1 Signaling Markers

There were no significant cluster effects or cluster × time interactions for p-mTOR (Ser2448) (**Figure 4A**), p-p70s6k (Thr389) (**Figure 4B**), p-4EBP1 (Thr37/46) (**Figure 4C**), or p-AMPKα (Thr172) (**Figure 4D**). p-4EBP1 exhibited a significant time effect (PRE > W3&W6, $p < 0.05$ at each comparison) as did p-mTOR (PRE > W6 > W3, $p < 0.05$ at each comparison). There were no significant cluster effects or cluster × time interactions for pan mTOR (**Figure 4E**), pan p70s6k (**Figure 4F**), pan 4EBP1 (**Figure 4G**), or pan AMPKα (**Figure 4H**). However, there were significant time effects whereby pan mTOR exhibited a decrease at W3 relative to PRE and an increase from W3 to W6 ($p < 0.05$ at each comparison), pan p70s6k exhibited a decrease at W3 and W6 relative to PRE ($p < 0.05$ at each comparison), and pan AMPKα exhibited a decrease at W3 relative to PRE and an increase from W3 to W6 ($p < 0.05$ at each comparison). Representative Western blots for these targets are presented in **Figure 4I**.

Cluster Differences in Biomarkers Related to Muscle Damage and Proteolysis

There were no significant cluster effects or cluster × time interactions for serum CK activity (**Figure 5A**), muscle 20S proteasome activity (**Figure 5B**), or muscle ubiquitin-labeled protein levels (**Figure 5C**). A significant time effect was observed for muscle ubiquitin-labeled protein levels where levels were lower at W6 than PRE ($p = 0.044$).

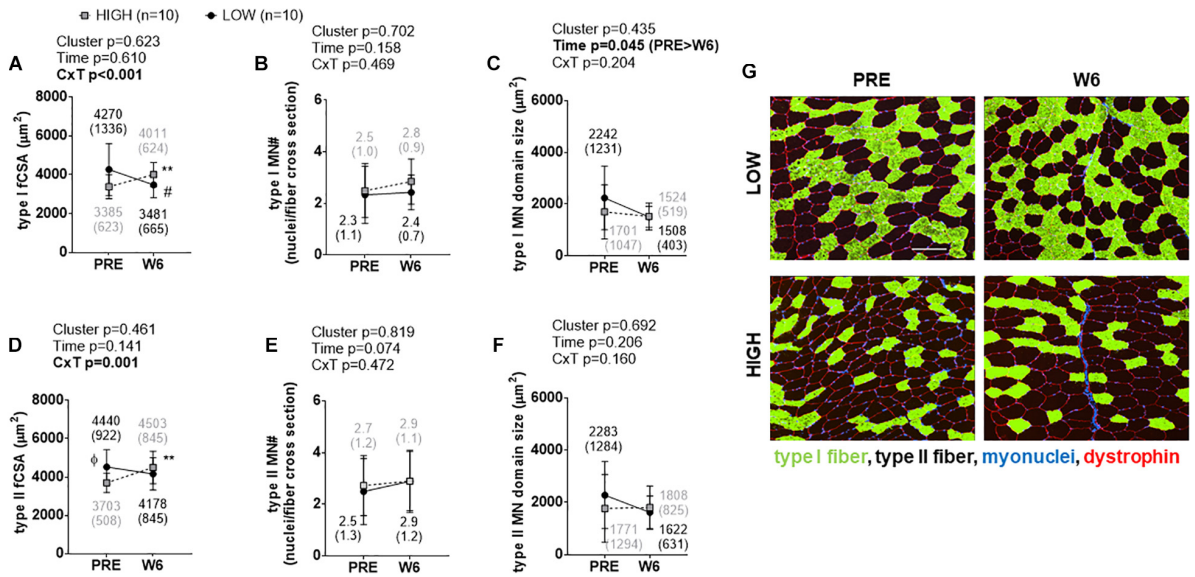

FIGURE 2 | PRE to W6 changes in type I and type II fCSA and myonuclear number as well as myonuclear domain size between LOW and HIGH responders. **(A)** Type I muscle fCSA in LOW versus HIGH responders; values decreased in LOW responders from PRE to W6 ($^{\#}p = 0.019$) and increased in HIGH responders from PRE to W6 ($^{**}p = 0.003$). **(B)** No significant time effect, cluster effect, or cluster × time (C × T) interaction existed for type I fiber myonuclear number (MN#). **(C)** A significant time effect, but not cluster effect or C × T interaction existed for type I fiber myonuclear domain size. **(D)** Type II muscle fCSA was 18% greater at PRE in LOW versus HIGH responders (Φ, $p = 0.022$), but values only increased in HIGH responders from PRE to W6 ($^{**}p = 0.005$). **(E)** No main effects or interaction existed for PRE to W6 changes in type II fiber MN#. **(F)** No significant main effects or C × T interaction existed for type II fiber myonuclear domain size. Values presented in line graphs are mean (standard deviation) values. **(G)** Representative 10x objective images from a low and high responder (white scale bar = 200 μm).

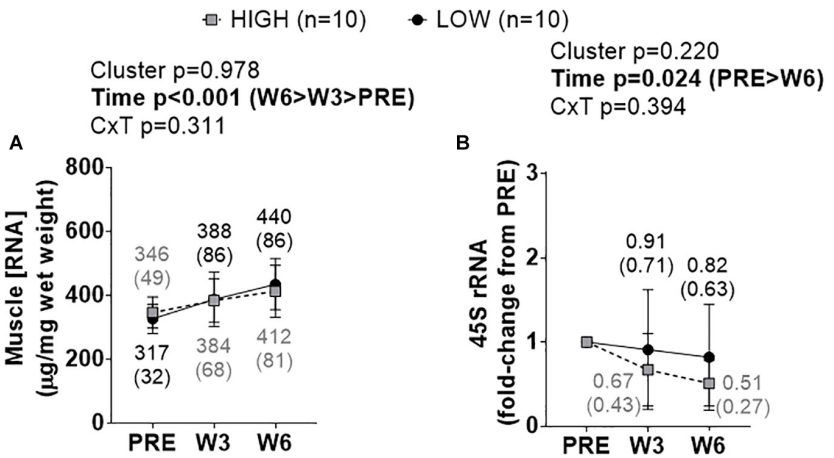

FIGURE 3 | PRE, W3, and W6 ribosome biogenesis marker differences between LOW and HIGH responders. **(A)** A significant time effect existed for increases in total RNA density (i.e., ribosome density). **(B)** A significant time effect existed for decreases in 45S rRNA fold-change scores. Values presented in line graphs are mean (standard deviation) values.

Representative Western blots for ubiquitin-labeled proteins are presented in **Figure 5D**.

Cluster Differences in MGF and MSTN mRNA Levels

There were no significant cluster effects or cluster × time interactions for fold-change scores in muscle MGF mRNA (**Figure 6A**) or MSTN mRNA (**Figure 6B**). There was a significant time effect for MGF mRNA whereby W6 values were greater than PRE ($p = 0.020$).

Cluster Differences in Androgen Signaling Markers

There were no significant cluster effects, time effects or cluster × time interactions for serum total testosterone levels (**Figure 7A**) or muscle AR protein levels (**Figure 7B**). Representative Western blots for AR are presented in **Figure 7C**.

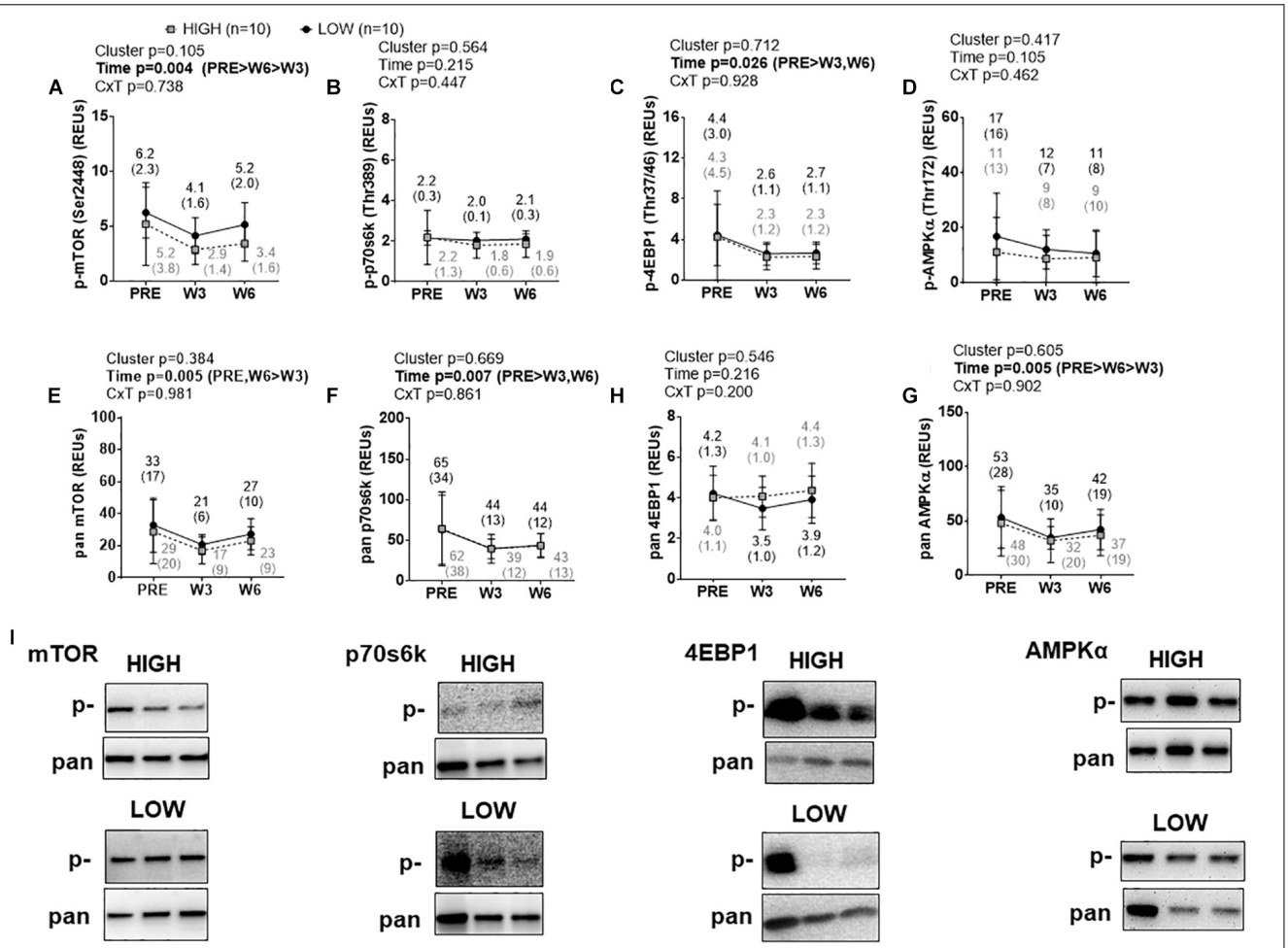

FIGURE 4 | PRE, W3, and W6 mTORc1 signaling marker differences between LOW and HIGH responders. **(A–D)** Phosphorylated (p-) mTORc1 targets. There were no significant cluster effects or cluster × time interactions for said markers. p-mTOR and p-4EBP1 demonstrated significant time effects. **(E–H)** Total (pan) mTOR, p70s6k, 4EBP1, and AMPKα. Again, there were no significant cluster effects or cluster × time interactions for said markers, although there were significant time effects for pan mTOR, pan p70s6k, and pan AMPKα. Values presented in line graphs are mean (standard deviation) values. **(I)** Representative Western blot images for a low and high responder.

Cluster Differences in Skeletal Muscle Glycogen and Citrate Synthase Activity Levels

There was no significant cluster effect, time effect, or cluster × time interaction for muscle glycogen levels (**Figure 8A**). Likewise, there was no significant cluster effect or cluster × time interaction for muscle citrate synthase activity levels (**Figure 8B**), although there was a significant time effect whereby W3 values and W6 values were lower than PRE ($p < 0.05$ at each comparison).

Stepwise Linear Regression to Establish Predictors of Hypertrophy

As stated prior, our second layer of analysis included performing stepwise linear regression in order to ascertain significant predictors of hypertrophy (i.e., the prediction variable in each model was the composite mean percent change score of the four clustering variables displayed in **Figure 1**). Notably, the strongest predictors are reported for each level of time with individual associated R^2 values, standardized beta coefficients, and p-values as (R^2 = β = p =). For the overall analysis (n = 30), PRE to W3 variables that qualified for the model included PRE type II fiber percentage (r = 0.488), PRE type I fiber percentage (r = −0.488), PRE type II fCSA (r = −0.480), PRE type I fCSA (r = −0.462), PRE ubiquitin-labeled protein levels (r = 0.330), PRE-W3 type I fiber myonuclear number percent change (r = 0.381), and PRE-W3 muscle pan AMPKα percent change (r = −0.345). The strongest positive predictors from PRE-W3 were: (a) PRE type II fiber percentage (R^2 = 0.238, β = 0.488, p = 0.006) and (b) PRE-W3 type I fiber myonuclear number percent change (R^2 = 0.144, β = 0.379, p = 0.018). The strongest negative predictors from PRE-W3 were: (a) PRE type I fiber percentage (R^2 = 0.238, β = −0.488, p = 0.006), (b) PRE type II fCSA (R^2 = 0.241, β = −0.491, p = 0.001), and (c) PRE-W3 muscle pan AMPK percent change (R^2 = 0.102, β = −0.332, p = 0.019). W3 to

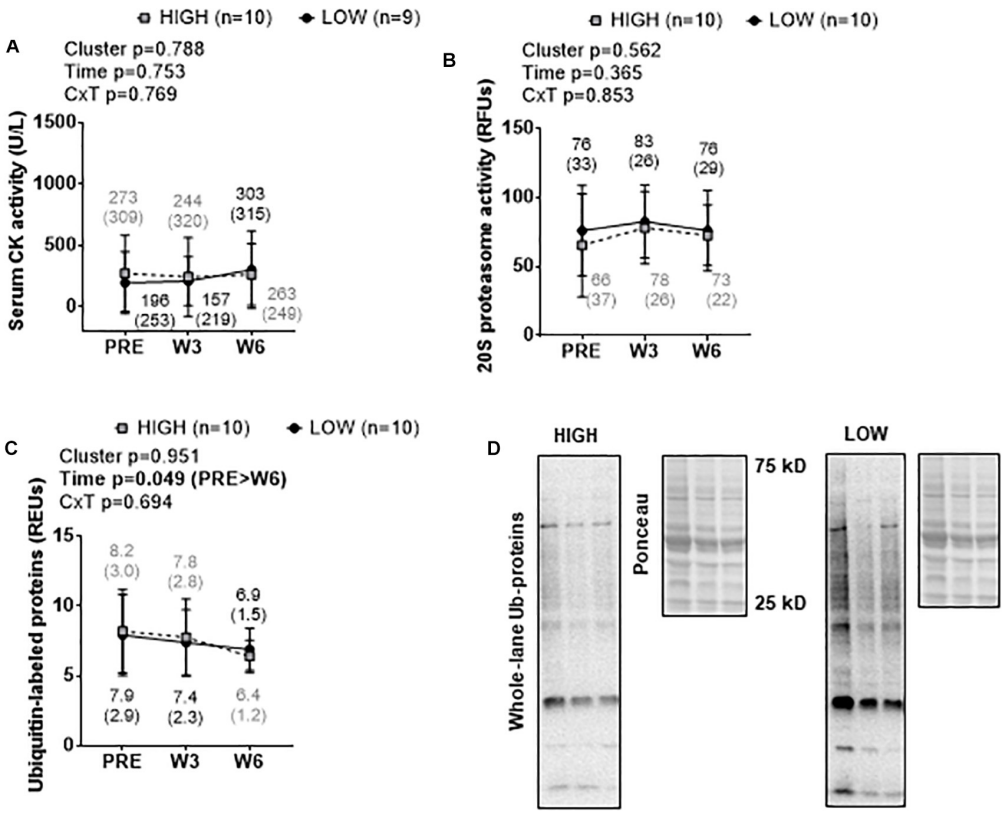

FIGURE 5 | PRE, W3, and W6 muscle damage and proteolysis marker differences between LOW and HIGH responders. No significant cluster effects or cluster × time interactions existed for serum CK activity **(A)**, muscle 20S proteasome activity **(B)**, or muscle ubiquitin-labeled protein levels **(C)**. A significant time effect existed for muscle ubiquitin-labeled protein levels. Values presented in line graphs are mean (standard deviation) values. **(D)** Representative Western blot image for a low and high responder.

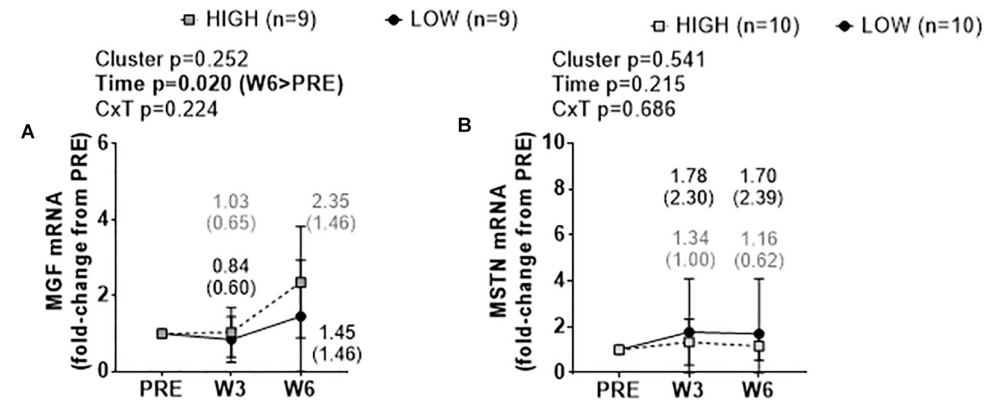

FIGURE 6 | PRE, W3, and W6 growth factor mRNA expression differences between LOW and HIGH responders. No significant cluster effects or cluster × time interactions existed for fold-change scores in muscle mechano growth factor (MGF) mRNA **(A)** or myostatin (MSTN) mRNA **(B)**. There was a significant time effect for MGF mRNA. Values presented in line graphs are mean (standard deviation) values.

W6 variables that qualified for the model were: PRE muscle p-mTOR ($r = -0.327$), W3–W6 type I fiber myonuclear number percent change ($r = 0.412$), W3–W6 type II fiber myonuclear number percent change ($r = 0.387$), W3–W6 serum cortisol percent change ($r = 0.33$), W3–W6 muscle p-p70s6k percent change ($r = 0.473$), W3–W6 ubiquitin-labeled protein levels percent change ($r = 0.318$), and W3–W6 muscle MGF mRNA percent change ($r = 0.353$). The only significant positive predictor from W3 to W6 was the W3–W6 percent change in p-p70s6k ($R^2 = 0.223$, $\beta = 0.473$, $p = 0.020$); although W3–W6 type I fiber

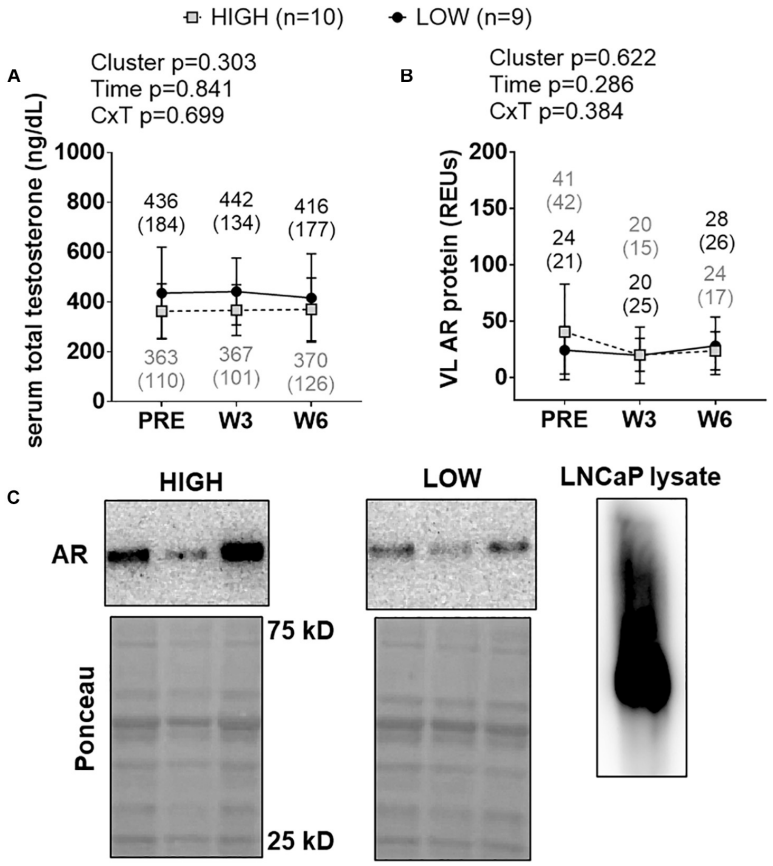

FIGURE 7 | PRE, W3, and W6 androgen signaling biomarker differences between LOW and HIGH responders. There were no significant cluster effects, time effects or cluster × time interactions for serum total testosterone levels **(A)** or muscle androgen receptor (AR) protein levels **(B)**. Cluster means at each time point are presented above line graphs as mean (standard deviation) values. Values presented in line graphs are mean (standard deviation) values. **(C)** Representative Western blot image for a low and high responder. Right inset of **(C)** demonstrates that our AR antibody yielded one prominent band for LNCaP cell lysates, and this band was in line with muscle AR content that was quantified.

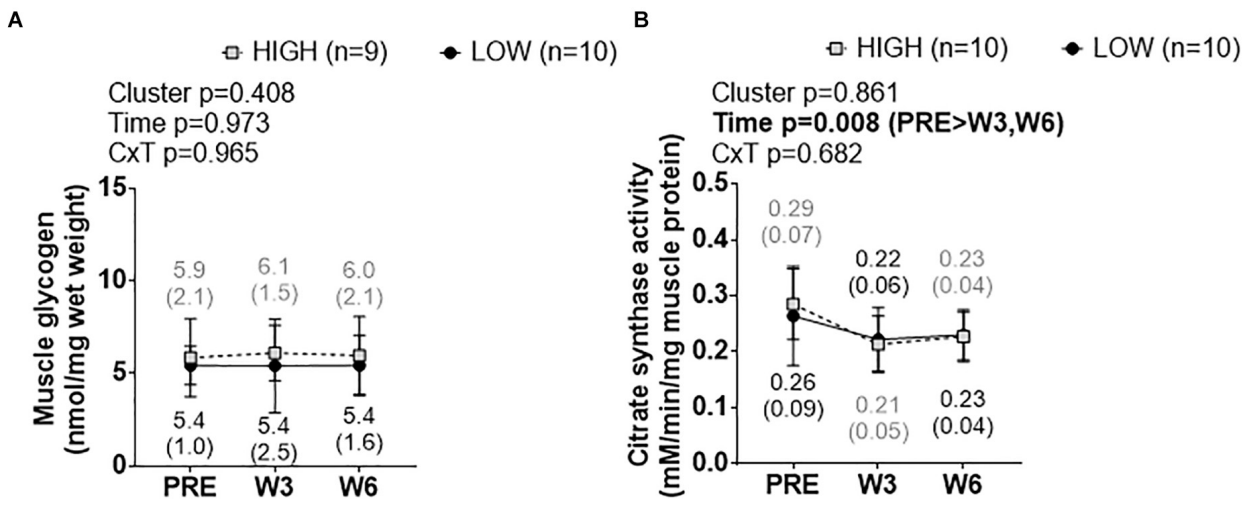

FIGURE 8 | PRE, W3, and W6 muscle glycogen and citrate synthase activity level differences between LOW and HIGH responders. **(A)** No significant cluster effect, time effect or cluster × time interaction existed for muscle glycogen levels. **(B)** No significant cluster effect or cluster × time interaction existed for muscle citrate synthase activity levels, although there was a significant time effect. Values presented in line graphs are mean (standard deviation) values.

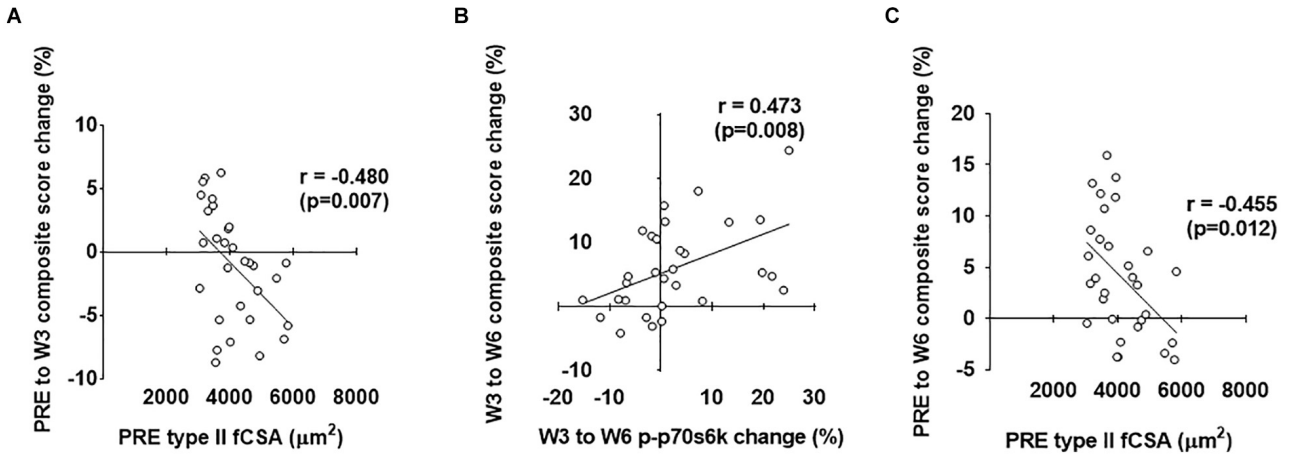

FIGURE 9 | Strongest predictors of PRE to W3, W3 to W6, and PRE to W6 composite hypertrophy scores. The strongest predictor of the PRE to W3 composite hypertrophy score was PRE type II fCSA values **(A)**. The strongest predictor of the W3 to W6 composite hypertrophy score was the percent change in type I fiber myonuclear number (MN#) **(B)**. The strongest predictor of the PRE to W6 composite hypertrophy score was PRE type II fCSA values **(C)**.

myonuclear percent change neared significance ($p = 0.067$). No models including significantly strong negative predictors were surmised from W3 to W6 ($p > 0.05$). PRE-W6 variables that qualified for the model included PRE type II fiber percentage ($r = 0.390$), PRE type I fiber percentage ($r = -0.390$), PRE type II fCSA ($r = -0.455$), PRE type I fCSA ($r = -0.431$), and PRE muscle p-AMPKα ($r = -0.301$). The strongest positive predictor from PRE-W6 was PRE type II fiber percentage ($R^2 = 0.152$, $\beta = 0.390$, $p = 0.033$). The strongest negative predictors were PRE type I fiber percentage ($R^2 = 0.160$, $\beta = -0.401$, $p = 0.024$) and PRE type II fCSA ($R^2 = 0.207$, $\beta = -0.455$, $p = 0.019$). The strongest predictors of the PRE to W3, W3 to W6, and PRE to W6 composite hypertrophy scores are illustrated in **Figure 9** below.

DISCUSSION

This study continues to highlight differences in biomarkers that exist between low and high hypertrophic responders following weeks of high-volume resistance training. While our sample size is limited, this study is unique given that most of the previous reports in this area have examined untrained individuals, whereas this is only the second study to examine well-trained college-aged males. Additionally, we feel a strength of the current study is classifying clusters using a combination of hypertrophic indices.

The most provocative finding herein was that, relative to high responders, low responders exhibited greater PRE type II fCSA (+18%, $p = 0.022$) values. Our regression analysis also suggested that lower PRE type II fCSA values and a greater PRE type II fiber percentage were the strongest predictors of the PRE-W6 hypertrophic response. In agreement with past data in untrained individuals demonstrating that low responders possessed higher pre-training VL thickness values (Mobley et al., 2018), these current data also imply that well-trained high responders may possess more growth potential given that they began training with lower fCSA values relative to low responders. Admittedly, none of the assayed biomarkers provide clear insight as to why pre-training fCSA values were greater in low versus high responders or how PRE type II fiber percentage mechanistically relates to the hypertrophic response. Thus, future work in this area is needed to determine genes or biomarkers (e.g., connective tissue thickness, DNA methylation status, etc.) which may affect inherent muscle size and fiber type-specific responses, and whether they are altered between low versus high responders during periods of resistance training. Notwithstanding, this is the first study to our knowledge suggesting that pre-training fCSA values are greater in low versus high hypertrophic responders. Likewise, we are the first to suggest that pre-training muscle fiber type has predictive bearing on the hypertrophic response. Indeed, the fiber type data herein are not in agreement with prior research which has suggested that pre-training muscle fiber type percentages were similar between low and high response clusters (Bamman et al., 2007; Stec et al., 2016; Mobley et al., 2018). However, these past reports all examined untrained participants. Hence, the potential for predominant muscle fiber type to influence the hypertrophic response to high volume training in previously trained participants should be further explored.

Several studies have reported that, in previously untrained participants, ribosome biogenesis is greater in high versus low hypertrophic responders to resistance training (Figueiredo et al., 2015; Stec et al., 2016; Mobley et al., 2018). Our finding indicating that changes in ribosome biogenesis markers were similar between high and low responders which were previously trained, however, suggests that this process may be less critical in stimulating muscle hypertrophy over a 6-week training period.

Satellite cell-mediated myonuclear accretion seemingly occurs during longer-term periods of resistance exercise training, and data from Bamman's and Phillips' laboratories suggests that

increases in satellite cell proliferation in response to one bout or weeks of resistance training are greater in high versus low responders (Petrella et al., 2008; Bellamy et al., 2014). In contrast, we recently reported that training-induced increases in satellite cell number and increases in types I and II fiber myonuclear number were similar between high versus low responders following 12 weeks of resistance exercise training (Mobley et al., 2018). While we did not assess satellite cell counts in the current study, no significant main effects or interactions were observed for either type I or type II myonuclear number per fiber which indicates that satellite-cell mediated myonuclear accretion may not differentiate skeletal muscle hypertrophy in previously trained college-aged males. However, it is interesting that the PRE-W3 percent change in type I fiber myonuclear number was a significant predictor of the percent change in the composite hypertrophy score. Hence, our regression results indicate that satellite cell-mediated type I fiber myonuclear addition could be involved with hypertrophy to a certain degree.

Human and rodent studies suggest the magnitude increase of mTORC1 signaling markers (e.g., p-p70s6k and p-4EBP1) following a resistance exercise bout are predictive of longer-term skeletal muscle hypertrophy (Baar and Esser, 1999; Terzis et al., 2008; Mitchell et al., 2014; Damas et al., 2016). While the W3–W6 percent change in p-p70s6k was a significant predictor of the W3–W6 change in the composite hypertrophy score, we observed no between-cluster differences in phosphorylated mTOR, p70s6k, 4EBP1, or AMPKα levels. These data suggest that mTORc1 signaling may not be critical in differentiating the hypertrophic response in previously-trained individuals, although these results should be interpreted with caution given that the W3 and W6 sampling time points were 24 h following a prior exercise bout. Alternatively stated, researchers typically assay these markers within the first 1–6 h following exercise given that these post-exercise time points yield robust changes (Farnfield et al., 2009; Haun et al., 2017), although there is data to suggest that p-mTOR (Ser2448), p-p70s6k (Thr289), and p-4EBP-1 (Thr37/46) are significantly elevated 24 h following a resistance exercise bout (Burd et al., 2011; Gundermann et al., 2014). Notwithstanding, it remains possible that these markers could have differed between clusters if biopsies were obtained in a more acute post-exercise time frame. Likewise, basal or post-exercise muscle protein and/or myofibrillar protein synthesis rates were not directly assessed in this investigation and could have differed between low and high responders.

We also sought to examine cluster differences in biomarkers related to muscle damage and proteolysis. Herein, we observed no between-cluster differences in serum CK activity levels, muscle 20S proteasome activity levels, or muscle ubiquinated protein levels. Likewise, our regression models indicated that none of these biomarkers significantly explained the variance in the composite hypertrophy score. These data agree with our prior data suggesting that neither muscle 20S proteasome activity nor MuRF-1 protein content differ prior to or following 12 weeks of resistance training in previously untrained males (Mobley et al., 2018). However, as with the lack of protein synthesis data herein, these data are limited in that we did not directly assess whether protein breakdown rates differed between clusters.

Androgen receptors operate as transcription factors to alter the mRNA expression of hundreds to thousands of genes (Jiang et al., 2009). A high level of enthusiasm exists regarding the hypertrophic effects of AR signaling given that the administration of anabolic steroids increases satellite cell proliferation (Sinha-Hikim et al., 2002; Sinha-Hikim et al., 2003) and MPS (Griggs et al., 1989; Ferrando et al., 1998). Two studies have reported that changes in skeletal muscle AR protein content correlate with increases in skeletal muscle hypertrophy. Ahtiainen et al. (2011) reported skeletal muscle AR protein increases correlated with fCSA and lean body mass increases in younger and older men following 21 weeks of resistance training. Mitchell et al. (2013) subsequently reported increases in muscle AR protein content correlate with muscle hypertrophy following 16 weeks of resistance training. More recently, Phillips' laboratory reported that AR protein content was greater prior to and following 16 weeks of resistance training in previously trained high versus low responders (Morton et al., 2018). However, we recently reported that 12 weeks of resistance training downregulated AR content in high and low responders who were previously untrained (Mobley et al., 2018), and suggested this was a negative feedback phenomena regarding resistance training and AR protein expression. Given that the paper by Morton et al. (2018) was published during the writing of this manuscript, we decided to perform a *post hoc* analysis on muscle AR levels in only the HIGH and LOW clusters. In the current study we observed no between cluster effect or interaction for serum testosterone or AR protein content. Our findings are in partial agreement with other literature (Mobley et al., 2018; Morton et al., 2018) in that there was no training effect, between-cluster effect, or interaction regarding serum total testosterone levels. However, it is difficult to reconcile why our AR protein findings differ from Morton et al. (2018), and we took extra precaution to ensure that our antibody was valid using a positive control LNCaP lysate. Thus, more research is needed in determining if muscle AR protein content does separate high versus low hypertrophic responders.

We recently proposed high responders may possess a greater mitochondrial volume given that muscle anabolism requires high amounts of cellular energy for protein turnover (Roberts et al., 2018a). Likewise, we recently reported that high responders that were previously untrained possessed greater muscle citrate synthase activity levels than low responders (Roberts et al., 2018b). However, the current citrate synthase activity data suggest that mitochondrial content is similar in both clusters, and similarly decreases in both clusters with training. Notwithstanding, while these data suggest mitochondrial volume decrements occur in high and low responders that were previously trained, it does not rule out the potential that the low or high response clusters experienced alterations in mitochondrial function (e.g., states 3 and 4 respiration levels, complex activity levels, etc.).

It should be noted that back squat training volume did not differ between response clusters, and this finding is also in agreement with past data we collected in previously untrained college-aged male low versus high responder cohorts (Mobley et al., 2018). Additionally, self-reported macronutrient intakes did not differ between clusters, and our laboratory as well as Bamman's laboratory have reported similar findings (Thalacker-Mercer et al., 2009; Mobley et al., 2018). Finally, while the number of participants in the LOW and HIGH clusters did not significantly or practically differ with regard to protein supplement consumption reported by Haun et al. (2018b), this does not rule out the potential that consuming large amounts of supplemental whey protein was primarily responsible for the hypertrophic response in the three HIGH responders assigned to this group.

Experimental Limitations

There are limitations to this study. First, the original intent of this project was to utilize high volume resistance training to enhance hypertrophy. As such, we did not obtain a post-intervention strength metric. Thus, it is currently unclear as to whether pre- to post-changes in lower body strength differed between the HIGH and LOW clusters herein. A second limitation of this study is the lack of Pax7 staining for histologically detecting satellite cell changes with training between clusters. While this staining would have been informative, we chose to allocate our resources toward analyzing other biomarkers and depended solely upon detecting changes in myonuclear number through DAPI staining in order to make inferences regarding satellite cell-mediated fusion events. However, we cannot rule out the potential that the HIGH and LOW clusters herein contained different satellite cell numbers prior to or after the training intervention. Another histology limitation herein is the lack of discrimination between type IIa and type IIx fibers. While Bamman's laboratory has reported that no baseline differences in types IIa and IIx fiber percentage exist between low and high hypertrophic responders (Bamman et al., 2007), this does not to rule out the possibility that differences existed in the currently analyzed clusters. Another limitation is that blood and biopsy sampling at the week 3 and week 6 time points occurred 24 h following the last training bout due to study logistics. This may have had an impact on some of the assayed biomarkers (e.g., serum CK and mRNA/rRNA expression patterns) and unfortunately is an unresolved limitation. Finally, like other similar studies examining hypertrophic responders and non-responders to resistance training (Davidsen et al., 2011; Stec et al., 2016; Morton et al., 2018), it is notable that this study was also limited in scope regarding n-size. In this regard, meta-analytical interrogations which combine all of these studies will likely be needed to obtain enough power in order to decipher which biomarkers differ between clusters.

CONCLUSION

This study continues to delineate biomarkers that exist between low versus high responders to resistance training, but is unique in that it is only the second study to date that has examined subjects that were well-trained prior to engaging in the training protocol. As stated in a recent perspective on the topic (Roberts et al., 2018a), research identifying intrinsic factors that regulate differential hypertrophic responses to resistance exercise training will generate future research which examines if these factors can be modulated by altering extrinsic variables such as nutrition, exercise dosing, or recovery strategies. Furthermore, these series studies will ultimately improve our understanding of factors that optimize resistance exercise training adaptations, and such research will likely be useful for individuals seeking to apply this knowledge in a practical setting.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

CH and MDR were primarily responsible for the current research question, statistical analyses, and writing of the manuscript. CV critically assisted with all aspects of execution and analysis. MDR was the principal investigator of the laboratory where the work for this study was performed. All other co-authors assisted in multiple aspects of data collection as well as the preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00297/full#supplementary-material

FILE S1 | Raw data from this study.

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Phenotypic Responses to a Lifestyle Intervention Do Not Account for Inter-Individual Variability in Glucose Tolerance for Individuals at High Risk of Type 2 Diabetes

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Background: Lifestyle interventions have been shown to delay or prevent the onset of type 2 diabetes among high risk adults. A better understanding of the variability in physiological responses would support the matching of individuals with the best type of intervention in future prevention programmes, in order to optimize risk reduction. The purpose of this study was to determine if phenotypic characteristics at baseline or following a 12 weeks lifestyle intervention could explain the inter-individual variability in change in glucose tolerance in individuals with high risk for type 2 diabetes.

Methods: In total, 285 subjects with normal glucose tolerance (NGT, FINDRISC score > 12), impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) were recruited for a 12 weeks lifestyle intervention. Glucose tolerance, insulin sensitivity, anthropometric characteristics and aerobic fitness were measured. Variability of responses was examined by grouping participants by baseline glycemic status, by cluster analysis based on the change in glucose tolerance and by Principal Component Analysis (PCA).

Results: In agreement with other studies, the mean response to the 12 weeks intervention was positive for the majority of parameters. Overall, 89% improved BMI, 80% waist circumference, and 81% body fat while only 64% improved fasting plasma glucose and 60% 2 h glucose. The impact of the intervention by glycaemic group did not show any phenotypic differences in response between NGT, IFG, and IGT. A hierarchical cluster analysis of change in glucose tolerance identified four sub-groups of "responders" (high and moderate) and "non-responders" (no response or deteriorated) but there were few differences in baseline clincal and physiological parameters or in response to the intervention to explain the overall variance. A further PCA analysis of 19 clinical and physiological univariables could explain less than half (48%) of total variability.

Conclusion: We found that phenotypic characteristics from standard clinical and physiological parameters were not sufficient to account for the inter-individual variability in glucose tolerance following a 12 weeks lifestyle intervention in inidivuals at high risk for type 2 diabetes. Further work is required to identify biomarkers that complement phenotypic traits and better predict the response to glucose tolerance.

Keywords: lifestyle intervention, risk of type 2 diabetes, glucose tolerance, inter-individual variability, prevention

INTRODUCTION

Several large clinical trials have shown that the onset of type 2 diabetes can be prevented or delayed among adults at high risk by a combination of diet and exercise (Tuomilehto et al., 2001; Knowler et al., 2002; Lindström et al., 2006; Ramachandran et al., 2006; Li et al., 2008). Even so, individual responses to lifestyle interventions are variable and further investigation is required to optimize risk reduction strategies. A better understanding of the variability in physiological responses would help match individuals with the best type of intervention in personalized prevention programs (Glauber and Karnieli, 2013; Stefan et al., 2016).

One of the factors that could influence the response to a lifestyle intervention in pre-diabetes is the underlying pathophysiology (Faerch et al., 2016). Previous diabetes prevention programs included mainly subjects with Impaired Glucose Tolerance (IGT) whereas few studies have included subjects with Impaired Fasting Glucose (IFG) or Normal Glucose Tolerance (NGT) who were nonetheless at high risk for progression. Differences in the physiological responses to the same exercise or dietary interventon, or indeed the type and amount of either, could also account for variability (Bohm et al., 2016). These factors may explain why some parameters, for example body fat, may improve following a lifestyle intervention while others may be unchanged or even deteriorate (Stephens and Sparks, 2015; Sparks, 2017).

The principal aim of the DEXLIFE (Diet and Exercise for Life) project is to identify novel biomarkers that complement clinical and physiological variables to better predict improvements in glycemic status following a lifestyle intervention (Andersen et al., 2014). As a first step, a 12 weeks lifestyle intervention was designed to investigate the range of physiological responses in a group of individuals who were at risk for type 2 diabetes (O'Donoghue et al., 2015). The purpose of this study was to determine if phenotypic characteristics at baseline or following a 12 weeks lifestyle intervention could explain the inter-individual variability in glucose tolerance in high risk individuals for type 2 diabetes.

MATERIALS AND METHODS

Setting

The study was conducted at Dublin City University (DCU), Ireland. The DEXLIFE intervention (12 weeks lifestyle program) was delivered in Dublin City Sport, an on-campus gym. This study was carried out in accordance with the recommendations of Declaration of Helsinki with written informed consent from all subjects. The protocol was approved by the Research Ethics Committee at DCU (DCUREC/2012/080) and all subjects provided written informed consent.

Participant Eligibility

Adults aged 18–75 years, who were inactive (<150 min of physical activity per week) and displaying at least one of the following diabetes risk factors were eligible to participate; (i) impaired fasting glucose (FPG levels ≥5.6 to <7 mmol/L (ii) impaired glucose tolerance (2 h plasma glucose levels ≥7.8 to <11.1 mmol/L following an oral glucose tolerance test) and/or (iii) normal glucose tolerance with a FINDRISC (Lindstrom and Tuomilehto, 2003) score >12 (1 in 6 chance of developing type 2 diabetes in the next 10 years). Individuals were excluded if they had previously diagnosed type 2 diabetes, severe cardiovascular, respiratory or renal disease, active cancer, neuromuscular, musculoskeletal or rheumatoid disorders exacerbated by exercise, significant cognitive or mental illness, if they were receiving any medication that could affect glucose metabolism, if they had a peak aerobic capacity >50 $ml \cdot kg^{-1} \cdot min^{-1}$ or >5% change in body weight in the previous 3 months.

Recruitment

Participants were identified in three ways. Information sessions were held locally; within the university, in local sports clubs, pharmacies and general practices within a 10 km radius of Dublin City University. An online screening tool (FINDRISC) was accessible on the DEXLIFE website. If an individual scored >12, an email was automatically generated to the DCU recruitment team and the potential participant contacted. Finally, Vhi Healthcare, Ireland's largest health insurance company, and one of the partners in DEXLIFE, identified eligible participants from their database of policy holders. All potential participants were provided with research study information sheets and consent forms.

Procedures

At baseline and following the 12 weeks lifestyle intervention, participants completed a number of clinical and physiological assessments.

Anthropometrics

Body weight was assessed on a digital platform with minimal clothing, and height was recorded on a stadiometer (SECA, Hamburg). Dual X-ray absorptiometry (Stratos, BMD Medical Systems) was used to quantify total body fat and fat-free mass

while subcutaneous and visceral fat depth was measured by ultrasonography (Aquila, Pie Medical).

Glucose Tolerance

A standard 75 g Oral Glucose Tolerance Test (OGTT) was performed in the morning after an overnight fast. Baseline blood samples were taken for glucose, insulin and lipids followed by samples at 30, 60, 90, 120, and 180 min post-glucose ingestion. The area under the glucose curve ($AUC_{glucose}$) and insulin ($AUC_{insulin}$) were calculated using the trapezoidal method. Insulin secretion was estimated by the insulinogenic index (Goedecke et al., 2009) and the insulin AUC from 0 to 30 min while insulin sensitivity was estimated by the Matsuda index (Matsuda and DeFronzo, 1999).

Cardiorespiratory Fitness

A 12-lead ECG stress test using a modified Bruce protocol was used to assess maximal oxygen consumption (VO_2max). Participants walked on a treadmill with either the speed or gradient increasing every 3 min until volitional fatigue or symptoms that warranted termination. Blood pressure was taken at each stage and heart rate was measured continuously. Oxygen consumption was measured using breath-by-breath analysis of expired air by indirect calorimetry (Vmax 29C, SensorMedics, Yorba Linda, CA, United States).

Laboratory Analyses

Serum insulin was measured with a commercially available fluoroimmunoassay (Roche Diagnostics, Mannheim, Germany). Plasma glucose was measured using a glucose oxidase method (Randox Laboratories, Crumlin, Co. Antrim, United Kingdom). Serum triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol were measured using enzymatic methods (Randox Laboratories, Crumlin, Co. Antrim, United Kingdom).

Lifestyle Intervention

The DEXLIFE lifestyle intervention was a 12 weeks supervised exercise training program accompanied with dietary advice.

Exercise Program

Participants were given access to DCU Sport. A qualified sports scientist or physiotherapist accompanied each individual to the gym for an induction session prior to commencing the intervention. The induction session included familiarization with the gym equipment and specific individual instruction relating to frequency, intensity, time and type of exercise to be performed. Participants performed 4 × 45 min exercise sessions per week at a moderate intensity, focusing on a combination of cardiovascular and resistance exercise. Exercise supervision was provided by the gym instructors based in DCU Sport. They were present during the exercise sessions, answered any questions and provided support to assist participants achieve the optimal exercise intensity. A personal online exercise diary was also made available for participants to track their individual progress and record any additional information, including other exercise.

Dietary Advice

A 3 day estimated food diary was used to assess dietary intake. Once completed, the participant met with a dietician to review the diary, identifying unhealthy food choices and to develop a plan to modify those choices. The concept of energy balance and restricting energy intake from fat was introduced. The energy goals were calculated by estimating the daily calories needed to maintain the participant's starting weight and if weight loss was indicated, 500–1000 calories were subtracted per day (depending on body weight) to achieve a 0.5 kg decrease in weight per week. Common to all food plans was <10% energy intake from saturated fat intake as well as a dietary fiber intake of >15 g/1000 kcal.

Adherence

To optimize adherence, an electronic exercise diary was employed and regular follow-up telephone calls were used. Participants were asked to record all exercise sessions in the diary, providing details of the frequency, time, intensity and type of exercise completed. Alongside the electronic diary, participants signed in each time they attended the gym and this information was provided to the research team. Participants were weighed by a gym instructor on a weekly basis in DCU Sport and they then entered their weight into their electronic exercise diary. The research team monitored the diary entries closely and contact was made if the diary was not completed for more than 2 days in a row or if body weight was not decreasing. Adherence rates were based on the number of completed exercise sessions with 100% adherence being 48 sessions (4 sessions × 12 weeks).

Data Analysis

The proportion of missing data ranged from <5% to 20% for the covariates. To avoid exclusion of participants with missing values which may infer biased results (Janssen et al., 2010), missing data on the covariates were imputed using the Multivariate Imputations by Chained Equations (MICE) method (van Buuren, 2007) with missing-at-random assumptions (R software). Fifty copies of the data, each with missing values suitably imputed, were independently assessed in the analyses described below. Estimates of parameters of interest were averaged across the copies according to Rubin's rules (Marshall et al., 2009).

In order to investigate the variability in response to the lifestyle intervention, a number of analytical steps were included. The first step was to determine the impact of the intervention on the group as a whole using Paired *t*-tests. The second step examined individual variability for each of the parameters. Participants were ranked and sorted according to the change from baseline for each parameter and the individual responses were represented as waterfall plots (Gillespie, 2012).

Step 3 grouped participants by their baseline glycaemic status; NGT, i-IFG, IGT, or screen-detected type 2 diabetes (T2DM). Mean values of change with 95% CI for the covariates were calculated and the corresponding differences between groups were tested in a linear regression analysis, adjusted for age, gender, BMI and the baseline level of the covariate.

Step 4 examined the change in glucose tolerance ($AUC_{glucose}$) by dividing participants into clusters of similar response. We used a hierarchical clustering approach based on the Euclidean distance (the absolute difference in change in outcome variables) between the observations and forming clusters using the Ward's

method (Hastie et al., 2009). The Ward's method forms clusters where the total within-cluster variance is minimized (compact clusters), and tends to produce clusters of more equal size than others. Linear regression analysis was conducted on each of the parameters at baseline firstly to determine if it was possible to predict responsiveness to the intervention from the change in glucose tolerance and secondly to identify differences in the response to the intervention for body composition, clinical parameters and fitness between the clusters.

With the final step, we further explored the individual responses using unbiased principal component analysis (PCA) to summarize and visualize the responses to all the observed variables (Zhang and Castello, 2017). The multivariate data was grouped into principal components in an attempt to identify the parameters that account for most of the variance. Statistical analyses were performed in R version 3.3.1 (The R Foundation for Statistical Computing)[1].

RESULTS

Participant Characteristics

Of the 285 participants recruited to the lifestyle intervention, 28 (9.3%) did not participate in the follow-up examination, leaving 257 for analysis. At baseline, the mean age was 54.2 ± 10.8 years with half of the participants being female (50.2%; n = 129). Almost half of the participants (48.2%; n = 123) drank alcohol but only 8% were smokers (n = 20). Participants were excluded if they were receiving any medication that could affect glucose metabolism but 98 participants (38.2%) were taking other prescribed medication at the time of the intervention. The most commonly prescribed were anti-hypertensive (18.6%; n = 46), lipid lowering (15.4%; n = 39) and analgesic (8.7%; n = 22) medication. Of these, 22.2% were taking both anti-hypertensive and lipid lowering medication and 9.8% were taking all three.

The exercise programme adherence rate was high, with participants completing 46.2 ± 8.0 of the prescribed 48 exercise sessions (96%). Several beneficial changes in clinical and metabolic parameters were observed following the 12 weeks lifestyle intervention (**Table 1**). As expected, there was a significant reduction ($p < 0.001$) in a broad range of parameters including body weight (−3.9 kg: 95% CI −4.3; −3.4), waist circumference (−5.1 cm: 95% CI −6.1; −4.1), body fat (−2.0%: 95% CI −2.3; −1.7), fasting (−0.2 mmol/l/l: 95% CI −0.28; −0.13) and 2 h glucose (−0.48 mmol/l/l: 95% CI −0.70; −0.26). There were no significant differences in HDL cholesterol and fasting insulin. We also had a parallel but not randomized group (n = 80) that were provided with physical activity and dietary recommendations. This data is not presented as the focus of this paper is to examine variability within the intervention group and provided guidelines was itself an intervention. However, the pre and post-data from this group are presented in **Supplementary Material** to demonstrate that most of the physiological variables did not change, in line with findings from previous diabetes prevention studies.

[1]www.R-project.org

TABLE 1 | Baseline characteristics of the study population (original data) and estimated impact of intervention (imputed data).

	Original data		Imputed data	
	n	Level	Change (95% CI)	*P*
Age (year)	257	54.2 (10.9)		
Male sex (%)	257	49.8		
Body composition				
Weight (kg)	257	89.7 (17.9)	−3.9 (−4.3; −3.4)	<0.001
BMI (kg/m^2)	257	31.1 (5.5)	−1.3 (−1.5; −1.2)	<0.001
Waist (cm)	224	104.4 (12.4)	−5.1 (−6.1; −4.1)	<0.001
Fat %	257	37.8 (8.6)	−2.0 (−2.3; −1.7)	<0.001
Subcutaneous fat (cm)	216	2.6 (1.97; 3.52)	−0.45 (−0.58; −0.32)	<0.001
Visceral fat (cm)	203	7.09 (5.72; 8.56)	−1.04 (−1.34; −0.74)	<0.001
Clinical measurements				
Fasting plasma glucose (mmol/l)	254	5.88 (0.87)	−0.20 (−0.28; −0.13)	<0.001
2 h plasma glucose (mmol/l)	249	6.83 (2.24)	−0.48 (−0.70; −0.26)	<0.001
Insulin (pmol/l)	255	76.6 (49.6; 116.9)	−8.1 (−19.6; 3.32.0)	0.164
AUC glucose (mmol min/L)	227	1158 (1002; 1344)	−65.0 (−87.4; −42.7)	<0.001
AUC insulin (·10^3 pmol min/L)	237	73.0 (48.8; 106.2)	−19.4 (−23.6; −15.3)	<0.001
Matsuda index	232	2.9 (2.0; 4.6)	0.72 (0.49; 0.96)	<0.001
Insulinogenic index	257	113.5 (70.4; 176.1)	−19.6 (−69.1; 30.0)	0.437
Sys blood pressure (mm/Hg)	249	134.8 (14.9)	−4.7 (−6.7; −2.6)	<0.001
Dia blood pressure (mm/Hg)	249	82.8 (10.2)	−4.0 (−5.5; −2.5)	<0.001
Triglycerides (mmol/L)	237	1.2 (0.9−1. 7)	−0.18 (−0.27; −0.09)	<0.001
Total Cholesterol (mmol/L)	234	5.4 (1.4)	−0.24 (−0.38; −0.11)	<0.001
HDL cholesterol (mmol/L)	235	1.3 (0.4)	0.00 (−0.04; 0.03)	0.780
Aerobic fitness				
VO_2max (ml/kg/min)	257	29.0 (7.7)	2.8 (2.2; 3.4)	<0.001

Data are means (SD), medians (interquartile range) or estimated changes (95% CI). P: p-value for overall unadjusted test of change.

Variability in Individual Responses

While the overall responses to the intervention were positive, there was a broad range of individual responses in the measured parameters, as shown in the waterfall plots (**Figure 1**). BMI and body fat decreased in 80–90% of participants while 70% increased VO_{2max}. Fasting and 2 h glucose as well as $AUC_{glucose}$ decreased in 60–64% of participants while insulin sensitivity improved in ∼70% of participants.

Glycemic Status at Baseline and Response to the Intervention

Participants were divided into those with (i) NGT (32.6%), (ii) i-IFG (40.8%), (iii) IGT (isolated IGT and combined IFG& IGT: 17.3%), and (iv) T2DM (9.3%). Participants were excluded if they had diabetes at the time of recruitment but a number were subsequently found to have screen-detected diabetes at the first OGTT. There was an overall improvement in the response, as determined by the difference between pre- and post-intervention scores, for each group (**Table 2**). After adjusting for age, gender and BMI, differences between baseline glycaemic status groups were identified in the fasting and 2 h blood glucose responses. With additional adjustment for baseline variability, there were no differences in the response between groups (**Table 2**).

Cluster Analysis Based on Change in Area Under the Curve for Glucose

A hierarchical cluster analysis based on the change in the $AUC_{glucose}$ identified four sub-groups, which could be

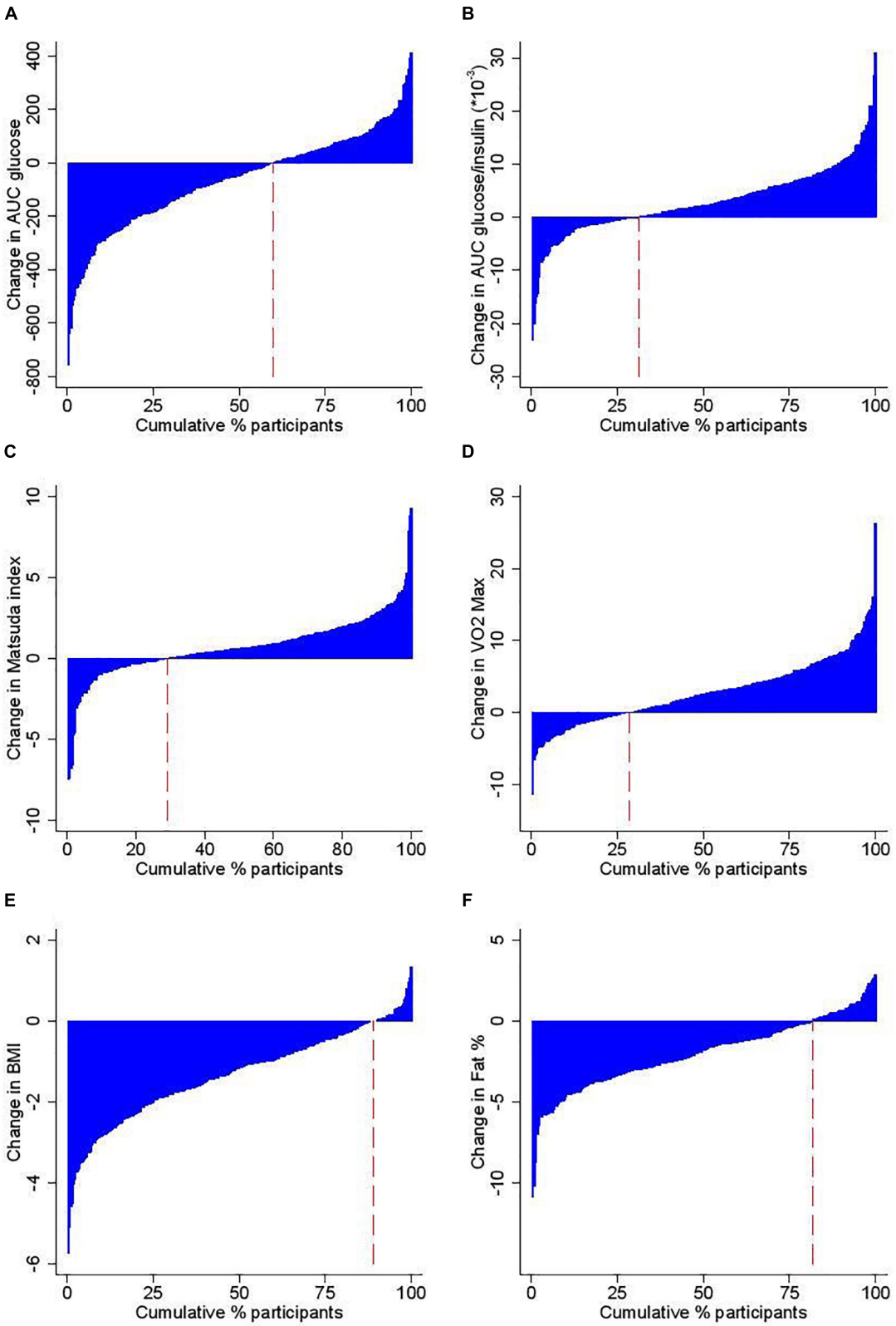

FIGURE 1 | Study participants according to change following the 12 week intervention in **(A)** Area Under the Curve (AUC) for Glucose during the OGTT, **(B)** AUC for Glucose/Insulin during the OGTT, **(C)** insulin sensitivity as estimated by the Matsuda index, **(D)** VO_2max, **(E)** BMI, and **(F)** % body fat. The red dashed line indicates the proportion of individuals with a lowered outcome level after the intervention.

TABLE 2 | Estimated impact of the intervention (imputed data) by glycemic group.

	NGT (n = 84)	i-IFG (n = 105)	i-IGT and IFG/IGT (n = 44)	T2DM (n = 24)	P_1	P_2
Body composition						
Weight (kg)	−4.0 (−4.7; −3.3)	−3.6 (−4.4; −2.9)	−3.3 (−4.4; −2.3)	−5.0 (−6.4; −3.6)	0.471	0.469
BMI (kg/m^2)	−1.4 (−1.6; −1.1)	−1.3 (−1.5; −1.0)	−1.1 (−1.5; −0.8)	−1.7 (−2.2; −1.3)	0.248	0.359
Waist circumference (cm)	−6.0 (−7.5; −4.5)	−5.0 (−6.7; −3.2)	−4.8 (−7.1; −2.5)	−5.8 (−9.0; −2.7)	0.692	0.877
Fat (%)	−1.9 (−2.3; −1.4)	−2.0 (−2.5; −1.5)	−1.8 (−2.5; −1.1)	−2.8 (−3.7; −1.9)	0.240	0.349
Subcutanous fat (cm)	−0.38 (−0.57; −0.18)	−0.38 (−0.58; −0.17)	−0.59 (−0.87; −0.30)	−0.43 (−0.8; −0.07)	0.648	0.572
Visceral fat (cm)	−1.24 (−1.71; −0.76)	−0.74 (−1.26; −0.22)	−0.91 (−1.66; −0.16)	−1.27 (−2.2; −0.35)	0.701	0.621
Clinical measurements						
Fasting plasma glucose (mmol/L)	−0.30 (−0.42; −0.18)	0.08 (−0.05; 0.22)	−0.14 (−0.32; 0.05)	−0.93 (−1.18; −0.67)	<0.001*	0.055
2 h plasma glucose (mmol/L)	−0.08 (−0.42; 0.25)	−0.04 (−0.42; 0.34)	−1.62 (−2.14; −1.10)	−1.50 (−2.24; −0.76)	< 0.001$^{\infty}$	0.306
Fasting Insulin (pmol/l)	−11.8 (−31.6; 8.0)	10.9 (−11.3; 33.2)	−21.5 (−51.8; 8.8)	−33.8 (−75.2; 7.6)	0.110	0.284
AUC glucose (mmol min/L)	−48.2 (−82.2; −14.1)	0.5 (−37.9; 38.9)	−149.9 (−202.9; −96.9)	−203.9 (−277.2; −130.6)	<0.001¥	0.985
AUC insulin (·10^3 pmol min/L)	−21.0 (−27.8; −14.3)	−13.3 (−20.8; −5.7)	−23.5 (−33.9; −13.1)	−26.3 (−40.4; −12.2)	0.508	0.529
Matsuda index	0.88 (0.49; 1.26)	0.38 (−0.06; 0.81)	0.66 (0.07; 1.25)	1.37 (0.56; 2.17)	0.345	0.278
Insulinogenic Index	−22.2 (−105.9; 61.4)	−27.6 (−85.6; 30.4)	−2.4 (−81.1; 76.3)	−42.7 (−152.0; 66.6)	0.824	0.736
Systolic blood pressure (mmHg)	−4.1 (−7.3; −1.0)	−5.5 (−9.0; −1.9)	−5.4 (−10.3; −0.5)	−1.0 (−7.5; 5.5)	0.505	0.129
Diastolic blood pressure (mmHg)	−3.4 (−5.7; −1.1)	−5.1 (−7.8; −2.5)	−3.2 (−6.8; 0.4)	−4.4 (−9.3; 0.5)	0.832	0.921
Triglycerides (mmol/L)	−0.25 (−0.39; −0.11)	−0.10 (−0.26; 0.05)	−0.10 (−0.32; 0.13)	−0.30 (−0.59; −0.01)	0.566	0.158
Total cholesterol (mmol/L)	−0.27 (−0.47; −0.07)	−0.11 (−0.33; 0.11)	−0.44 (−0.75; −0.12)	−0.23 (−0.65; 0.20)	0.326	0.627
HDL cholesterol (mmol/L)	0.01 (−0.04; 0.06)	0.00 (−0.06; 0.05)	−0.05 (−0.13; 0.03)	0.01 (−0.10; 0.11)	0.626	0.427
Aerobic fitness						
VO_2max (ml/kg/min)	3.2 (2.3; 4.2)	2.7 (1.6; 3.8)	2.4 (1.0; 3.9)	2.0 (0.0; 4.0)	0.535	0.238

Data are estimated changes (95% CI).

P_1, p-value for overall test of difference in change between subgroups of glycaemia, adjusted for age, sex, BMI; P_2, additional adjusting for the baseline level of the covariate.

**Fasting plasma glucose (mmol/L): i-IFG vs. NGT: $p < 0.001$; T2DM vs. other groups: $p < 0.001$.*

$^{\infty}$ *2 h plasma glucose (mmol/L): IGT vs. NGT: $p < 0.001$; IGT vs. i-IFG: $p < 0.001$; T2DM vs. NGT: $p < 0.001$; T2DM vs. i-IFG: $p < 0.001$.*

¥ *AUC glucose (mmol min/L): IGT vs. NGT: $p < 0.001$; IGT vs. i-IFG: $p < 0.001$; T2DM vs. NGT: $p < 0.001$; T2DM vs. i-IFG: $p < 0.001$.*

categorized as either responders or non-responders (**Table 3**). Cluster 1 (n = 17) and Cluster 2 (n = 57) showed a High (HI-RES) or Moderate (MOD-RES) improvement in $AUC_{glucose}$ while Cluster 3 (n = 126) and Cluster 4 (n = 57) did not show any change (NO-RES) or had deteriorated (DET-RES) $AUC_{glucose}$. Almost 50% of the high responders (Cluster 1) had T2DM while those with i-IFG formed the largest proportion in the moderate responder (Cluster 2) and no response (Cluster 3) groups. Of those that deteriorated (Cluster 4) just under 50% had NGT (**Table 3**).

The physiological and clinical characteristics of participants in the four clusters are presented in terms of their response to the intervention (**Table 3**) and their baseline data prior to the intervention (**Table 4**). There were few differences in baseline characteristics between the clusters. DET-RES (Cluster 4) had lower body weight at baseline compared with HI-RES (Cluster 1) and NO-RES (Cluster 3) but not MOD-RES (Cluster 2). HI-RES (Cluster 1) had higher visceral fat and triglycerides than the other clusters while HDL Cholesterol was higher in DET-RES (Cluster 4) than the groups that responded (Cluster 1 and 2). There were no differences in total body fat, BMI, waist circumference, VO_{2max} or subcutaneous body fat. The non-responder groups (Cluster 3 and 4) had better baseline glycemic characteristics than those that responded (Clusters 1 and 2), as expected (**Table 4**).

The change in each variable following the intervention was also assessed to determine if the cluster analysis could identify physiological or clinical characteristics to differentiate the groups (**Table 3**). Cluster 1 (HI-RES) lost more weight and body fat than the other groups but there were no differences between cluster 2 (MOD-RES) and cluster 3 (NO-RES). All groups improved to a similar degree in waist circumference, abdominal fat, blood pressure, lipids and VO_{2max}. There was no significant difference in the number of minutes of exercise completed during the intervention.

Principal Component Analysis of Individual Variability

PCA was applied to explore individual variation in response using all measured clinical and physiological parameters (**Figure 2**). This multivariate visualization is complementary to the univariate waterfall plots. There was a rightward shift in standard deviation ellipses that represent the overall variance, indicating a positive response to the intervention. Individual changes in all measured parameters (n = 19) were subjected to PCA resulting in a two component solution that only accounted for 48% of total variation. The loadings are presented in **Table 5**. The highest loadings in the first principal component (PC1), which explained 36.7% of variation, were BMI (−0.33), waist

TABLE 3 | Response to the intervention based on clusters of change in $AUC_{glucose}$ (imputed data).

	Responders		Non-responders			
Baseline	**C1 HI-RES**	**C2 MOD-RES**	**C3 NO-RES**	**C4 DET-RES**	P_1	P_2
N	17	57	126	57		
Mean (SD) of change in $AUC_{glucose}$	−485 (101)	−236 (52)	−34 (63)	161 (82)		
Range of change in $AUC_{glucose}$	−756; −368	−367; −157	−156; 70	71; 401		
Baseline						
Age (year)	53.4 (11.0)	53.6 (11.5)	52.9 (11.2)	57.9 (8.7)		
Male sex (%)	65	53	51	40		
NGT (%)	6	18	37	47		
i-IFG (%)	18	42	44	39		
IGT (%)	29	26	15	7		
T2DM (%)	47	14	4	7		
Change						
Body composition						
Weight (kg)	−7.5 (4.7)	−4.4 (3.4)[a]	−3.6 (3.3)[a]	−2.7 (2.8)[a,b]	<0.001	<0.001
BMI (kg/m^2)	−2.6 (1.5)	−1.5 (1.1)[a]	−1.2 (1.1)[a]	−1.0 (1.0)[a,b]	<0.001	<0.001
Waist circumference (cm)	−9.7 (6.7)	−5.2 (6.9)	−4.6 (7.6)	−4.8 (6.6)	0.092	0.058
Fat (%)	−3.8 (2.3)	−2.5 (2.4)[a]	−1.7 (2.2)[a,b]	−1.6 (1.7)[a,b]	<0.001	<0.001
Subcutanous fat (cm)	−0.68 (1.0)	−0.59 (0.72)	−0.37 (0.95)	−0.40 (0.80)	0.372	0.172
Visceral fat (cm)	−2.17 (2.41)	−1.27 (2.06)	−0.84 (2.22)	−0.91 (1.96)	0.137	0.566
Clinical measurements						
Fasting plasma glucose (mmol/L)	−0.74 (1.14)	−0.48 (0.81)	−0.11 (0.47)	0.02 (0.59)[a,b,c]	<0.001	0.010
2 h plasma glucose (mmol/L)	−3.7 (1.9)	−1.6 (1.2)[a]	−0.3 (1.1)[a,b]	1.1 (1.8)[a,b,c]	<0.001	<0.001
AUC glucose (mmol min/L)	−484 (113)	−239 (56)[a]	−35 (65)[a,b]	155 (72)[a,b,c]	<0.001	<0.001
AUC insulin ($\cdot 10^3$ pmol min/L)	−60.2 (54.1)	−31.4 (38.0)[a]	−16.1 (26.7)[a,b]	−2.9 (25.7)[a,b,c]	<0.001	<0.001
Matsuda index	2.47 (2.61)	1.45 (1.85)	0.53 (1.62)[a,b]	−0.11 (2.14)[a,b]	<0.001	<0.001
Insulinogenic index	17.7 (44.2)	31.8 (121.8)	−31.9 (149.1)	−55.1 (204.7)[b]	0.038	0.041
Systolic blood pressure (mmHg)	−1.2 (11.3)	−5.1 (17.2)	−5.4 (15.6)	−3.6 (16.1)	0.761	0.139
Diastolic blood pressure (mmHg)	−3.0 (10.5)	−4.4 (12.1)	−3.7 (12.3)	−4.5 (11.0)	0.957	0.735
Total cholesterol (mmol/L)	−0.71 (1.08)	−0.35 (0.97)	−0.16 (1.05)	−0.17 (0.82)	0.186	0.113
Triglycerides (mmol/L)	−0.56 (1.02)	−0.26 (0.66)	−0.15 (0.70)	−0.08 (0.52)	0.090	0.930
HDL cholesterol (mmol/L)	−0.01 (0.21)	0.01 (0.26)	0.02 (0.26)	−0.06 (0.22)	0.382	0.465
Fitness and physical activity						
VO_2max (ml/kg/min)	3.8 (8.0)	2.7 (3.7)	3.1 (4.6)	1.9 (4.5)	0.359	0.304
Exercise time (mins)	3604 (2035; 5827)	2585 (1485; 3560)	2318 (1271; 3615)	2120 (1328; 3550)	0.123	–

Data are estimated changes (95% CI). P_1, p-value for overall unadjusted test of difference between clusters. P_2, overall test adjusted for the baseline level of the covariate. Pairwise tests of difference between clusters adjusted for the baseline level of the covariate: [a]*$P < 0.05$ vs. C1.*
[b]*$P < 0.05$ vs. C2.*
[c]*$P < 0.05$ vs. C3.*

circumference (−0.33) and visceral fat (−0.31). The highest loadings in the second principal component (PC2), comprising 11.4% of total variation, were $AUC_{glucose}$ (−0.52) and 2 h blood glucose (−0.48), fasting plasma glucose (0.45), and insulinogenic index (0.31).

DISCUSSION

The main findings from this study confirm the overall positive impact of a lifestyle intervention on a group at high risk of developing type 2 diabetes (Tuomilehto et al., 2001; Knowler et al., 2002; Lindström et al., 2006; Ramachandran et al., 2006; Li et al., 2008) but highlight the challenges identifying sub-groups of individuals that are likely to respond or not respond. Our data demonstrate that when participants were categorized by glycemic status or changes in glucose tolerance following the intervention, it was not possible to identify a set of phenotypic characteristics that could differentiate sub-groups.

Following the 12 weeks intervention, most subjects had decreased BMI, waist circumference and body fat (80–89%) and increased aerobic fitness (72%). However, in agreement with others (Solomon et al., 2013; Stefan et al., 2015), we found a lesser proportion of subjects with improved fasting plasma glucose (64%), 2 h glucose (60%), and AUC glucose (62%). Sparks (2017) argues that a sizeable proportion of individuals do not respond to exercise training and the outcome depends on the variable selected. It is also possible that changes in total daily activity or

TABLE 4 | Baseline characteristics based on the clusters of change in $AUC_{glucose}$ (imputed data).

	Responders		Non-responders		
	C1 HI-RES	**C2 MOD-RES**	**C3 NO-RES**	**C4 DET-RES**	***P***
N	17	57	126	57	
Mean (SD) of change in $AUC_{glucose}$	−485 (101)	−236 (52)	−34 (63)	161 (82)	
Range of change in $AUC_{glucose}$	−756; −368	−367; −157	−156; 70	71; 401	
Age (year)	53.4 (11.0)	53.6 (11.5)	52.9 (11.2)	57.9 (8.7)	
Male sex (%)	65	53	51	40	
NGT (%)	6	18	37	47	
i-IFG (%)	18	42	44	39	
IGT (%)	29	26	15	7	
T2DM (%)	47	14	4	7	
Body composition					
Weight (kg)	96.0 (13.7)	89.5 (16.5)	91.3 (18.9)	84.2 (17.2) [a,c]	0.038
BMI (kg/m^2)	33.4 (4.1)	30.9 (4.9)	31.2 (5.6)	30.4 (6.0)	0.262
Waist circumference (cm)	111.2 (11.3)	103.2 (10.7)	105.1 (12.3)	101.9 (14.0)	0.070
Fat (%)	39.7 (6.9)	37.9 (8.8)	37.2 (8.4)	38.4 (9.4)	0.656
Subcutanous fat (cm)	3.0 (1.2)	3.0 (1.3)	2.9 (1.2)	2.7 (1.0)	0.679
Visceral fat (cm)	9.4 (2.1)	7.5 (2.3)[a]	7.0 (2.1)[a]	7.0 (2.2)[a]	0.002
Clinical measurements					
Fasting plasma glucose (mmol/L)	6.6 (1.7)	6.2 (1.0)	5.7 (0.6)[a,b]	5.8 (0.7)[a,b]	<0.001
2 h plasma glucose (mmol/L)	10.3 (3.7)	7.7 (2.3)[a]	6.3 (1.7)[a,b]	6.2 (1.5)[a,b]	<0.001
AUC glucose (mmol min/L)	1668 (469)	1332 (295)[a]	1127 (214)[a,b]	1118 (220)[a,b]	<0.001
AUC insulin ($\cdot 10^3$ pmol min/L)	110.0 (89.1; 128.9)	76.7 (58.8; 122.8)	71.5 (45.9; 103.4)[a]	61.0 (42.5; 91.2)[a,b]	0.006
Matsuda index	1.7 (1.3; 2.3)	2.3 (1.7; 3.9)[a]	3.0 (2.1; 4.7)[a,b]	3.6 (2.8; 5.3)[a,b]	<0.001
Insulinogenic index	112.7 (68.9; 130.4)	113.5 (66.7; 168.1)	124.8 (83.8; 203.2)	103.5 (69.3; 185.9)	0.162
Systolic blood pressure (mmHg)	141.6 (12.9)	136.5 (16.6)	133.7 (13.4)	133.5 (16.6)	0.174
Diastolic blood pressure (mmHg)	84.6 (8.5)	83.3 (10.6)	82.3 (10.4)	82.6 (10.0)	0.832
Total cholesterol (mmol/L)	5.5 (1.0)	5.3 (1.3)	5.4 (1.6)	5.3 (1.0)	0.958
Triglycerides (mmol/L)	1.8 (1.3; 2.8)	1.4 (0.9; 1.9)[a]	1.2 (0.9; 1.6)[a]	1.1 (0.8; 1.5)[a,b]	0.004
HDL cholesterol (mmol/L)	1.1 (0.2)	1.2 (0.3)	1.3 (0.4)	1.4 (0.3)[a,b]	0.007
Fitness and physical activity					
VO_2max (ml/kg/min)	28.8 (6.2)	29.8 (7.3)	29.2 (8.2)	28.0 (7.5)	0.635
Exercise time (mins)	3604 (2035; 5827)	2585 (1485; 3560)	2318 (1271; 3615)	2120 (1328; 3550)	0.123

Data are means (SD) or medians (interquartile range). P, p-value for overall unadjusted test of difference between clusters. Skewed distribution data were log-transformed prior to the test.
[a] $P < 0.05$ vs. C1.
[b] $P < 0.05$ vs. C2.
[c] $P < 0.05$ vs. C3.

sedentary time over the course of the intervention might play a role. However, there are many factors that can influence the inter-individual variability in response to a lifestyle intervention (Solomon, 2018) and it is still a matter of discussion whether variables should be isolated and their contribution analyzed or if it would be more effective to identify molecular or metabolic biomarkers to collectively account for the overall variance.

Previous research has suggested the differences in response to a lifestyle intervention could be due to the inclusion of different prediabetic glycemic categories (Schafer et al., 2007; Malin et al., 2012, 2013; Solomon et al., 2015) since the pathophysiology of progression to type 2 diabetes may differ based on the glycemic status (Faerch et al., 2016). However, despite differences in the baseline glycemic status of our participants, the average improvement was similar between groups and comparable with diabetes prevention studies in the literature (Tuomilehto et al., 2001; Lindström et al., 2006). These data are supported by Malin et al. (2012, 2013) who found no differences in the responses between glycemic groups to an exercise intervention (Malin et al., 2012) and that the change in fasting plasma glucose was the only variable to differ between groups (Malin et al., 2013). Similar results have been reported for a 9 month intervention (Schafer et al., 2007) where NGT and IGT subjects decreased body weight, visceral and liver fat with improved insulin sensitivity. Collectively, these findings demonstrate a similar clinical and metabolic responses to a lifestyle intervention in different glycemic groups.

The cluster analysis identified four distinct sub-groups including high (HI-RES) and moderate (MOD-RES) responders, a group that were unchanged (NO-RES) and one that had

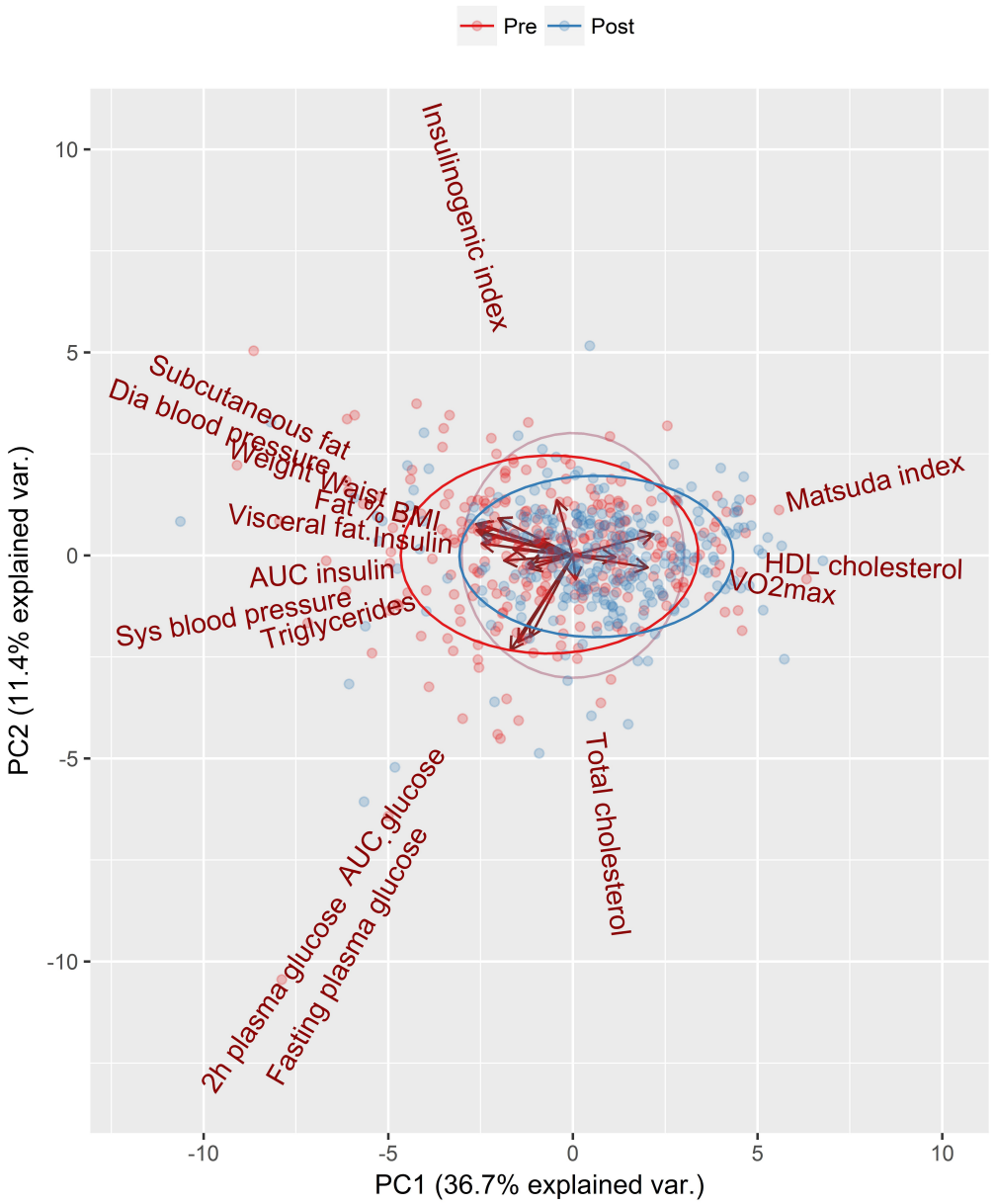

FIGURE 2 | The first two principal components of the data, showing a multivariate change over the 12 weeks intervention. The first and second principal components (PC1 and PC2) are shown on the x-axis and y-axis, respectively. The loadings of the variables are shown with arrows, and the scores of the participants are shown as dots in the background with red (pre) and blue (post-intervention). Arrows pointing to the right (on the x-axis) and upwards (on the y-axis) illustrate a positive change score, e.g., Matsuda index and VO_2max (l/min). Arrows pointing to the left (on the x-axis) and downwards indicate a negative change score.

deteriorated (DET-RES) glucose tolerance. Other studies found that 15–20% of individuals with type 2 diabetes do not improve glucose tolerance (Sparks et al., 2013) and that ∼30% with IGT/T2DM do not change blood glucose following exercise training (Solomon et al., 2013; Stefan et al., 2015). Our results are comparable despite the larger number of NGT and i-IFG subjects in the present study. The baseline characteristics were similar between the four clusters, despite differences in $AUC_{glucose}$ following the intervention. Those that deteriorated (Cluster 4) had a lower body weight, triglycerides and HDL cholesterol but were not sufficient to identify a set of characteristics that would predict a change in glucose tolerance following a lifestyle intervention.

The main differences in response to the intervention were noted between the HI-RES group (Cluster 1) who achieved the greatest amount of weight loss but only accounted for 6% of subjects (**Table 3**). Approximately 75% of the participants fell into the MOD-RES (Cluster 2) and NO-RES (Cluster 3) groups. Apart from body fat there were no differences in the clinical or physiological variables between these two groups. The DET-RES

TABLE 5 | Loading of the variables in the two principal components, sorted by the magnitude of the loadings in PC 1.

Variable	PC1	PC2
Waist circumference	−0.330	0.150
BMI	−0.328	0.142
Weight	−0.318	0.175
% fat	−0.316	0.122
Visceral fat	−0.310	0.0675
Matsuda index	0.274	0.118
VO_{2max} ml/kg/min	0.255	−0.0664
Subcutaneous fat	−0.254	0.206
AUC insulin	−0.234	−0.0281
AUC glucose	−0.213	−0.524
Insulin	−0.202	0.0343
Blood glucose_120 min	−0.187	−0.481
BP Systolic	−0.152	−0.0484
Triglycerides	−0.148	−0.0717
Fasting plasma glucose	−0.145	−0.452
Cholesterol (HDL)	0.144	−0.0089
BP Diastolic	−0.128	0.0833
Insulinogenic index	−0.0540	0.306
Cholesterol (total)	−0.0097	−0.135

group had a smaller weight and body fat reduction than the HI-RES and MOD-RES groups. However, these "non-responders," in terms of glucose tolerance, still had an improvement in all clinical and physiological variables, highlighting the difficulties differentiating individuals most likely to improve their glycemia. It was notable that there was a greater proportion of men in the HI-RES cluster (65%) and women in the DET-RES cluster (60%), with similar sex distribution in MOD-RES and NO-RES. Further research will be required to determine if men and women are more likely to respond to a lifestyle intervention but we cannot rule out a potential confounding effect on the findings.

Other possible explanations for variability of response phenotypes have included baseline insulin secretory capacity (Solomon et al., 2013), insulin resistance or the presence of non-alcoholic fatty liver disease (Stefan et al., 2015, 2016). Using estimates derived from the OGTT we did not observe differences in the response of insulin sensitivity or insulin secretion between the glycemic groups, however, a hyperglycemic clamp is more sensitive than an OGTT in this regard. The 4 clusters were based on change in glucose tolerance so it is not surprising the two "responder" groups were more insulin sensitive. There was no pattern in the insulinogenic index at baseline, or in response to the intervention, to suggest a primary role for compromised insulin secretion.

Principal Component Analysis was used to summarize the multivariate data. The standard deviation ellipses support the overall positive group response with the observed shift to the right (**Figure 2**). All parameters were included in this analysis, yet only 48% of the variance could be explained. The changes in body weight or composition are often reported to demonstrate the effectiveness of a lifestyle intervention, or explain the response, but PC1, made up mostly of body composition variables, only accounted for 37% of the overall variance. PC2 contained a mix of variables linked to glucose tolerance and VO_2max that only accounted for ∼11% of the variance.

These findings are consistent with several published studies (Thamer et al., 2007; Totsikas et al., 2011; Solomon et al., 2013; Mann et al., 2014). Solomon et al. (2013) reported correlations between changes in insulin secretion and changes in glycemia that explain ∼6–16% of the variance. Thamer et al. (2007) found associations with BMI, visceral adipose tissue and leg fat that explain ∼8–16% of the variance in insulin sensitivity, while Totsikas et al. (2011) found an association between a high anaerobic threshold at baseline and the prediction of improvements in insulin sensitivity that explained ∼4% of the variation. Thus, while there are significant associations between changes in phenotypic characteristics and glycemic outcomes, only small amounts of the overall variance can be explained; and even when combined in multivariate analysis still less than half of the variance is accounted for, highlighting the need for better predictors of improvements in glycemic status.

CONCLUSION

In conclusion, we report a broad range of individual responses in individuals at high risk for type 2 diabetes following a 12 weeks lifestyle intervention. We found that the standard clinical and physiological variables were not sufficient to predict the responsiveness to an intervention in the majority of individuals. In agreement with Solomon (2018) we believe there is a need for additional biomarkers to complement standard clinical measures that help predict blood glucose responses to a lifestyle intervention.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

JJN and DO'G conceived and designed the study. GO'D and AK were involved in trial project management, recruitment, and acquisition of all data. SC, ED, HD, PF, HK, NM, EM, JN, MW were involved in data acquisition. GO'D, DO'G, and JJN wrote the manuscript. AK and KF researched data and contributed to the discussion. GA, DV, and TS performed the statistical analysis. All authors reviewed and edited the manuscript. DO'G was the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00317/full#supplementary-material

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Cellular Aspects of Muscle Specialization Demonstrate Genotype – Phenotype Interaction Effects in Athletes

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Introduction: Gene polymorphisms are associated with athletic phenotypes relying on maximal or continued power production and affect the specialization of skeletal muscle composition with endurance or strength training of untrained subjects. We tested whether prominent polymorphisms in genes for angiotensin converting enzyme (ACE), tenascin-C (TNC), and actinin-3 (ACTN3) are associated with the differentiation of cellular hallmarks of muscle metabolism and contraction in high level athletes.

Methods: Muscle biopsies were collected from *m. vastus lateralis* of three distinct phenotypes; endurance athletes (n = 29), power athletes (n = 17), and untrained non-athletes (n = 63). Metabolism-, and contraction-related cellular parameters (such as capillary-to-fiber ratio, capillary length density, volume densities of mitochondria and intramyocellular lipid, fiber mean cross sectional area (MCSA) and volume densities of myofibrils) and the volume densities of sarcoplasma were analyzed by quantitative electron microscopy of the biopsies. Gene polymorphisms of ACE (I/D (insertion/deletion), rs1799752), TNC (A/T, rs2104772), and ACTN3 (C/T, rs1815739) were determined using high-resolution melting polymerase chain reaction (HRM-PCR). Genotype distribution was assessed using Chi^2 tests. Genotype and phenotype effects were analyzed by univariate or multivariate analysis of variance and *post hoc* test of Fisher. P-values below 0.05 were considered statistically significant.

Results: The athletes demonstrated the specialization of metabolism- and contraction-related cellular parameters. Differences in cellular parameters could be identified for genotypes rs1799752 and rs2104772, and localized *post hoc* when taking the interaction with the phenotype into account. Between endurance and power athletes these concerned effects on capillary length density for rs1799752 and rs2104772, fiber type distribution and volume densities of myofibrils (rs1799752), and MSCA (rs2104772). Endurance athletes carrying the I-allele of rs1799752 demonstrated 50%-higher volume densities of mitochondria and sarcoplasma, when power athletes that carried only the D-allele showed the highest fiber MCSAs and a lower percentage of slow type muscle fibers.

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Discussion: ACE and tenascin-C gene polymorphisms are associated with differences in cellular aspects of muscle metabolism and contraction in specifically-trained high level athletes. Quantitative differences in muscle fiber type distribution and composition, and capillarization in knee extensor muscle explain, in part, identified associations of the insertion/deletion genotypes of ACE (rs1799752) with endurance- and power-type Sports.

Keywords: muscle, athlete, myofibril, mitochondria, capillary, gene, angiotensin, tenascin

INTRODUCTION

Skeletal muscle is a critical determinant of Sports performance. This influence has its origin in the force producing capacity of contracting muscle which is fueled by the conversion of metabolic substrates. These relationships are specifically evident for muscle output at the extremes of the power spectrum, which characterize the capacity for producing maximal power and the capacity to sustain power output (i.e., fatigue resistance) (Flueck, 2010). In a healthy, able-bodied and motivated subject (Hegner, 2012; Speckmann et al., 2015), both capacities are to a considerable degree set by the structural characteristics of skeletal muscle: on one side maximal power and force is related to cross-sectional area and the volume density of myofibrils of muscle fiber (types), and on the other fatigue resistance (or endurance) is related to the capillarization, and the volume densities of mitochondria and intramycellular lipid in muscle fibers (Vock et al., 1996; Weibel, 1996).

The performance of skeletal muscles can be specifically developed (or maximized) by physical training-through a feed forward mechanism that remodels muscle composition. In this respect, maximal performance is usually improved with a high-load, low-repetitive type of training whereas fatigue resistance is ameliorated to a considerable extent by a highly-repetitive low-load type of training (Wernbom et al., 2007; Flueck and Eilers, 2010). The respective functional improvement can be explained in terms of quantitative and qualitative structural adaptations at the level of muscle organelles that set contractile function and metabolic supply. Accordingly, an improvement in maximal power can represent an increase in the cross-sectional area of muscle fiber (types) and increased volume density of myofibrils (Speckmann et al., 2015). On the contrary, an increase in fatigue resistance can be explained by increases in capillary-to-fiber ratio or capillary length density in locomotor muscle, in conjunction with an elevated volume density of mitochondria and intramyocellular lipid (Oberholzer et al., 1976; Ingjer, 1979; Speckmann et al., 2015).

There is classical evidence that muscle composition and muscle performance (i.e., peak force and endurance) and systemic components of endurance performance (i.e., maximal oxygen uptake, VO_2max) are influenced by heritable factors (Klissouras et al., 1973; Simoneau and Bouchard, 1995; Bouchard et al., 1999; Silventoinen et al., 2008). Natural sequence variants in genes, i.e., gene polymorphisms, have been identified to be associated with specific athletic traits, i.e., power and endurance (Yang et al., 2003; Bray et al., 2009; Aleksandra et al., 2016). Polymorphisms in the genes for actinin-3 (ACTN3; rs1815739), myostatin (rs1805086 and rs1805065), angiotensin converting enzyme (ACE; rs1799752), and tenascin-C (TNC; rs2104772) have been found to be associated with differences in muscle composition and/or muscle volume in untrained and moderately trained individuals and adaptations in muscle composition with endurance training (Vincent et al., 2007; Santiago et al., 2011; Vaughan et al., 2013; Li et al., 2014; Vaughan et al., 2016; Valdivieso et al., 2017a). Specifically the I-allele represented by polymorphism rs1799752, which characterizes the insertion of a silencer region within intron 16 of the ACE gene, is found to be associated with superior trainability of endurance performance compared to subject being characterized by the homozygous deletion of the silencer region (i.e., ACE-D/D genotypes; (Woods et al., 2000; Flueck et al., 2010). ACE I-allele carriers (i.e., ACE-I/I and ACE-I/D genotypes) have been found to demonstrate lower ACE transcript expression in skeletal muscle, and reduced serum levels of the encoded ACE, and its product, the major vasoconstrictor angiotensin 2, than ACE-D/D genotypes (Rigat et al., 1990; Vaughan et al., 2013; Mathes et al., 2015; Vaughan et al., 2016). Consequently ACE-I/I and ACE-I/D genotypes show increased capillary perfusion during exhaustive endurance exercise when capillary perfusion is not improved in ACE-D/D genotypes (van Ginkel et al., 2015). The reportedly reduced improvement of blood supply in ACE-D/D compared to ACE-I/I and I/D genotypes is related to an inefficient import of serum glucose, perturbed mitochondrial metabolism (Vaughan et al., 2016), and lower transcript expression of lipid and glucose metabolism-associated factors in knee extensor muscle of ACE-D/D genotypes at the end of exhaustive endurance exercise (Mathes et al., 2015). Consistently, ACE I-allele carriers are found to demonstrate greater increases in the volume density of subsarcolemmal mitochondria, and intramyocellular lipid stores in *m. vastus lateralis* after endurance training (Vaughan et al., 2013). The relevance of the ACE-related response of *m. vastus lateralis* to endurance exercise, and the contribution of its genetic inhibition via the ACE I-allele, is corroborated by the effects of pharmacological ACE inhibition on metabolism-related transcript in *m. vastus lateralis* post endurance exercise and training (Zoll et al., 2006; van Ginkel et al., 2016), and the fact that this shift in transcript expression is modulated by the ACE-I/D gene polymorphism (Mathes et al., 2015). For instance, oral intake of the ACE inhibitor lisinopril increased

Abbreviations: ACE, angiotensin converting enzyme; ACTN3, actinin-3; HRM-PCR, high resolution melt analysis polymerase chain reaction; MCSA, mean cross sectional area; TNC, tenascin-C; VO_2max, maximal oxygen uptake.

transcript levels of the shear stress-related pro-angiogenic factors VEGF and tenascin-C in *m. vastus lateralis* when the levels of hypoxia-related mitochondrial transcripts were lowered (van Ginkel et al., 2015). Additionally, ACE I-allele carriers are found to demonstrate a higher cross-sectional area of *m. vastus lateralis* and embedded muscle fibers compared to endurance-trained ACE-D/D genotypes (Vaughan et al., 2016; Valdivieso et al., 2017b). Similarly, polymorphism rs2104772 being characterized by the non-synonymous exchange of thymidine (T)-to-adenosine (A) in amino acid codon 1677 of tenascin-C has been found associated with higher volume densities of mitochondria and higher gains in capillary-to-fiber ratio in *m. vastus lateralis* with endurance exercise (Valdivieso et al., 2017a). These differences were related to lowered muscle levels of tenascin-C protein in T/T homozygotes respective to A-nucleotide carriers, reproducing the negative effects of a lowered tenascin-C expression on activity-induced angiogenesis as seen in anti-gravity muscles of tenascin-C deficiency transgenic mice (Fluck et al., 2008). As well, ACE I-allele carriers (i.e., polymorphism rs1799752; Zhang et al., 2003; Fluck et al., 2008) and T/T-genotypes of polymorphism rs1805086 in the ACTN3 gene (Vincent et al., 2007), being characterized by the absence of ACTN3 protein, have been found to demonstrate a higher percentage of slow type muscle fibers in healthy untrained subjects, and this corroborates with observations in the respective transgenic deficient animals (Zhang et al., 2005; MacArthur et al., 2008).

Endurance exercise during months of training has been identified to modify certain genotypic influences on muscle composition. For instance, in healthy subjects endurance training has been found to affect – and in part overrule – the influence of the ACE-I/D genotype on the concentration of metabolic substrates and transcript expression (Valdivieso et al., 2017b). As this involves certain signaling processes of muscle plasticity, i.e., the pro-angiogenic factor VEGF (discussed in Valdivieso et al., 2017b), genetic influences being reported in untrained individuals may not linearly translate to well- and highly-trained subjects. This may specifically apply to the adaptive mechanisms in high level athletes, whose muscles are subjected to the impact of high loads and intensities during years of training and competition. This influence may outweigh the influence of a single genetic factor (discussed in Frese et al., 2016; Leonska-Duniec et al., 2016).

It has not been investigated whether differences in muscular performance in an elite athlete population are related to genetic influences on the composition of muscle fibers. Taking into account the immediate relevance of muscle fiber composition for muscle performance (Hoppeler, 1986; Weibel, 1996; Vock et al., 1996; Flück and Hoppeler, 2003; Zierath and Hawley, 2004; Harber and Trappe, 2008), adding insights into the interrelationships between fitness-associated gene polymorphisms and muscle composition would be an important complement to understand individual responses to training. It may allow developing a future personalized approach to physical training which takes the trainability of metabolic and contractile traits of muscle performance into account. The aim of our investigation was therefore to test whether differences in cellular aspects of muscle metabolism and contraction in high level endurance- and power-athletes are associated with either of the three genotypes (rs1799752, rs2104772, rs1815739), as this has in part been shown for untrained or moderately-trained subjects, and transgenic animals, and to assess to what extent this depends on the athletic phenotype. We chose to investigate the knee extensor muscle, *m. vastus lateralis*, due to its involvement in propulsion and established biologically relevant linear relationships between its composition and systemic/physiological measures of exercise performance (Tihanyi et al., 1982; Valdivieso et al., 2017a; Pollock et al., 2018; van der Zwaard et al., 2018). Based on previous findings in healthy subjects we expected that the ACE-I/D and TNC gene polymorphisms, respectively rs1799752 and rs2104772, would affect (aerobic) metabolism- and contraction-related structural aspects of muscle, which are conditioned by endurance training. Conversely, we expect that the ACTN3 genotype (rs1815739) would affect the cellular composition of muscle fibers related to contractile function, assuming that this would be more evident in power athletes.

MATERIALS AND METHODS

Study Design

The present study was carried out in the Laboratory for Muscle Plasticity at the research center Balgrist Campus of the Balgrist University Hospital. Frozen biopsies from the *m. vastus lateralis* muscle of high level competitive athletes from endurance- and power-type Sports were collected during the athletes' active phase, and healthy untrained individuals (i.e., non-athletes) were genotyped according to the polymorphisms for the insertion/deletion allele of ACE gene (ACE-I/D, rs1799752) and single nucleotide polymorphisms in the genes for ACTN3 (C/T rs1815739) and TNC (A/T, rs2104772). The samples stem from various published and unpublished studies for which data on muscle composition, age, gender, and aerobic characteristics of performance, and the level of athletic specialization were available (**Supplemental Table S1**; Hoppeler, 1986; Luthi et al., 1986; Hochli et al., 1995; Suter et al., 1995; Billeter et al., 2003). Additionally, fiber typing has been performed for the purpose of this investigation on frozen material.

Ethics Statement

The study was conducted in accordance with the Helsinki declaration for research on humans. The gathering of physiological and cell biological meta-data was conducted with permission of the Ethics committee of the canton of Berne. Genotyping was carried out and analyzed anonymously under application of the non-competence rule of the Human Research Act after clarification by the ethics committee of the canton of Zurich.

Subjects

The sample consisted of 30 elite white Caucasian athletes from endurance-type (professional cyclists, 100 km runners) and 17 white Caucasian power- type (shot-putters, weight lifters) Sports that lived in Switzerland. The athletes included

participants and winners of international competitions, world championships and Olympic Games; and ranked at the top of their respective disciplines. The untrained individuals (i.e., non-athletes) were recruited previously from employees and students being associated with the Universities of Berne or Fribourg. Further prerequisites for inclusion in the study were the presence of sufficient biopsy material for genotyping and well-documented data on skeletal muscle composition. **Table 1** shows the subject characteristics.

Muscle Composition

Biopsies were characterized for muscle ultrastructure with an established morphometric technique based on electron micrography of glutaraldehyde-fixed muscle biopsies (Schmutz et al., 2006). The following parameters of the athletes were analyzed and have in part been reported (Hoppeler, 1986; Hochli et al., 1995; Billeter et al., 2003): Capillary-to-fiber ratio, capillary length density, mean fiber cross sectional area, myofibrillar volume density, mitochondrial volume density, sarcoplasmic volume density, intramyocellular lipid density. Sarcoplasmic volume density was defined as structures internal to muscle fibers which did not represent myofibrils, mitochondria and intramyocellular lipid; therefore representing the sum of the membranous structures (sarcoplasmic reticulum, Golgi-apparatus), cytosol and myonuclei (Horber et al., 1987; Vock et al., 1996; Weibel, 1996). The distribution of slow and fast type muscle fiber types was determined based on histochemical ATPase staining or immunofluorescence as described (**Supplemental Figure S1**; Billeter et al., 2003; Fluck et al., 2019). The fast fiber population was not differentiated into the different subtypes. The comparison of values for the histochemical and immunological detection of slow and fast fiber types in consecutive sections revealed a mean error or measurement of less than 1%.

DNA Extraction and Genotyping

For DNA extraction and genotyping, the collected muscle tissue from the *m. vastus lateralis* was used. If for some reason the biopsy from the *m. vastus lateralis* was not usable, samples from the *m. deltoideus* were used. All muscle biopsies were stored at minus 80°C. In order to collect 5 mg of muscle tissue, 25 μm cryosections were taken at −25°C. If it was not possible to collect 5 mg of tissue in certain biopsies, an attempt was made to continue with the available amount of muscle tissue in the next working steps. Experience shows that reliable genotyping can also be carried out with a smaller amount of muscle tissue. The samples were then stored at minus 80°C until the DNA extraction step. The DNA was extracted using DNeasy Blood & Tissue Kit (Cat. No 69504, Qiagen, Basel, Switzerland). In order to extract a high quantity of DNA for genotyping, several lysis, and washing steps followed by filtration by centrifugation were carried out.

TABLE 1 | Characteristics of the three phenotypes.

Parameter	Age (years)	Body mass (kg)
Endurance athlete (n = 30)	30.1 ± 5.7	64.5 ± 6.0
Power athlete (n = 17)	25.4 ± 10.1	88.6 ± 25.2
Non-athlete (n = 63)	29.5 ± 9.3	76.5 ± 12.8
p-value (3 phenotypes)	0.147	0.001
p-value (Endurance vs. Power)	0.455	< 0.001

Mean ± standard deviation of age and body mass of the studied endurance and power athletes, and non-athletes. Statistical analysis was conducted with univariate ANOVA with post hoc test of least significant difference.

For the high HRM-PCR, an identical procedure was used for all three investigated genotypes. The reaction mix was prepared with the KAPA HRM FAST Master Mix (KAPA BIOSYSTEMS, Labgene Scientific, Châtel-St-Denis, Switzerland) and specifically designed primers.

For detecting TNC SNP rs210477, two primers (5′-CAAAAAAAGCAGTCTCTGAGCCAC-3′ and 5′-TTCAGTAGTCTCTCTCTGAGAC-3′) were used as established (Valdivieso et al., 2017a) to amplify a 85 base pair long DNA fragment. For detecting ACTN3 SNP rs1815739, the primers ACTN3 forward (5′-CTGTTTGCCTGTGTGTAAGTGGGGGGG-3) and ACTN3 reverse (5'-TGTCACAGTATGCAGGAGGGG-3′) were used as established by North et al. (1999) to produce a 291 base pair long DNA fragment. For detecting the ACE gene I/D gene polymorphism rs1799752, two primer combinations were used to amplify the I- and D-allele as established (Vaughan et al., 2013). For the identification of the D-allele, the primer combination (ACE1 5′-CATCCTTTTCTCCCATTTCTC-3′ and ACE3 5′-ATTTCAGAGCTGGAATAAAATT-3′) amplified a gene fragment of 83 base pairs. The I-allele was detected by the primer combination (ACE2 5′-TGGGGATTACAGGCGTGATACAG-3′ and ACE3 5′-ATTTCAGAGCTGGAATAAAATT-3′), which amplified a gene fragment of 66 base pairs.

The specificity of the method was underpinned by controls. As positive controls, the genetic material of people already genotyped for these polymorphisms was used. The negative control was a non-template control reaction with H_2O. In addition, the reactions were always carried out in duplicate. HRM-PCR was performed using an Illumina ECO^{TM} real-time PCR system (Labgene Scientific, Châtel-St-Denis, Switzerland) with 42 cycles and described thermal settings for the PCR cycle for the respective primer pairs (North et al., 1999; Vaughan et al., 2013; Valdivieso et al., 2017a).

Gene polymorphisms were analyzed using EcoStudy software (Illumina, Labgene Scientific, Labgene Scientific, Châtel-St-Denis, Switzerland). Genotypes were identified by comparing the specific melting profiles of the samples to reference curves from the wild type, mutant and heterozygous allele, which genotypes had been identified by HRM-PCR and microsequencing. In the analysis of the ACE I/D PCR results, the amplification of the reaction mixtures with ACE I/I or ACE D/D primers was investigated. If a subject's sample was amplified in both primer mixtures, it was identified as a heterozygous ACE-I/D genotype. However, if the sample was amplified in only one of the primers, it could be assigned to the respective homozygous genotype. If no clear result was obtained despite repetition, the amplified samples were sent to an external laboratory (Microsynth, Balgach, Switzerland) for sequencing.

Statistics

Compliance with Hardy-Weinberg equilibrium was assessed using an MS-Excel based online calculator[1]. Other statistical analyses were performed with statistical software SPSS 19.0 (IBM, Chicago, IL, United States). The distribution of the investigated genotypes in relation to the phenotype (power athlete, endurance athlete, non-athlete) was compared with Chi^2 tests. Differences in muscle composition between genotypes (rs1799752, rs210477, rs1815739) and the phenotype were assessed with univariate analysis of variance (ANOVA) for single structural variables [capillary length density, capillary-to-fiber ratio, mean cross sectional area (MCSA)] and multivariate ANOVA (MANOVA) for inter-related structural variables (i.e., volume densities), respectively. Effects were localized using a *post hoc* test for the least significant difference. A p-value below 0.05 was considered as statistically significant. Sample size calculations were carried out with G*Power for a one-way ANOVA with fixed effects (Faul et al., 2007). Linear relationships were calculated based on Pearson correlations.

RESULTS

Subject Characteristics

Table 1 shows the characteristics of the endurance athletes, power athletes and non-athletes which muscle composition and genotypes were assessed. Body mass was 37%-higher in the power athletes than the endurance athletes, with the values for the non-athletes residing in between, i.e., 18% higher than in the endurance athletes.

The distribution of genotypes is shown in **Figure 1**. Over all phenotypes, rs1799752 ($p = 0.723$), but not rs2104772 ($p = 0.003$) and rs1815739 ($p = 0.036$), met the Hardy-Weinberg equilibrium. For rs2104772 the deviation from the equilibrium was also identified for the endurance athletes and non-athletes whereas for rs1815739 the deviation was identified only in non-athletes.

Neither genotype demonstrated a significant association with the phenotype.

Knee Extensor Muscle Composition Between Phenotypes

We subsequently tested whether differences would exist between phenotypes and genotypes for muscle fibers composition and associated capillaries in *m. vastus lateralis*. Significant differences between the phenotypes, especially when only the two athlete types were considered (**Table 2**), were identified for all assessed muscle parameters except capillary-to-fiber (**Figure 2**). Capillary length density, mitochondrial volume density, intramyofibrillar lipid droplet volume density, and sarcoplasmic volume density were 26, 54, 165, and 79%, respectively higher in endurance than in power athletes. Conversely, the MCSA of muscle fibers and the myofibrillar volume density were 17, and 13%, respectively higher in power athletes. The percentage of slow type muscle fibers in the endurance athletes tended to be 11% higher than in power athletes ($p = 0.07$).

[1]www.dr-petrek.eu/documents/HWE.xls

Differences in the cellular composition of muscle fibers and capillaries of athletes respective to non-athletes pointed in opposite direction for the endurance and power athletes for the MCSA of muscle fibers, and the volume density of myofibrils and sarcoplasma, respectively. The percentage of slow type muscle fibers in the endurance athletes was 17% higher than in non-athletes.

Genotype Effects on Muscle Specialization Are Influenced by the Phenotype

Figure 3 visualizes the statistical significance of genotype-related differences of muscle fiber composition and associated capillaries in *m. vastus lateralis* over the three genotypes and phenotypes. rs1799752 and rs2104772 gene polymorphisms affected the multivariate volume densities. Significant associations revealed for the interaction between the phenotype and gene polymorphism rs1799752 respective to the single cellular parameters, volume densities of myofibrils and sarcoplasma, and fiber type distribution (**Supplemental Table S2**).

Genotype × Phenotype Interactions in Athletes

When only considering the muscle composition of athletes, no differences between a genotype was identified (**Supplemental Table S3**). Power calculations exposed that the prospective replica number to identify differences in muscle composition between a genotype alone would have been in the hundreds.

When considering interaction effects between the athlete type and genotype, differences were identified for the two gene polymorphisms rs1799752 (fiber type distribution, volume densities of myofibrils and sarcoplasma, respectively) and rs2104772 (capillary length density and MCSA), but not rs1815739 (**Table 3**).

For rs1799752 the interaction effect on myofibrillar volume density could be localized to higher values in homozygous non-carriers of the ACE-I-allele (i.e., ACE-D/D genotypes) respective to ACE-I-allele carriers (i.e., heterozygous ACE-I/D and ACE-I/I genotypes) in endurance-trained athletes (**Figure 4**). Power-trained ACE-I-allele carriers demonstrated a higher percentage of slow type muscle fibers than those that were non-carriers of the I-allele. For capillary length density all three ACE-I/D genotypes showed higher values in those who were endurance-trained than the respective power-trained genotypes. On the *post hoc* level MCSA was higher in power-trained D/D genotypes than power-trained ACE-I-allele carriers and the respective endurance-trained D/D genotype. Sarcoplasmic volume density was higher in endurance-trained I/I genotypes than endurance-trained ACE-D-allele carriers and respective power-trained I/I genotype. As well higher volume densities of mitochondria were identified at the *post hoc* level in endurance athletes carrying the I-allele of rs1799752.

For rs2104772 the interaction effect on capillary length density could be localized to higher values in power-trained A/T than T/T genotypes and higher values for endurance-trained A/A

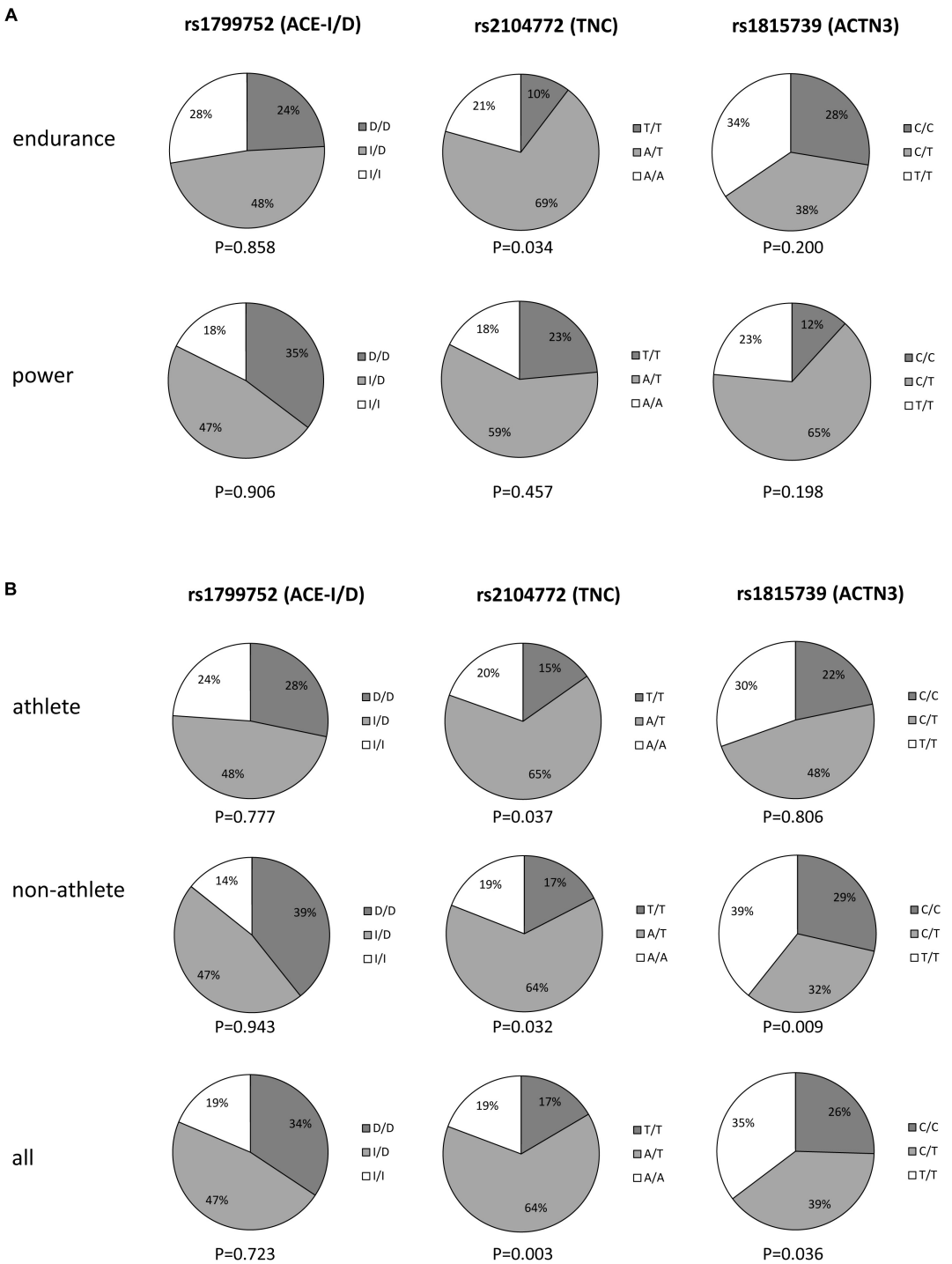

FIGURE 1 | Fractions of the individual genotypes within the phenotypes. Pie chart displaying the distribution of the three genotypes in the two athlete types **(A)**, and athletes, non-athletes and all subjects combined **(B)**. *p*-values below each chart refer to the respective significance of the test for Hardy–Weinberg equilibrium.

and T/T genotypes than the respective power-trained genotypes. For MCSA the effect could be localized to higher values in power-trained T/T compared to A/T genotypes (**Figure 5**). Additionally, MCSA was higher in power-trained T/T genotypes than the respective endurance-trained genotype.

A *post hoc* difference in the distribution of muscle fiber types was identified for the interaction between the rs1815739 (ACTN3) genotype and the athlete type. Thereby the percentage of slow type muscle fibers was higher in endurance-trained than power-trained T/T

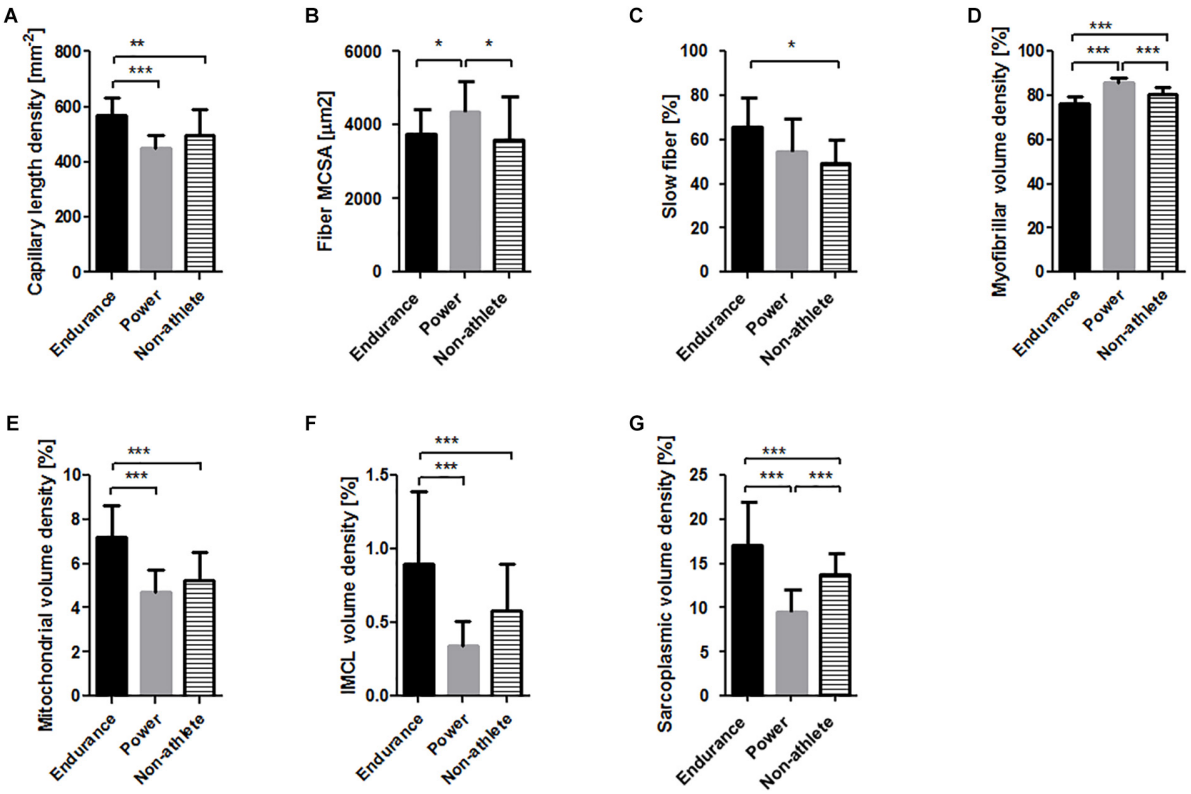

FIGURE 2 | *M. vastus* lateralis composition in the three phenotypes. Bar graphs visualizing the mean and standard deviation of the **(A)** capillary length density, **(B)** muscle fiber MCSA, **(C)** slow type fiber percentage, and volume densities of myofibrils **(D)**, mitochondria **(E)**, intramyofibrillar lipid **(F)** and sarcoplasma **(G)**. Asterisk indicates significant difference. $*p < 0.05$, $***p < 0.001$.

TABLE 2 | Differences between power and endurance athletes for the assessed muscle parameters.

Parameter	*p*-value	*F*-value	Effect size (η^2)
Capillary-to-fiber ratio	0.440	0.615	0.024
Capillary length density	**<0.001**	22.725	0.476
Fiber MCSA	**<0.001**	62.986	0.716
Fiber type distribution	0.069	3.671	0.149
Myofibrillar volume density	**0.035**	4.971	0.166
Mitochondrial volume density	**<0.001**	23.159	0.481
Intramyocellular lipid volume density	**0.002**	12.094	0.302
Sarcoplasmic volume density	**<0.001**	21.034	0.429

Statistical analysis was conducted with ANOVA or MANOVA. Significant p-values are printed in bold font.

genotypes ($p = 0.039$) but this was not revealed for the C/C genotypes ($p = 0.975$).

DISCUSSION

In the past decades it has become clear that genetic predisposition may present an advantage for training-induced muscle plasticity and this may affect strategies to enhance sports-specific performance, especially in athletes (Ghosh and Mahajan, 2016). Our investigation tested a number of implicit hypotheses on associations between selected genotype × phenotype interactions and the composition of knee extensor muscle (see **Supplemental Table S4**). We identified differences in muscle composition that were associated with an interaction between the phenotype and gene polymorphisms rs1799752 and rs2104772. With the current sample size genotype differences for individual cellular parameters were not resolved when the phenotype was not considered (**Supplemental Table S3**). The results, such as those for gene polymorphism rs1799752, confirm the higher volume density for mitochondria and intramyocellular lipid in I/I vs. D/D genotypes that was previously identified in endurance-trained Swiss white Caucasian subjects (Vaughan et al., 2013). Also, phenotype-related *post hoc* differences were identified for the distribution of muscle fiber types between genotypes of polymorphism rs1799752 and rs1815739 that are in line with the reported associations with the single genotypes in untrained subjects' muscle (see **Supplemental Table S4**; Zhang et al., 2003; Vincent et al., 2007). As well, we identified interaction effects between the phenotype and the gene polymorphism rs2104772, which for power athletes reproduced the expected lower muscle capillarity in T/T genotypes (Fluck et al., 2008). Interestingly, we also identified that the Hardy-Weinberg was not met for gene polymorphism rs2104772 ($p = 0.003$) in endurance athletes and non-athletes and for gene polymorphism rs1815739

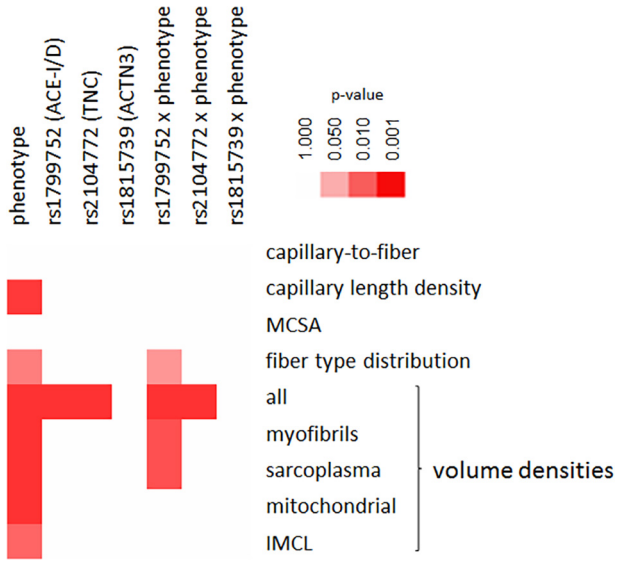

FIGURE 3 | Genotype × phenotype interactions on muscle composition. Color coded heat map visualizing the statistical significance of differences between the three studied genotypes × three phenotypes for parameters of muscle composition. The color scale used to rate the level of significance is indicated to the top right.

in non-athletes (p = 0.036). The latter observations indicate an influence of mitochondria-encoded traits that are asymmetrically inherited via the maternal line on the selection and/or genotype distribution for the studied non-athletes (Dionne et al., 1991; Eynon et al., 2011). This is the first investigation addressing interaction effects between athletic phenotypes and genotypes on muscle ultrastructure in a population of highly trained subjects. Our results indicate that in the investigated population of high level athletes, alike in moderately trained subjects (Vaughan et al., 2013, 2016; Valdivieso et al., 2017b), there is a genotype related difference in the specialization of critical cellular hallmarks of muscle performance with years of training and competition.

We identified a number of differences in the composition of muscle fibers in *m. vastus lateralis* between high level endurance and power athletes and relative to untrained healthy subjects (**Figure 2**). In general the findings confirmed previous results on muscle specialization with types of training and are in line with the fact that certain relative variables of muscle fiber composition (such as volume densities) in either athlete population do also distinguish from the respective values in the non-athletes (see **Figure 2**; Hoppeler, 1986). This concerned for instance, the higher percentage of slow type muscle fibers, and higher volume densities of mitochondria, intramyocellular lipid and sarcoplasma in the muscle fibers of endurance athletes. Together with the increased capillarization, these metabolism-related adjustments reflect the cellular factors underlying the locally enhanced aerobic capacity that explain the improved fatigue resistance, and higher velocity and specific power of slow and fast type muscle fibers in elite endurance-trained subjects (Flück and Hoppeler, 2003; Zierath and Hawley, 2004; Harber and Trappe, 2008). Conversely, the elevated volume density of myofibrils and MCSA of muscle fibers in power athletes exemplifies the cellular aspects that enhance power output with (single) muscle contractions. In this regard, we note that the electron microscopically-estimated fiber MCSAs, especially those of the power athletes, were smaller than reported previously based on light microscopy (Billeter et al., 2003), reflecting methodological differences related due to tissue processing where fiber diameter is reduced by 30% vs. the original size (Carlsen et al., 1961). As well, we note that the biopsies for the studied shot-putters and weight lifters were from a knee extensor muscle where larger differences in the muscle size and fiber MCSA would probably be evident in the arm muscles compared to the non-athletes.

While there is solid evidence for the enrichment of certain genotypes of polymorphisms rs1799752 and rs1815739 in athletic cohorts compared to the general population (Yang et al., 2003; Bray et al., 2009; Ma et al., 2013; Aleksandra et al., 2016), it has been concluded lately that genotypes rs1799752 and rs1815739 may not necessarily associate at the level of statistical significance when relevant parameters for endurance athletes, such as running times in competition, are considered or when athletes are matched to fit controls (Hruskovicova et al., 2006; Ash et al., 2011; Papadimitriou et al., 2018). The athlete type-dependent differences between genotypes of polymorphisms rs1799752 and rs1815739 (and rs2104772) indicate that the enrichment of the implicated genotypes in athletic populations is related to influences on the differentiation of cellular hallmarks of mechanical and metabolic function of muscle fibers (**Table 3** and **Figures 4**, **5**). In this respect a number of the identified genotype differences are in line with expectations on the association of the three gene polymorphisms with muscle composition seen previously in untrained or moderately trained subjects (see **Supplemental Table S4**). For instance, for rs1815739 a higher slow type muscle fiber percentage was identified at the *post hoc* level in endurance- respective to power-trained T/T-genotypes, which was not evident in the CC-genotypes. This observation is consistent with the reported association of the T/T-rs1815739 genotype with endurance athletes and fiber type distribution in untrained subjects (Yang et al., 2003; Vincent et al., 2007). Importantly, because identified at the level of a main statistical effect, for the endurance-trained homozygous I-allele carriers of rs1799752, being associated with endurance performance (Woods et al., 2001; Flueck et al., 2010), we detected higher mitochondrial volume densities than respective D-allele carriers (**Figure 4E**). As well, we localized the main association of the muscle fiber type distribution with the interaction of rs1799752 and the athlete phenotype to higher percentages of slow type fiber percentage in the *m. vastii* of I-allele carrying power athletes. However, in the endurance athletes we did not identify a rs1799752 related difference in muscle fiber MCSA of *m. vastus lateralis* (**Figure 4C**) as previously reported for moderately endurance-trained I-allele carriers (Valdivieso et al., 2017b), and identify in contrast to our expectation (see **Supplemental Table S4**) a lower volume density of myofibrils in endurance athletes being homozygous for I-allele carriers compared to D-allele

TABLE 3 | Interactions between genotype × athlete type on muscle composition.

Comparison	Parameter	*p*-value	*F*-value	Effect size (Eta^2)
Athlete type ×	Capillary-to-fiber ratio	0.582	0.555	0.048
rs1799752 (ACE)	**Capillary length density**	**0.023**	4.516	**0.291**
	Fiber MCSA	0.159	2.004	0.154
	Fiber type distribution	0.126	2.345	0.216
	Myofibrillar volume density	**0.025**	4.387	**0.285**
	Mitochondrial volume density	0.832	0.186	0.017
	Intramyocellular lipid volume density	0.234	1.545	0.114
	Sarcoplasmic volume density	0.056	3.258	0.214
Athlete type ×	Capillary-to-fiber ratio	0.879	0.130	0.012
rs2104772 (TNC)	**Capillary length density**	**0.012**	5.468	**0.332**
	Fiber **MCSA**	**0.029**	4.198	**0.276**
	Fiber type distribution	0.997	0.003	0.001
	Myofibrillar volume density	0.851	0.163	0.015
	Mitochondrial volume density	0.695	0.370	0.033
	Intramyocellular lipid volume density	0.952	0.050	0.004
	Sarcoplasmic volume density	0.938	0.065	0.005
Athlete type ×	Capillary-to-fiber ratio	0.985	0.015	0.001
rs1815739 (ACTN3)	Capillary length density	0.995	0.005	0.000
	Fiber MCSA	0.991	0.009	0.001
	Fiber type distribution	0.233	1.524	0.078
	Myofibrillar volume density	0.756	0.283	0.023
	Mitochondrial volume density	0.759	0.279	0.023
	Intramyocellular lipid volume density	0.884	0.124	0.010
	Sarcoplasmic volume density	0.550	0.013	0.049

Statistical analysis was conducted over the two athlete types with multivariate ANOVA or univariate ANOVAs with post hoc test of least significant difference. Significant p-values and their effect sizes are printed in bold.

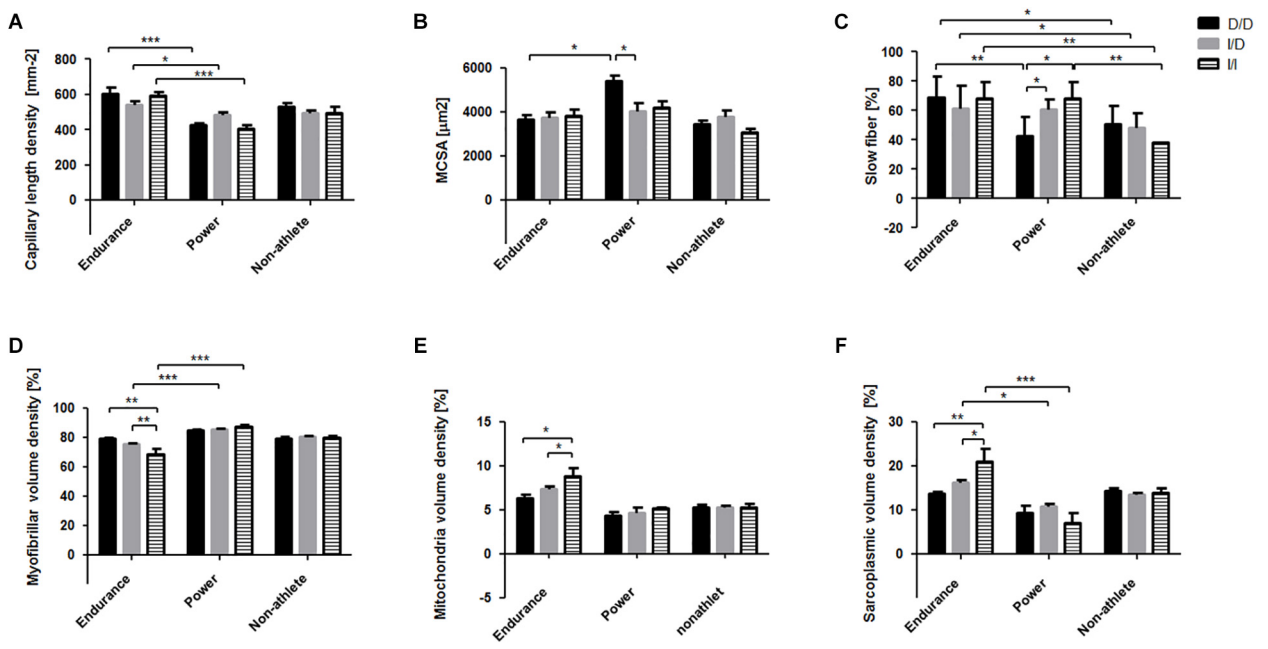

FIGURE 4 | Interaction effects between the phenotype and the rs1799752 (ACE) gene polymorphism on muscle composition. Bar graph of the mean and standard deviation for the investigated muscle characteristics for the rs1799752 genotypes in the two athlete types. For comparison, the values in the non-athletes are shown as well, but without the indication of statistically significant differences. **(A)** capillary length density, **(B)** fiber mean cross sectional area (MCSA), **(C)** slow type fiber percentage, and volume densities of myofibrils **(D)**, mitochondria **(E)**, and sarcoplasmia **(F)**. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ for the indicated differences.

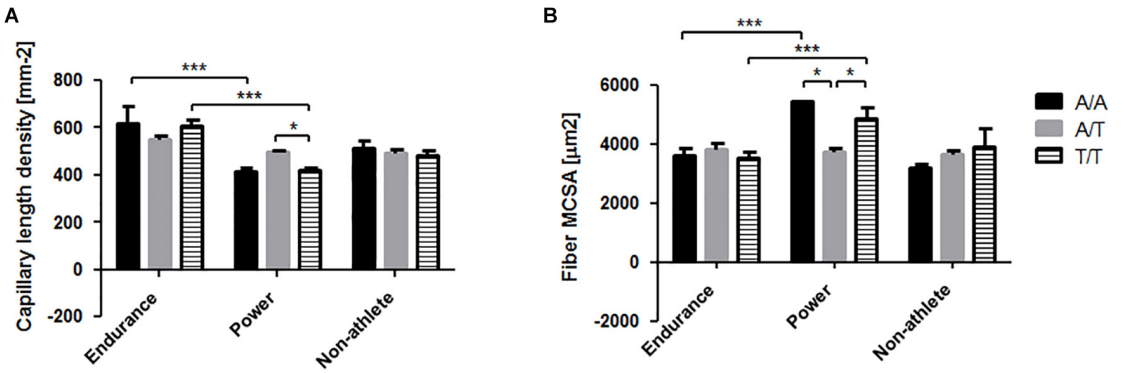

FIGURE 5 | Interaction effects between the phenotype and the rs2104772 (TNC) gene polymorphism on muscle composition. Bar graph of the mean and standard deviation for the investigated muscle characteristics for the distinct rs2104772 genotypes in the two athlete types. For comparison, the values in the non-athletes are shown as well, but without the indication of statistically significant differences. **(A)** capillary length density, **(B)** fiber MCSA. $*p < 0.05$ and $***p < 0.001$ for the indicated differences.

carriers. Consistently with the former association, a higher MCSA of muscle fibers was identified for power athletes with the ACE-D/D respective to the ACE-I/D genotype (**Figure 4D**). These findings suggest that the over-representation of D/D genotypes in power-type athletes (Woods et al., 2001; Flueck et al., 2010), is related to a negative association between the ACE-I allele and the concentration of myofibrils in muscle fibers in this phenotype, which reflects the main influence of the encoded ACE system on muscle fiber growth (Verbrugge et al., 2018). Meanwhile the rejection of the hypothesis, that endurance athletes carrying the I-allele would demonstrate an elevated fiber MCSA, emphasize that other factors explain the previous rs1799752-related differences in fiber MCSA of moderately endurance-trained British subject (Valdivieso et al., 2017b). It has been shown before that leg muscles (i.e., *m. vastus lateralis* and *m. gastrocnemius*) of world class shot-putters and hammer throwers are known to display a considerable range of muscle fiber compositions (Coyle et al., 1978; Terzis et al., 2010). Our observations in *m. vastus lateralis* extend this notion to suggest an association of the fiber type distribution in power athletes with gene polymorphisms in ACE and ACTN3. Collectively, our observations hint that the influence of gene polymorphism rs1799752 on the cross-sectional area (and volume) of muscle fibers and entire muscle depend on training-type modulated factors (Valdivieso et al., 2017b).

Furthermore, we identified a higher capillary length density in the *m. vastus lateralis* of A/T compared to T/T genotypes of rs2104772 for power athletes, reproducing similar genotype differences in the capillary-to-fiber ratio of moderately endurance-trained subjects (**Figure 5B**; Valdivieso et al., 2017a). As well, fiber MCSA was higher in T/T than A/T genotypes of rs2104772 (**Figure 5B**). The T/T genotype had previously been shown to demonstrate lower increases in tenascin-C protein levels in *m. vastus lateralis* with endurance training, highlighting a possible influence of tenascin-C on fiber MCSA that had been pointed out in anti-gravity muscles of tenascin-C deficient transgenic mice (Fluck et al., 2008). For rs1799752, only trends for a difference between genotypes of a given phenotype could be identified at the *post hoc* level for capillary length density in *m. vastus lateralis* (**Figure 4A**). For instance, in endurance athletes we observed tendinously lower values for capillary length density in I/D compared to D/D genotypes ($p = 0.069$). These observations in Swiss power athletes are consistent with our observations on similar genotype differences in capillary length density in *m. vastus lateralis* of endurance-trained healthy individuals of Swiss descent (Vaughan et al., 2013); they do however contrast findings on opposite associations in moderately endurance-trained white British subjects (Valdivieso et al., 2017b). Conversely, in power athletes capillary length density nearly tended to be lower in D/D genotypes than I/I genotypes ($p = 0.128$) of rs1799752. Overall, the athlete type modulated differences in capillary length density between genotypes of rs2104772 supports the notion of a mutual association between tenascin-C and the training-type specific adaptations in muscle capillarity. Specifically, the findings show that in power-trained A/T genotypes of rs2104772 there is a shift toward smaller fibers with better perfusion that are expected to have a metabolic advantage to carry out repeated contractions under a high metabolic flux (**Figure 5**). Meanwhile, the present findings do not allow us to reject the hypothesis that the ACE/angiotensin 2 system modifies vascular processes in exercised muscle, especially as this occurs in an interdependent manner with tenascin-C (reviewed in van Ginkel et al., 2016; Valdivieso et al., 2017a).

We identified that effects of the rs1799752 genotype were only resolved at the level of significance for muscle fiber type distribution, and the volume density of myofibrils and sarcoplasma in *m. vastus lateralis*, when interactions with phenotype were considered (**Table 3** and **Supplemental Table S2**). This observation reinforces that training-type related factors importantly modify the association of the studied ACE gene polymorphism with muscle composition (Valdivieso et al., 2017b). In this respect, it is important to consider that in moderately trained subjects both the rs1799752 (and rs2104772) gene polymorphisms are associated with a different expression response of angiogenic processes as well as those

of mitochondrial processes and amino acid metabolism in *m. vastus lateralis* subsequent to a single bout of endurance exercise (Vaughan et al., 2013, 2016; Valdivieso et al., 2017a,b). Collectively, the findings hint for a quantitative relevance of ACE-related genetic influences on the exercise-specific expression responses of skeletal muscle (Flueck, 2009; Vaughan et al., 2013; Mathes et al., 2015) which remains to be addressed.

Differences in the organellar composition of the knee extensor muscle, as they relate to the metabolic and mechanical properties of skeletal muscle (Weibel, 1996; Weibel and Hoppeler, 2005), may reflect to a considerable degree the variability of muscular aspects of physical performance (Reid and Fielding, 2012). In this respect it is worth to consider that correlations existed between genotype-associated cellular and subcellular parameters of knee extensor muscle and physiological parameters of aerobic performance over the entire population of endurance athletes and non-athletes (**Supplemental Figure S2**). This concerned positive linear relationships between VO_2max and aerobic peak power output, with capillary-to-fiber ratio (r = 0.56 and 0.53) and mitochondrial volume density (r = 0.76 and 0.47), and conversely negative linear relationships of the former two physiological parameters with the volume density of myofibrils (r = -0.74 and -0.40), all of which met p-values of below 0.02. Also, the percentage of slow type muscle fibers (r = 0.53, p = 0.001) and sarcoplasmic volume density (r = 0.64, $p < 0.001$) were linearly related to VO_2max, supporting the contribution of muscle composition to aerobic capacity and performance (Tihanyi et al., 1982; di Prampero, 2003; Pollock et al., 2018; van der Zwaard et al., 2018). In this respect it is worth to consider that certain differences in muscle composition between genotypes of rs1799752 and rs2104772 within a given athlete phenotype were considerable. For instance the volume density of mitochondria was approximately 50% higher in ACE I-allele carriers compared to ACE-D/D genotypes (**Figure 4E**). As well in ACE I-allele carriers, 50% higher values were identified for the volume density of sarcoplasma (**Figure 4F**), the functional relevance of which is not understood. Similarly, capillary length density, reflecting the capacity for substrate supply to working muscle (Vock et al., 1996), was nearly 25% higher in A/T genotypes of rs2104772 when MSCA demonstrated the inverse trend (**Figure 5**). These differences indicate that an increased capacity for capillary perfusion and mitochondrial metabolism in knee extensor muscle contributes to the reportedly elevated aerobic capacity for athletic I-allele carriers of polymorphism rs1799752 and suggests a similarly improved capacity for fueling substrate import in A/T carriers of rs2104772. Previous investigations found associations between the ACE and ACTN3 polymorphism and physical performance (Woods et al., 2001; Yang et al., 2003; Kikuchi et al., 2014) while others did not report an association between the ACE gene I/D polymorphism and the multisystemic variable VO_2max in endurance athletes *per se* (Rankinen et al., 2000). In this respect it has been shown that the incidence of the ACE-I/I genotype is increased in successful marathon runners and inline skaters (Hruskovicova et al., 2006). It remains to be elaborated to which degree the in here identified genotype x phenotype interaction effects on knee extensor muscle composition, such as the elevated volume density of mitochondria in the highly endurance-trained ACE-I/I genotypes (**Figure 4**), stand for differences in local strategies between genotypes to enhance muscle performance of athletes, and excel in competition.

All metadata on skeletal muscle composition were collected using standardized methods (**Supplemental Table S1**), legitimizing the direct comparison of the results from different data sets. In order to ensure that the study results are not biased, the selected athletes are only elite athletes, who rank among the national elite or even among the world leaders in their discipline. Our study however was subject to limitations which reduce the statistical and interpretative power of our results for the competitive athlete. For instance, because it is difficult to obtain biopsies from elite athletes, the study population of 46 elite athletes for a gene association study is rather small. The consequently lowered power explains why our investigation could not confirm (at the level of statistical significance) the reported associations for the single gene polymorphisms rs1799752 and rs1815739 in endurance- respective to power-athletes that have been shown in studies with large cohorts (Woods et al., 2000; Vincent et al., 2007; Bray et al., 2009; Kikuchi et al., 2014; Aleksandra et al., 2016). For fiber type distribution we could extend previous findings on the association of gene polymorphisms rs1799752 and rs1815739 and slow fiber percentage to elite athletes (Yang et al., 2003; Zhang et al., 2003; Vincent et al., 2007; Verbrugge et al., 2018). To counteract limitations due to sample size, and in line with others (Aleksandra et al., 2016) we pooled data from different type of endurance athletes (i.e., cyclist and runner) despite the possibly different degree of ultra-structural specialization in knee extensor muscle (Vaughan et al., 2013). Secondly, we do not have a complete set of physiological or performance records, especially as these were not recorded at the time of biopsy collection, and because the anonymous data assessment did not allow completing missing data. Thus, the relevance of volumetric differences in muscle organelles in the studied knee extensor muscle in setting variability of athletic performance cannot be fully appreciated or addressed. The identified differences in muscle composition between endurance and power-type athletes, and the interaction between genotype differences, are to a good degree in line with previous studies in non-athletes (Valdivieso et al., 2017a,b), and support that the pooling of *vastii* between different sports disciplines with similar "power" characteristics did not essentially interfere with the resolution of biologically relevant genetic influences. Nevertheless. the identified statistically significant interaction effects between the ACE and *post hoc* differences support the notion of a heritable influence on muscle composition depending on the athletic phenotype. The underlying mechanism, i.e., whether this reflects an adaptive response to the years of training and competition, and the extent to which the resulting genetic differences in muscle composition are of functional relevance to produce differences in muscle's capacity to produce power over short or long duration, remains to be elaborated. Possibly this also comprises the investigation of further gene polymorphisms to apprehend the breadth of the possible genetic influence on training-specific adaptations in muscle composition.

CONCLUSION

Polymorphisms in the genes for angiotensin-converting enzyme and tenascin-C are confirmed to be associated with the specialization of (sub)cellular aspects of metabolic and mechanical muscle functioning and show that this interacts with the athlete phenotype. Reported associations between the studied gene polymorphisms and differences in the acute effects of physical activity on muscle gene expression and muscle adaptation hint that the identified genotype × athlete phenotype interactions are due to the impact of physiological cues during years of training and competition. Our results may contribute to the development of a future personalized approach to physical training which takes the trainability of metabolic and contractile traits of muscle performance into account. Currently, however, the functional relevance of the identified genotype influence between endurance and power athletes for muscle performance remain to be further analyzed.

ETHICS STATEMENT

The study was conducted in accordance with the Helsinki declaration for research on humans. The gathering of physiological and cell biological meta-data was conducted with permission of the Ethics committee of the canton of Berne. Genotyping was carried out and analyzed anonymously under application of the non-competence rule of the Human Research Act after clarification by the ethics committee of the canton of Zurich.

AUTHOR CONTRIBUTIONS

MF designed the study. MVF acquired the funding. MVF, PV, SK, and MK performed the experiments. MK and PV analyzed the experiments. MVF, MK, SK, and PV analyzed the data. DF interpreted the results. MVF, MK, and DF drafted the manuscript. MF, MVF, and PV revised the manuscript. MF, MK, DF, SK, MVF, and PV endorsed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00526/full#supplementary-material

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Myokine/Adipokine Response to "Aerobic" Exercise: Is It Just a Matter of Exercise Load?

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Purpose: Exercise health benefits are partly mediated by exertional changes in several myokines/adipokines. This study aimed to compare the acute response of some of these biomarkers to aerobic exercise performed at the intensity corresponding to the maximum fat oxidation rate (FATmax) or the "anaerobic" threshold (AT).

Methods: Following a cross-over, counterbalanced design, 14 healthy untrained men (23 ± 1 years) performed a 45-min exercise bout at their FATmax or AT intensity (been previously determined through incremental exercise tests). The concentration of interleukin (IL)-15, follistatin, myostatin, fibroblast-growth factor (FGF)-21, irisin, resistin, and omentin was measured at baseline and 0, 1, 3, 24, 48, and 72 h post-exercise.

Results: AT exercise was performed at a higher intensity (85 ± 8 vs. 52 ± 14% of maximal oxygen uptake [$VO_{2\,max}$], $p < 0.001$) and induced a higher energy expenditure ($p < 0.001$) than FATmax, whereas a greater fat oxidation was observed in the latter ($p < 0.001$). A higher peak response of FGF-21 (+90%, $p < 0.01$) and follistatin (+49%, $p < 0.05$) was found after AT-exercise, as well as a trend toward a higher peak level of omentin (+13%, $p = 0.071$) and a greater decrease in resistin (−16%, $p = 0.073$).

Conclusion: Increasing exercise load (from FATmax to AT) results in a higher response of FGF-21, follistatin and omentin to aerobic exercise, with the subsequent potential cardiometabolic benefits. No effects were, however, observed on the remainder of biomarkers. Future research should address if manipulating other exercise variables (e.g., type, frequency) can promote a higher myokine/adipokine response.

Keywords: cytokines, metabolism, physical activity, training, responders

INTRODUCTION

Regular physical exercise is an effective lifestyle intervention for the prevention and treatment of numerous non-communicable diseases (Fiuza-Luces et al., 2013, 2018), with "aerobic" (or "endurance") exercise (e.g., brisk walking, jogging/running, swimming) being probably the most commonly prescribed modality (Warburton et al., 2006). Aerobic exercise has proven to reduce cardiovascular disease (CVD) risk factors such as high blood pressure, hyperlipidemia, or altered glucose homeostasis, among others (Whelton et al., 2002; Snowling and Hopkins, 2006; Kodama et al., 2007). However, the magnitude of the health benefits seem to depend on exercise loads (Duncan et al., 2005). For instance, a greater improvement in CVD risk factors and cardiorespiratory fitness (CRF) might be observed with vigorous exercise (i.e., >6 metabolic equivalents [METs], or >60% of maximal oxygen uptake [$VO_{2\,max}$]) than with less intense training (Swain and Franklin, 2006).

The prescription of aerobic training loads can therefore be modified to maximize health benefits. In this regard, there is a high inter-individual variability in the physiological responses and adaptations to exercise at a fixed relative intensity (i.e., expressed as a percentage of $VO_{2\,max}$ or maximal heart rate) (Mann et al., 2013). By contrast, prescribing exercise loads relative to individually determined specific physiological indicators ("thresholds") whose relative intensity varies between individuals might homogenize the elicited stress and thus reduce individual variability in metabolic responses (Mann et al., 2013). For instance, a commonly prescribed intensity is that associated to the so-called "anaerobic threshold" [AT, although the term "anaerobic" is physiologically inappropriate (Chamari and Padulo, 2015)], which is also termed "second ventilatory threshold" or "respiratory compensation point" (Meyer et al., 2005), and represents the maximum intensity that can be maintained in a steady state oxidative metabolism (Connolly, 2012). On the other hand, exercise prescription at the intensity corresponding to the point of maximal fat oxidation (FATmax) has gained popularity during the last decade (Jeukendrup and Achten, 2001). The latter strategy has proven beneficial for weight management and for improving several markers of cardiometabolic health (Venables and Jeukendrup, 2008; Romain et al., 2012).

Although both AT and FATmax training provide cardiometabolic benefits, the results of comparing these two exercise intensities remain to be reported. In this respect, the benefits of aerobic training on cardiometabolic health seem to be partly mediated by the cumulative effects of repeated, acute bouts of exercise-induced changes in several hormones and molecules such as the so-called myokines and adipokines, which are released from muscles and adipose tissue, respectively, to the blood and exert endocrine or paracrine effects in other cells, tissues or organs (Pedersen and Febbraio, 2012; Fiuza-Luces et al., 2018).

Several myokines have been proposed to mediate exercise-induced health benefits besides the most studied and well known myokine, interleukin (IL)-6 (Fiuza-Luces et al., 2013). Notably, IL-15, fibroblast growth factor (FGF)21, or irisin have been reported to induce cardiometabolic benefits through promoting the browning of white adipose tissue (WAT), and enhancing glucose homeostasis (Busquets et al., 2006; Nielsen et al., 2008; Elbelt et al., 2013; Kim H. J. et al., 2013; Woo et al., 2013; Fisher and Maratos-Flier, 2016; Perakakis et al., 2017). Other myokines, mainly myostatin or follistatin, are more related to muscle plasticity, with the former being a negative regulator of muscle growth (Huang et al., 2011) and the latter promoting skeletal muscle development through the activation of anabolic pathways (Winbanks et al., 2012). Evidence suggests that these myokines also exert metabolic benefits (Huang et al., 2011; Braga et al., 2014). In addition, exertion-related changes in the circulating levels of adipokines such as omentin and resistin have been proposed to mediate the cardiometabolic benefits of exercise, with the former enhancing glucose metabolism (Yang, 2006), and the latter – which has been reported to decrease in response to exercise (Prestes et al., 2009) – antagonizing insulin action and being associated with obesity (Steppan et al., 2001; Adeghate, 2004).

Thus, the magnitude of release of these factors in response to exercise might partly explain the different benefits obtained depending on the training stimulus. However, evidence is still lacking regarding the influence of different training variables, notably relative intensity, on this response. The main purpose of this study was to compare the response of several myokines/adipokines involved in cardiometabolic health to an exercise session of the same total duration performed at either AT or FATmax intensity. The effect of these two types of training sessions on resting metabolic rate (RMR) was also analyzed as a secondary endpoint. We hypothesized that, since the AT usually corresponds to a higher intensity than FATmax (∼80–85 vs. 50–60% of $VO_{2\,max}$, respectively), an exercise session at AT-intensity would result in a higher total exercise load and promote a more marked release of myokines/adipokines than a FATmax-session, with the subsequent potential cardiometabolic health benefits.

MATERIALS AND METHODS

Participants

Fourteen male subjects participated in this study ([mean ± SD] age, 23 ± 1 years; body mass index, 22 ± 2 kg · m^{-2}). Inclusion criteria were being healthy (i.e., free of CVD, diabetes or abnormal glucose tolerance, or any other acute/chronic disease), aged 18 years or older, and performing no regular physical exercise (i.e., <20 min twice a week). Participants were required to maintain the same dietary habits during the study, and to refrain from doing exercise, smoking or drinking coffee or alcohol during the 48 h prior to each visit. They were informed of the objects and procedures and provided both verbal and written informed consent. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board (Ethics Committee of the Chinese Institute of Sport Science).

Experimental Design and Measurements

Each participant attended our laboratory at 7.00 am on four different days after an overnight fast. Thereafter they ate a standardized breakfast (one cup of soy milk, one egg, and two ∼50 g steamed stuffed buns), and 2 h later they completed the exercise tests or sessions that are described below. All exercise tests and sessions were conducted on the same treadmill (pulsar4.0; H/P/cosmos; Traunstein, Germany).

Incremental Test for AT and $VO_{2\,max}$ Determination

The day of the first visit to the laboratory, participants performed an incremental treadmill exercise test in which speed was initially set at 7 km · h^{-1} and was increased by 1 km · h^{-1} every minute until volitional exhaustion, while treadmill inclination was kept constant at 0%. The test was deemed valid if at least three of the following four criteria were met: (a) a plateau in oxygen uptake (VO_2) was observed despite increasing treadmill speed; (b) the subject was no longer able to maintain the required speed; (c) the respiratory exchange ratio (RER) exceeded a value of 1.10; and (d) the age-predicted maximum heart rate (HR_{max}, 220 minus age, in years) was achieved. Gas-exchange data were consistently collected breath-by-breath with the same metabolic cart during the study (MetaMax 3B, Cortex, Biophysik; Leipzig, Germany). The $VO_{2\,max}$ was determined as the highest 30-s average VO_2 value whereas the AT was calculated with three validated methods using 30-s average data (Meyer et al., 2005): (a) a modified V-slope for the relationship between ventilation (VE, in the *y* axis) and carbon dioxide production (VCO_2, in the *x* axis), that is, a disproportionate increase in VE with respect to VCO_2 (i.e., two regression lines are fitted for the upper and lower part of the relationship and their intersection represents the AT); (b) the ventilatory equivalent method (i.e., the first systematic rise in the ventilatory equivalent for CO_2); and (c) the first decrease in the expiratory fraction of CO_2. Two experienced researchers independently determined the AT by visual inspection using the three methods, and the mean of the three measurements was entered for analysis. Of note, the second two methods (b and c) do not usually provide additional information with respect to the first one other than a different representation of the onset of exercise induced-hyperventilation (Meyer et al., 2005).

Incremental Test for FATmax Determination

After 4–7 days, participants performed another incremental exercise test (protocol modified from Suk et al., 2015) for the determination of the intensity associated with their individual FATmax. After a 3-min warm-up at 20% of the velocity associated to the $VO_{2\,max}$ (V_{max}), participants performed six 6-min stages at 25, 35, 45, 55, 65, and 75% of the V_{max}, with a 5-min rest between stages. Mean fat expenditure (g/h) was calculated from the last 3 min of each stage, and FATmax was determined as the highest point of the binomial parabola formed by the fat expenditure-velocity data.

AT and FATmax Exercise Sessions and Subsequent Measurements

After another 4–7-day period, participants performed two 45-min "aerobic" exercise training sessions at either FATmax or AT intensity, following a cross-over, counterbalanced design, and with a 7-day rest period between sessions. Each session was preceded by a 10-min general warm up consisting of mobility exercises, walking, and jogging at a self-selected intensity. They were required to not surpass an intensity above which they could not talk easily. Aerobic energy expenditure during the training sessions was determined through the analysis of VO_2 (MetaMax 3B, Cortex, Biophysik; Leipzig, Germany), and heart rate (HR) was also continuously assessed with a heart rate monitor (RS400, Polar, Kempele, Finland).

RMR was analyzed at baseline (before the standardized breakfast) and 24, 48, and 72 h after each session. During RMR measurements participants lied supine for 20 min in a room that had minimal light and noise, and constant ambient temperature (22 ± 1°C). Measurements were deemed valid if there was a variation <25 ml min^{-1}. The first 3 min were considered as the stabilization phase and were discarded from the analyses.

Blood variables were analyzed at baseline, immediately upon session termination (post 0 h), and 1, 3, 24, 48, and 72 h after each session, respectively. Blood samples (10-mL) were drawn from the antecubital vein and centrifuged at 3000 × *g* for 10 min. The obtained serum was then kept at −80°C until analysis with enzyme-linked immunosorbent assays (ELISA) of: follistatin (number: DFN00, R&D Systems; Minneapolis, MN, United States); myostatin (number: DGDF80, R&D Systems); FGF-21 (number: DF2100, R&D Systems); IL-15 (number: 0707170149, R&D Systems); irisin (number: EK-067-29, Phoenix Pharmaceuticals, Burlingame, CA, United States); omentin (number: EZH0MNTN1-29K, Millipore, Burlington, MA, United States); and resistin (number: DRSN00, R&D Systems). The intra-assay coefficient of variation was ≤5.3, ≤2.7, ≤5.4, ≤3.9, <10, ≤5.3, and ≤3.6% for IL-15, follistatin, myostatin, FGF-21, irisin, resistin, and omentin, respectively, whereas the inter-assay variability was <9.1, ≤9.2, ≤6.0, ≤10.9, <15, ≤9.2, and ≤6.9%. The standard curves were analyzed by double parallel tube. The peak concentration and the area under the curve (AUC) displayed by the concentration-time data (trapezoid rule) were analyzed for each molecule. All the data were analyzed as a percent of baseline values.

Statistical Analysis

Data are presented as mean ± SD unless otherwise stated. The normal distribution (Shapiro–Wilk test) and homoscedasticity (Levene's test) of the data were checked before any statistical treatment. A two-way (condition [AT, FATmax], time) repeated-measures ANOVA was used to compare the response over time of the blood variables and RMR between the two types of exercise sessions (AT or FATmax). A Greenhouse–Geisser correction was applied when Mauchly's test of sphericity was violated. Bonferroni *post hoc* test was conducted to analyze differences between conditions at each time point. To minimize the risk of statistical error type I, the level of significance was corrected for multiple comparisons by dividing 0.05 by the total number of time points (i.e., threshold *p*-value = 0.007 [0.05/7]). Student's paired *t* tests were conducted to analyze the differences in energy expenditure and peak and AUC concentration between sessions. The magnitude of the differences between conditions was

determined through the imputation of effect sizes (ES, Hedges'g). All statistical analyses were conducted using a statistical software package (SPSS 23.0, United States).

RESULTS

Subjects' VO_2max and maximum HR (HRmax) averaged 46 ± 4 ml kg min^{-1} and 192 ± 7 bpm, respectively. Although, all participants maintained the prescribed FATmax-associated velocity during the corresponding 45-min exercise bout an increase in RER could be observed in some of them by the end of exercise, with the subsequent variation in fat oxidation rate. By contrast, they could not exercise continuously for 45 min at the AT-associated velocity; accordingly, they were allowed to take one or two brief recovery periods (consisting of walking for 5 min at 3 km · h^{-1}) before continuing running at the prescribed velocity until completing 45 min.

Metabolic Response to Exercise

AT was performed at a higher intensity than FATmax (85 ± 8 vs. 52 ± 14% of $VO_{2\,max}$ and 90 ± 4 vs. 68 ± 9% of HRmax, both $p < 0.001$). As a consequence, AT resulted in a higher energy expenditure (762 ± 118 vs. 480 ± 160 kcal/h, $p < 0.001$). AT also induced a greater absolute and relative oxidation of carbohydrates, but a lower contribution of fat (**Figure 1**). No condition ($p = 0.674$), time (0.073) or condition by time effect ($p = 0.664$) was observed for post-exercise RMR (**Figure 2**).

Blood Variables

The time course and peak and AUC concentrations for each biomarker are displayed in **Figure 3** and **Table 1**, respectively.

Interleukin-15

Six participants presented a IL-15 concentration below the minimum detection levels and thus their results could not be analyzed (total *n* for analyses = 8 per condition). No significant condition ($p = 0.435$), time ($p = 0.550$) or condition by time effect ($p = 0.905$) was observed for IL-15 (**Figure 3A**), with no differences in peak or AUC concentration between conditions (**Table 1**). No significant changes in IL-15 levels were observed at any time point after AT or FATmax exercise compared with baseline.

Follistatin

A significant time ($p < 0.0001$ but no condition ($p = 0.073$) or condition by time effect ($p = 0.164$) was observed for follistatin (**Figure 3B**). A trend to an increase in follistatin levels above baseline values was observed up to 3 h after both AT and FATmax exercise ($p = 0.026$ and 0.007, respectively but above the corrected threshold *p*-value of 0.007). However, a higher peak follistatin was observed after the AT session, with no differences between conditions in AUC (**Table 1**).

Myostatin

A significant time ($p < 0.0001$) but no condition ($p = 0.870$) or condition by time effect ($p = 0.067$) was observed for myostatin (**Figure 3C**), with no differences between conditions in peak or AUC concentration (**Table 1**). No changes were observed in myostatin levels after FATmax exercise compared to baseline, but a significant increase was observed immediately after the AT session ($p < 0.001$).

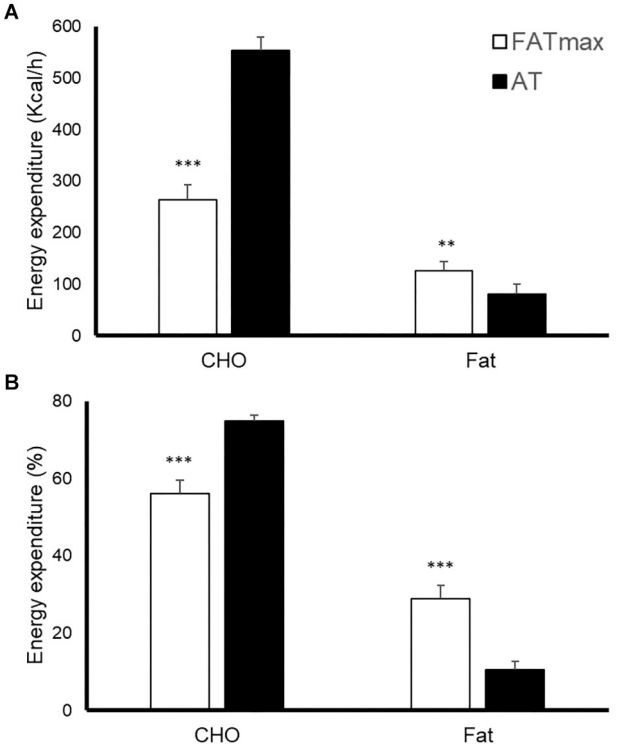

FIGURE 1 | Energy expenditure [expressed as absolute **(A)** and relative **(B)** values] in response to 45-min exercise at the intensity of maximal fat oxidation (FATmax) or the anaerobic threshold (AT). Data are mean ± standard error. Significant differences between conditions: $^{**}p < 0.01$, $^{***}p < 0.001$. CHO, carbohydrate.

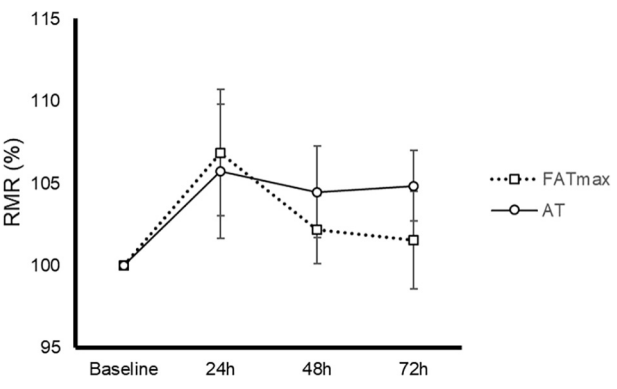

FIGURE 2 | Time-course of resting metabolic rate (RMR) in response to 45-min exercise at the intensity of maximal fat oxidation (FATmax) or the AT. Data are mean ± standard error. No significant condition, time, or condition by time effect ($p > 0.05$) was found.

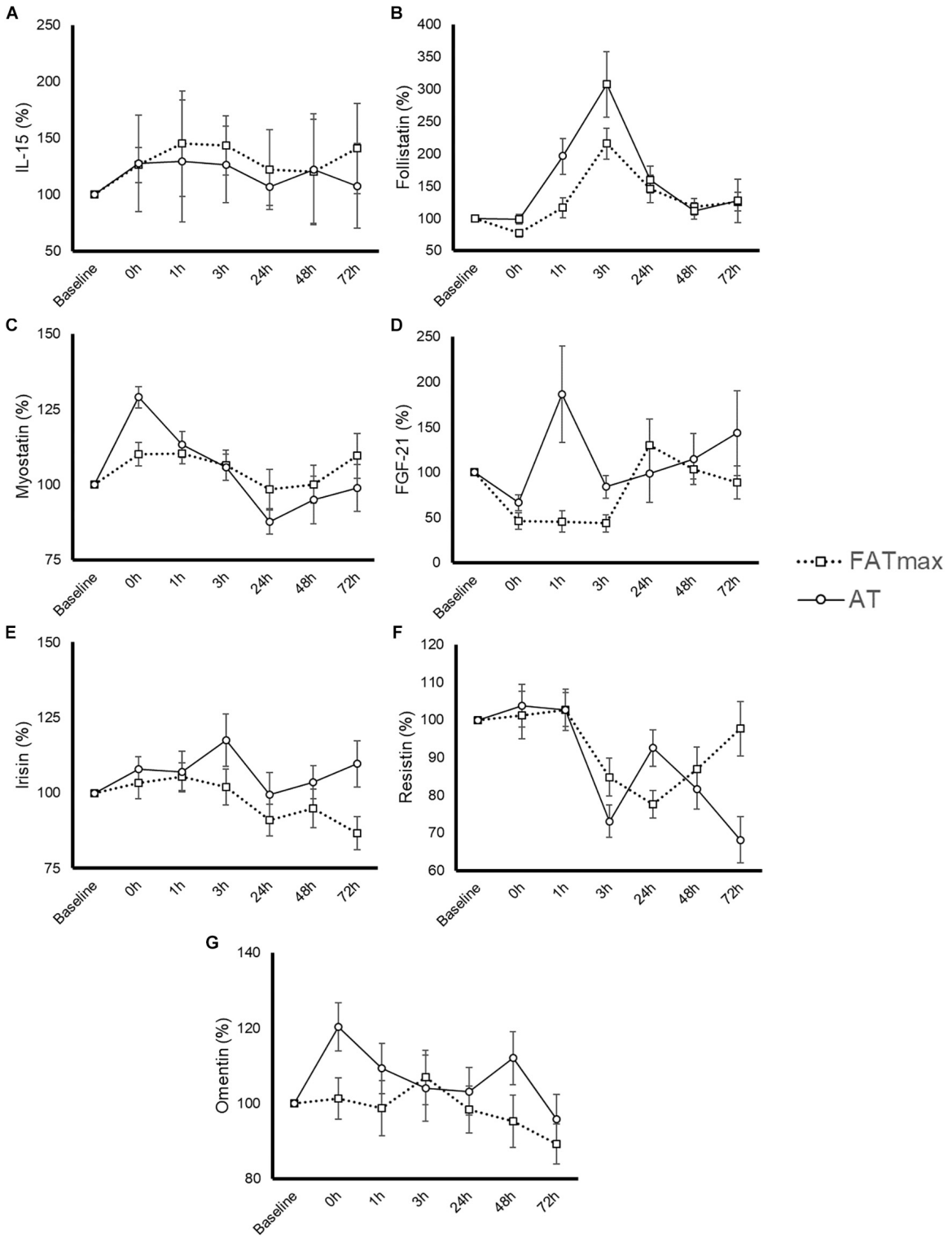

FIGURE 3 | Time-course response of interleukin (IL)-15 **(A)**, follistatin **(B)**, myostatin **(C)**, fibroblast growth factor-21 (FGF-21) **(D)**, irisin **(E)**, resistin **(F)**, and omentin **(G)** to 45-min exercise at the intensity of maximal fat oxidation (FATmax) or the AT. Data are mean ± standard error. All molecules were measured in 14 subjects except for IL-15 and FGF-21, which were measured in 8 and 13 of them, respectively. The significance threshold for *post hoc* analyses was adjusted for the number of time points (i.e., $p = 0.05/7 = 0.0071$). A significant condition by time effect was observed for resistin and omentin, but there were no significant differences between conditions at any specific time point ($p > 0.007$).

TABLE 1 | Myokine/adipokine response to aerobic exercise at the intensity of maximal fat oxidation (FATmax) or the anaerobic threshold (AT).

	Peak (%)				AUC (%)			
	FATmax	**AT**	***p*-value**	**ES**	**FATmax**	**AT**	***p*-value**	**ES**
IL-15	194 ± 118	160 ± 41	0.180	0.36	9251 ± 7374	8342 ± 6947	0.434	0.16
Follistatin	241 ± 86	359 ± 177	**0.024**	0.82	10302 ± 3451	11688 ± 3280	0.376	0.40
Myostatin	124 ± 23	136 ± 18	0.160	0.56	7382 ± 1540	6891 ± 1244	0.374	0.34
FGF-21	155 ± 92	294 ± 209	**0.009**	0.83	7073 ± 3464	7982 ± 4877	0.589	0.21
Irisin	116 ± 19	131 ± 30	0.121	0.58	6746 ± 1292	7603 ± 1661	0.081	0.56
Resistin	66 ± 13	57 ± 13	0.073	0.56	6184 ± 923	5902 ± 801	0.375	0.21
Omentin	114 ± 24	129 ± 24	0.071	0.75	6997 ± 1621	7582 ± 1735	0.283	0.44

Data are mean ± SD and % of baseline values. All molecules were measured in 14 subjects except for IL-15 and FGF-21, which were measured in 8 and 13 of them, respectively. Contrary to the other myokines/adipokines, peak concentration for resistin represents the lowest value recorded. Significant p-values ($p < 0.05$) are highlighted in bold. AT, anaerobic threshold; AUC, area under the curve concentration; ES, effect size; FGF-21, fibroblast growth factor-21; and IL-15, interleukin-15.

Fibroblast Growth Factor-21

One participant presented with a FGF-21 concentration below the minimum detection levels and thus his results could not be analyzed (total *n* for analyses = 13). A significant condition effect ($p = 0.009$) and a trend to a significant time effect ($p = 0.061$) was observed for FGF-21 (**Figure 3D**). A trend toward an increase in FGF-21 levels above baseline values was observed immediately after AT exercise ($p = 0.059$). By contrast, FGF-21 levels decreased above baseline values at 0 ($p = 0.002$), 1 ($p = 0.013$, yet above the threshold *p*-value) and 3 h ($p = 0.001$) after the FATmax session, respectively. We found no condition by time effect ($p = 0.112$), but a greater peak concentration was observed after AT exercise (**Table 1**). There were no differences between conditions in AUC concentration (**Table 1**).

Irisin

A significant time effect ($p = 0.011$) and a trend toward a significant condition by time effect ($p = 0.067$) was observed for irisin (**Figure 3E**). However, there were no differences between conditions in peak or AUC concentration (**Table 1**). No significant changes in irisin levels were observed at any time point after AT or FATmax exercise AT compared to baseline.

Resistin

A significant time ($p < 0.001$) and condition by time effect ($p < 0.001$) was observed for resistin. Resistin levels were significantly decreased 24 h after FATmax exercise compared to baseline ($p = 0.001$). In turn, they were significantly decreased 3 ($p = 0.001$) and 72 h ($p = 0.004$) after AT compared to baseline, and a trend toward lower levels was also found 48 h post-exercise ($p = 0.086$). No significant differences were found between conditions at any specific time point (**Figure 3F**). No differences between conditions were found in AUC concentration, but a trend toward a lower minimal concentration was found after AT compared to FATmax (**Table 1**).

Omentin

A significant time ($p = 0.033$) and condition by time effect ($p = 0.031$) was observed for omentin. *Post hoc* analysis revealed a trend toward higher values with AT compared to FATmax immediately after exercise (post 0 h, $p = 0.020$) as well as toward higher values 48 h later ($p = 0.070$) (**Figure 3G**). There were no differences between conditions in AUC concentration, but a trend toward a higher peak concentration was observed after AT compared to FATmax ($p = 0.071$) (**Table 1**).

DISCUSSION

The present study compared the response of several myokines/adipokines involved in metabolic regulation and weight management after two exercise sessions performed at the AT or FATmax AT (at mean intensities corresponding to ∼85 and ∼52% of VO_2max, respectively) in healthy subjects. We expected *a priori* to observe a greater response with the latter given that it was performed at a considerably higher intensity. In this respect, our results indeed showed that AT induced a ∼2 and ∼1.5-fold higher increase in FGF-21 and follistatin concentration, respectively, compared to FATmax, as well as a greater (20%) omentin response immediately post-exercise. However, no significant differences were observed for the rest of myokines/adipokines. No differences were found either in the time course of RMR post-exercise.

Previous research has analyzed the myokine/adipokine response to exercise. However, the influence of manipulating training variables such as exercise load on this response remains to be elucidated, and there is scarce information on the time-course of this response. In this respect, we recently observed a high individual variability in the myokine response to two popular exercise modalities such as high-intensity interval training (commonly known as "HIIT") and resistance training (He et al., 2018). In the present study, however, we assessed the myokine/adipokine response of the same individuals to two sessions of the same type of exercise (i.e., "aerobic" or "endurance") but performed at different relative intensities and thereby resulting in different total exercise loads. This design allowed us to study the role of exercise load on this response. To our knowledge, this is the first study to assess how manipulating training load modifies the myokine/adipokine response to exercise. Moreover, here we described the time-course response of these factors up to 3 days after exercise, which might help to gain insight into the duration of the metabolic effects of a

single exercise session. The observed trend to an exercise-induced increase in FGF-21 and follistatin levels is in agreement with previous research (Hansen et al., 2011; Kim K. H. et al., 2013; Tanimura et al., 2016). FGF-21 regulates glucose homeostasis and lipid utilization, augments brown fat thermogenesis, and has been related to improved insulin resistance, weight loss and to the browning of WAT (Lee et al., 2012, 2014; Woo et al., 2013; Fisher and Maratos-Flier, 2016). In turn, follistatin is a myostatin-binding protein that promotes skeletal muscle development through the activation of the mammalian target of rapamycin pathway (Winbanks et al., 2012). Follistatin also plays a role in metabolism (i.e., reduction of body fat, improvement of glucose homeostasis and WAT browning) (Braga et al., 2014). Thus, increases in these two myokines could be potentially related to some of the cardiometabolic health benefits usually obtained with exercise, and our results suggest that AT might be more effective for this purpose than FATmax.

Increasing exercise intensity also resulted in higher circulating levels of omentin, an adipokine that seems to counteract insulin resistance and obesity (De Souza Batista et al., 2007), improving glucose metabolism through the stimulation of Akt phosphorylation in muscle tissue (Yang, 2006). In line with these results, 12 weeks of aerobic exercise have proven superior to other types of exercise (i.e., resistance exercise and a combination of both resistance and aerobic exercise) for the promotion of omentin production in diabetic animals (de Castro et al., 2019). Similarly, 12 weeks of aerobic exercise have also been reported to result in improved cardiometabolic risk factors (waist circumference, insulin resistance, and lipid profile) together with increased circulating omentin levels in overweight/obese men (Saremi et al., 2010), which has been corroborated in later studies (Wilms et al., 2015; Ouerghi et al., 2017). Endurance exercise appears therefore as an effective strategy for the stimulation of omentin release by adipose tissue – with subsequent potential metabolic benefits – but the present findings suggest that the magnitude of these benefits might depend on exercise intensity.

Although no differences were observed between conditions for the myostatin response, only AT induced a transient increase of this protein immediately after exercise. Myostatin, a transforming growth factor (TGF)β family member and the first described myokine, is a negative regulator of muscle growth (Huang et al., 2011). Several studies have reported a down-regulation of myostatin expression after both endurance and resistance exercise (Louis et al., 2007; Hittel et al., 2010; Lundberg et al., 2012). In contrast, other authors found an increase in myostatin mRNA in contracting muscles after exercise, which was coupled to a decrease in its transcriptional activity due to the activation of Notch, a TGFβ inhibitor (MacKenzie et al., 2013). Thus, although one could hypothesize that the increase in myostatin observed after AT might reduce the anabolic response to exercise, increases in myostatin expression *per se* should not necessarily result in lower skeletal muscle hypertrophy.

By contrast, exercise induced no significant increases in one of the most studied myokines, IL-15 (Fiuza-Luces et al., 2018). IL-15 has been purported to have beneficial effects on metabolic homeostasis through a decrease in WAT (Nielsen et al., 2008) and an enhancement of glucose metabolism (Busquets et al., 2006; Kim H. J. et al., 2013). Previous studies have found acute increases in the circulating levels of this myokine after resistance exercise training (Riechman, 2004; Pérez-López et al., 2018), but mixed results have been reported regarding the effects of endurance exercise. In agreement with our findings, no changes in plasma levels of IL-15 were observed after 1 (Chan, 2004) or 3 h of cycling exercise (Rinnov et al., 2014), or after 2.5 h of running (Ostrowski et al., 1998). On the other hand, Tamura et al observed an immediate increase in IL-15 plasma levels in response to 30 min of treadmill running, although IL-15 returned to basal values 3 h later (Tamura et al., 2011). There is therefore controversy on whether endurance exercise stimulates the release of IL-15 from muscles to the bloodstream, and more research is warranted to elucidate potential factors that might influence the production of this myokine.

Both training sessions decreased resistin levels at least at one time point post-exercise, and although the minimum values registered tended to be lower after AT compared to FATmax, no consistent differences between sessions were found. Resistin is produced by WAT and brown adipose tissue, antagonizes insulin action, and is elevated in obesity (Steppan et al., 2001; Adeghate, 2004). In agreement with our findings, plasma resistin levels have been reported to decrease acutely after resistance training in sedentary post-menopausal women (24 and 48 h post-exercise), and reduced values of this adipokine were also found when this training program was maintained for 16 weeks (Prestes et al., 2009). Other authors have found decreased resistin levels after long-term aerobic exercise training (Kadoglou et al., 2007; Jones et al., 2009). By contrast, Jamurtas et al. (2006) showed no changes in plasma resistin levels up to 48 h after a 45-min bout of cycling at 65% of VO_2max, and no variations in the levels of this adipokine were found after 14 weeks of exercise with or without concomitant diet in post-menopausal women with type 2 diabetes (Giannopoulou et al., 2005). More research is thus needed to determine if intensity mediates exercise effects on this adipokine.

No exercise effects or differences between conditions were observed for irisin. Despite the attention given to irisin in recent years owing to its advocated role in WAT browning and energy expenditure (Pedersen and Febbraio, 2012; Elbelt et al., 2013), controversy exists on the biological relevance of this myokine (Raschke et al., 2013), and some methodological issues regarding its measurement have been raised (Albrecht et al., 2015). Further research is needed to confirm the effect of exercise on irisin concentration and the potential of this myokine as a therapeutic target against obesity and its related complications.

The present study has some limitations that must be noted, notably the lack of measurement of some important myokines such as IL-6. Moreover, although our findings suggest that AT might induce a greater peak response to exercise than FATmax in some myokines, we cannot discern if these differences would eventually translate into actual benefits in cardiometabolic health. Finally, the present results, obtained in young healthy individuals, might not be necessarily applicable to other populations (e.g., obese or older people).

In summary, a higher endurance exercise intensity (i.e., AT vs. FATmax) induced a higher response of

follistatin, FGF-21 and omentin in healthy subjects, which could have potential implications due to the role of these myokines/adipokines in muscle plasticity and metabolism. However, no consistent differences between sessions were observed for IL-15, myostatin, irisin, or resistin. To optimize exercise prescription future research should determine the effects of varying exercise loads in the myokine/adipokine response of other populations (e.g., including people with cardiometabolic conditions) and to assess whether manipulating other exercise variables such as exercise type or volume might increase the time-course response of myokines/adipokines.

ETHICS STATEMENT

Participants signed an informed consent form after having the procedures explained. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board (Ethics Committee of the Chinese Institute of Sport Science).

AUTHOR CONTRIBUTIONS

ZihH, YT, CH, JZ, PH, ZilH and SY conceived the study and performed the experiments. PV and AL analyzed the data and drafted the manuscript. All authors significantly contributed to the final version of the manuscript.

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Physical Exercise as Personalized Medicine for Dementia Prevention?

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Accumulating evidence mainly from observational studies supports the notion that lifestyle factors such as regular physical activity can modulate potential risk factors of dementia. Regarding a potential mechanism for this interaction, results from intervention studies show that exercising can induce neuroplastic changes in the human brain. However, a detailed look at the study results reveals a wide interindividual variability in the observed effects. This heterogeneity may originate from the fact that there are "responders" and "non-responders" with respect to the impact of physical exercise on physiological outcome parameters (i.e., VO_2 peak) and the brain. From this, it follows that recommendations for physical exercise programs should not follow a "one size fits all" approach. Instead, we propose that the exercises should be tailored to an individual in order to maximize the potential neuroplastic and preventive effects of regular exercise. These adaptations should take the individual performance levels into account and impact both the quality (i.e., type) and the quantity of exercises (i.e., intensity, duration, and volume).

Keywords: exercise, dementia, neuroplasticity, personalized medicine, responder

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INTRODUCTION

According to recent predictions, the global number of people affected by dementia will rise from currently 47 to 131.3 million by 2050 (Prince et al., 2015), whereby Alzheimer's disease (AD) as the most common cause of dementia accounts for up to 75% of cases (Masters et al., 2015). Hope for the imminent development of disease-modifying drug therapies has faded after more than 200 clinical trials with new drugs have failed in the recent past (Schneider et al., 2014). In this context, concepts of healthy aging are becoming increasingly important.

Due to the lack of prospect for causal pharmacological treatments, dementia research is currently directed toward modifiable risk and lifestyle factors serving as preventive strategies (Kivipelto et al., 2018a). Norton et al. (2014) postulate that one third of the global prevalence of Alzheimer's disease is related to modifiable risk factors. Among other factors especially physical inactivity, overweight, hypertension, and diabetes mellitus have been identified as modifiable risk factors. The latter open an opportunity for various preventive strategies. According to a computational model, a 10% reduction of the risk factors per decade could lead to a decrease in 8.3% of the global Alzheimer's prevalence by 2050 (Norton et al., 2014). Additionally, delaying the onset of dementia by 5 years could reduce the number of affected people by nearly 50% (Sperling et al., 2011) and would have a key public health impact.

Systematic reviews on epidemiological studies suggest a strong impact of regular physical activity (as opposed to mainly sedentary behavior) on dementia risk (Hamer and Chida, 2009; Sofi et al., 2011). For example, Hamer and Chida (2009) have shown in a meta-analysis including 16 prospective studies with 163,797 non-demented participants in which physical activity is associated with a reduced risk of dementia of all types of 26% and a reduced Alzheimer's disease risk of 45%.

However, randomized controlled interventions reported mixed findings regarding the effect of exercising on cognition and the brain, casting some doubt on its preventive power against dementia (Müller et al., 2017). In summary, current research indicates that interventions are more beneficial at preclinical and early clinical stages of Alzheimer's disease (Forbes et al., 2015; Brini et al., 2018). Regarding the multifactorial etiology of most dementia cases, current large multidomain trials [MAPT (Andrieu et al., 2017), PreDIVA (van Charante et al., 2016), and FINGER (Ngandu et al., 2015)] investigate the effect of lifestyle interventions on cognitive functions and, ultimately, dementia prevention. So far only the FINGER trial has revealed beneficial intervention effects on cognitive functions among participants at risk of dementia (Ngandu et al., 2015). Whether these effects have an impact on the later incidence of dementia in the participants is not yet clear. Actually, the global initiative *World Wide Fingers* aims to advance dementia prevention studies (Kivipelto et al., 2018b).

INDIVIDUAL RESPONSE TO PHYSICAL EXERCISE

Numerous epidemiological, cross-sectional, and interventional studies indicate that regular physical activity has positive effects on health in general and brain health in particular and has the potential to reduce the risk of dementia (Hamer and Chida, 2009; Sofi et al., 2011; Brini et al., 2018; Liu-Ambrose et al., 2018). However, a detailed analysis of the studies often reveals a wide interindividual variability of the results (Müller et al., 2018). The individual response to physical exercise has received attention in sport science since the 1980s (Rankinen and Bouchard, 2008). Especially in the context of endurance and strength training, there is strong evidence for different individual physiological adaptations to identical exercise and training variables (Buford et al., 2013; Weatherwax et al., 2016). Based on these wide interindividual variations, humans have been divided into "responders" or "non-responders" with respect to a specific exercise (Buford et al., 2013). Here "responders" are defined as subjects who achieve a benefit, while "non-responders" may exhibit an unchanged or even worsened performance under the same stimulus (Bouchard and Rankinen, 2001). However, the term "responder" is currently under discussion. For example, Booth and Laye (2010) proposed that the term "non-responding" should be replaced by the term "low sensitivity" accounting for the fact that usually so-called non-responders show some training effects after all albeit to a lower extent, indicating that they may "convert" to responders if the training is adapted for them, i.e., by increasing training frequency.

PHYSICAL EXERCISE AS PERSONALIZED MEDICINE

Personalized medicine is an approach for pharmacological drug treatment and preventive interventions based on individual variability in genetics, anthropometrics, biomarkers, environment, and other factors. Thereby, the terms "personalized medicine," "precision medicine," and "individualized medicine" are often used synonym. Especially in oncology, personalized therapies have been successfully used for years (Shin et al., 2017). Currently, this approach has become popular in dementia research as well, e.g., Hampel et al. (2017).

Referring to the observation of the interindividual variety in physiological adaptions in response to physical exercise from sport science, the concept of "responders" and "non-responders" would have fundamental implications on the proposed neuroprotective and preventive factors of physical activity on dementia, too. In this regard, the following questions arise: (1) Which factors cause the large interindividual heterogeneity in response to physical training? (2) Are all outcomes affected equally by the individual responsiveness? and (3) How can we overcome non-responsiveness so that (almost) all individuals experience benefits? In the following, we will briefly discuss these questions.

1. Which factors cause this large interindividual heterogeneity in response to physical training?

Like with the risk for dementia (and other diseases), the individual physiological response to physical exercise is modulated by concomitant modifiable (e.g., diet) and non-modifiable factors (e.g., genetics, gender; Bouchard and Rankinen, 2001; Rankinen and Bouchard, 2008; Booth and Laye, 2010; Sparks, 2017). Regarding the latter, as of now over 150 genetic markers have been associated with elite athlete status (Ahmetov et al., 2016) and trainings response (Bray et al., 2009). Additionally, several single-nucleotide polymorphisms were identified as being related to the training response. Results of the HERITAGE (HEalth, RIsk factors, exercise Training And Genetics) study (Bouchard et al., 1999; Timmons et al., 2010) indicate that the interindividual variation in physiological responses to exercise is based, among other causes, on genetic factors. Here, 21 single nucleotide polymorphisms accounted for 49% of VO_2 peak variation (Bouchard et al., 2011). Interestingly, VO_2 peak has been associated with brain function in older adults (Erickson et al., 2009, 2011). Timmons et al. (2010) suggested molecular classifications based on 29 RNA signatures that predicted VO_2 peak, whereby 11 single-nucleotide polymorphisms explain 23% of the variance in VO_2 peak. However, only one intervention study has used a genetic-based algorithm for personalized resistance training (Jones et al., 2016). Altogether, several aspects

of the connection between genotype and exercise response are still unclear (Mann et al., 2014).

2. Are all outcomes affected equally by individual responsiveness status?

Both resistance and endurance trainings have yielded strongly varying results across individuals and, moreover, within an individual, the results are often inconsistent across different variables, so that the same person may present training induced benefits in one domain but not the other (Vellers et al., 2018). For example, after a 12-week resistance training, Hubal et al. (2005) reported that on average muscle size and strength increased in young adults. However, a closer look reveals that the gains in muscle size varied from −2 to +59% and those in strength between −32 and 149%. Similar results were reported following endurance interventions (Bouchard et al., 1999; Bouchard and Rankinen, 2001). Moreover, Karavirta et al. (2011) observed a wide range of individual responses to a combined endurance and strength training in older adults. After the 21 weeks of intervention, cardiorespiratory fitness (VO_2 peak) gains varied from −8 to 42% and strength (maximal isometric bilateral leg extension) from −12 to 87%. Other trials have shown a similar interindividual heterogeneity regarding the VO_2 peak response to exercise training in young (Kohrt et al., 1991; Bouchard and Rankinen, 2001) and old adults (Chmelo et al., 2015; Ross et al., 2015). Furthermore, other cardiorespiratory (e.g., blood pressure, heart rate at work load) and metabolic (e.g., insulin sensitivity, cholesterol) parameters have also shown strong interindividual differences in adaptation to exercise (Bouchard and Rankinen, 2001; Fritz et al., 2006). This interindividual variability could have a fundamental influence on the effect of physical exercise on neuroprotection and prevention of dementia, because especially cardiorespiratory and metabolic parameters are high-risk factors of dementia. A non-response to physical exercise on these risk factors could thus avert the positive effects of physical exercise on dementia risk in general. Other aspects such as gender (Barha et al., 2017a) or APOE (Berkowitz et al., 2018) are likely to influence the effects of physical activity.

Thereby, cardiovascular, neuromuscular, and balance training improve differently cognitive performance and neuroplasticity in elderly (Voelcker-Rehage et al., 2011; Voelcker-Rehage and Niemann, 2013; Levin et al., 2017). There are only a few studies regarding interindividual variability following physical exercise interventions on cognitive functions. Heisz et al. (2017) reported that a 6-week exercise, cognitive, or combined training led to general improvements in memory functions in young adults. In more detail, individuals with greater cardiovascular improvements had also larger increases in levels of the brain-derived neurotrophic factor (BDNF) and the insulin-like growth factor-1 (IGF-1). Furthermore, high responders to exercise in the combined training group had better memory performance compared with exercise alone.

3. How can we account for the interindividual heterogeneity to achieve optimal results in (almost) all individuals?

Following the hypothesis that there is a clinical relevant group of "non-responders" or "low sensitivity," the question raises whether modifications of a given exercise program (e.g., type of exercise, exercise durations, exercise volume, exercise intensity) can overcome the lack of training effects. Some current studies indicate that the non-responder status can be mitigated by increasing the exercise intensity and/or dose (Bonafiglia et al., 2016; Lundby et al., 2017; Montero and Lundby, 2017). For example, Montero and Lundby have shown that the percentage of non-responders is lower when the training is accomplished four to five times per week as opposed to only one to two times. Regarding effects on cognitive function, the optimal dose-response relationship is still largely unknown. One potential mediator of training effects on brain function is BDNF, which enhances neuroplasticity *via* different pathways (Brigadski and Leßmann, 2014). BDNF excretion is induced by lactate (Schiffer et al., 2011), and peripheral blood lactate levels have also been shown to correlate with cognitive improvements (Lee et al., 2014; Tsukamoto et al., 2016). Consequently, exercise interventions should be intensive enough to increase lactate and, as a consequence, BDNF. However, especially in older adults, classical exercise interventions rarely achieve the second ventilatory threshold (VT2) level, which is associated with an accumulation of lactate. Using High Intensity Interval Training (HIIT) with higher intensities and lower volumes could be a method to achieve higher numbers of responders. Considerable evidence is accumulating regarding the positive effects and safety of HIIT training strategies even for older adults and patients with chronic diseases (Ross et al., 2016) (e.g., chronic heart failure, COPD, diabetes).

FUTURE RECOMMENDATIONS

Actually, personalized medicine is primary concerned with heterogeneity in an individual's response to medical drugs. But, there is also an urgent need for personalized preventive exercise strategies (Buford et al., 2013; Berkowitz et al., 2018). Thereby, personalized exercise programs would enhance training efficiency and improve more outcome variables in a larger number of individuals. For future intervention studies, genetic analyses could help to identify potential "responders" and "non-responders." Furthermore, a personalized exercise training program should be based on a complex performance analysis, especially to identify specific individual weaknesses. More research is needed to provide more detailed recommendations for the suggested personalized exercise programs and to overcome the one-size-fits-all approach (Barha et al., 2017b).

AUTHOR CONTRIBUTIONS

PM wrote the manuscript. PM, MT, and NM revised the manuscript.

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Association Between Hematological Parameters and Iron Metabolism Response After Marathon Race and *ACTN3* Genotype

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α-Actinin-3 (*ACTN3* R577X, rs.1815739) polymorphism is a genetic variation that shows the most consistent influence on metabolic pathway and muscle phenotype. XX genotype is associated with higher metabolic efficiency of skeletal muscle; however, the role of *ACTN3* polymorphism in oxygen transport and utilization system has not yet been investigated. Therefore, the aim of this study was to determine the influence of *ACTN3* polymorphisms on hematological and iron metabolism response induced by marathon race. Eighty-one Brazilian amateur male endurance runners participated in the study. Blood samples and urine were collected before; immediately after; and 1, 3, and 15 days after the marathon race. Urine, hematological parameters, iron metabolism, and *ACTN3* genotyping analyses were performed. The marathon race induced a decrease in erythrocytes, Hb, and Ht, and an increase in hematuria, creatinine, myoglobin, red cell distribution width, mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, direct and indirect bilirubin and erythropoietin. Moreover, an elevation immediately or 1 day after the marathon race follows a reduction 3 or 15 days after the marathon race were observed on transferrin saturation and iron and transferrin levels. Hematological parameters and iron metabolism changes induced by marathon race were not observed in XX genotypes. Hematuria and decreased erythrocytes, Hb, Ht, and iron and transferrin levels were observed only in RR and/or RX genotypes but not in XX genotypes. The percentage of runners with hematuria, leukocyturia, iron deficiency, creatinine, myoglobin, and bilirubin imbalance was higher in RR compared to XX genotypes. *ACTN3* polymorphism is associated with iron metabolism and hematological responses after endurance exercise. Despite these results being based on a small sample, they highlight a protective role of the XX genotype on hematological and renal changes induced by long-distance exercise. Therefore, these findings should be further replicated.

Keywords: long-distance exercise, iron metabolism, polymorphism, hematological parameters, actinin-3

INTRODUCTION

Iron deficiency is associated with impairment in the transport and use of oxygen, and consequently increases the risk to sports anemia and may affect athletic performance (Peeling et al., 2008, 2014). The potential mechanisms involved in iron deficiency induced by long-distance exercise are intravascular and extravascular hemolysis, hematuria, and sweat and gastrointestinal iron loss and inflammation (Kobayashi et al., 2014; Peeling et al., 2014). Hemolysis is caused by many factors such as oxidative stress, inflammation, erythrocyte deformability, hemodilution, and mechanical rupture from foot strike or from compression of muscle on capillaries (Telford et al., 2003; Robinson et al., 2006; Yusof et al., 2007; Lippi et al., 2012; Mairbaurl et al., 2013; Peeling et al., 2014; Robach et al., 2014). High body temperature and catecholamine levels, hypotension after ischemia, foot strike, and bladder trauma also contribute to hematuria after intense exercise (Bellinghieri et al., 2008; Shephard, 2016).

Previous studies suggest that hemolysis is a heritable trait and is correlated with many energy metabolites (Van 't Erve et al., 2015). Hypoxia is the major stimulus for the release of ATP from erythrocytes, hemolysis, and hematuria (Sikora et al., 2014). *ACTN3* (α-Actinin-3) R577X polymorphism has consistently been shown to influence metabolic pathway and muscle performance (MacArthur and North, 2004, 2007; Eynon et al., 2013; Lee et al., 2016). The sarcomeric α-actinins (ACTN2 and ACTN3) are major components of the muscle Z-line but expression of ACTN3 is restricted to Z-lines of fast-twitch fibers while ACTN2 is expressed in all fibers (North et al., 1999; Mills et al., 2001; Eynon et al., 2013). *ACTN3* R577X polymorphism is a single-nucleotide polymorphism (SNP) at codon 577 (rs 1,815,739) due to replacement of an arginine (R) with a stop codon (X); homozygosity for the X allele results in a lack of production of functional ACTN3 protein (North et al., 1999; Mills et al., 2001; Eynon et al., 2013). Approximately, 18% of Caucasians present XX genotype and are completely deficient in ACTN3 protein (Eynon et al., 2014). The functional properties of ACTN2 and ACTN3 seem to be determined by capacity to interact with key proteins involved in biological processes (Seto et al., 2011a,b; Lee et al., 2016). ACTN3 deficiency and XX genotype has been associated with decreased muscle strength, muscle mass, and fast-twitch fiber, however, to improve the metabolic efficiency of skeletal muscle in humans and in the *Actn3* knockout mouse model (Seto et al., 2013; Erskine et al., 2014; Norman et al., 2014; Kikuchi and Nakazato, 2015; Houweling et al., 2018; Del Coso et al., 2019). Our hypothesis is that the metabolic efficiency attributed to XX genotype may improve parameters of oxygen transport and utilization system (red blood cells and iron metabolism) after long-distance exercise. Blood flow and oxygen demand increase in muscle cells during exercise, promoting changes in some metabolites such as 2,3-diphosphoglycerate (2,3-DPG) and adenosine triphosphate (ATP), H^+, CO_2, Cl^-, and/or leading to hypoxia that modulates oxygen transport and utilization system and hematuria (renal hypoxia) (Mairbaurl, 2013).

On this basis, the aim of this study is to determine the influence of *ACTN3* R577X polymorphisms on hematological parameters and iron metabolism response induced by amateur marathon running.

MATERIALS AND METHODS

Subjects

Eighty-one Brazilian amateur male endurance runners that completed the São Paulo International Marathon 2015 participated in the current study. The recruitment of the volunteers was performed by the São Paulo International Marathon Organization (2015) by mailing. Runners were randomized after medical history and examination and training history. The noninclusion criteria were: the use of medication for cardiac, metabolic, pulmonary, or kidney injury; use of alcohol or any kind of drugs; pathologies including systemic arterial hypertension, liver, kidney, metabolic, inflammatory, or neoplastic diseases; not having participated in a half-marathon or marathon previously; and training volume less than 40 km per week. Subjects were informed of the experimental procedures and possible risks and signed the written informed consent before participating. The study and written informed consent were approved by the Ethics Committee of Dante Pazzanese Institute of Cardiology, Brazil (Permit Number: 979/2010), in accordance with the Declaration of Helsinki.

São Paulo International Marathon 2015 was performed on 17 May at 08:00 am. Every 2–3 km during the run, water was allowed *ad libitum*; sports drinks on 18 and 36 km; and potato and gel on 30 km. The weather parameters at São Paulo International Marathon in 2015 between 8 am and 2 pm were: average temperature was 19.8°C (maximum = 22.6°C, minimum = 16.7°C) and average relative humidity was 72.8% (maximum = 86%, minimum = 61%) (National Institute of Meteorology, Ministry of Agriculture, Livestock and Supply).

Total body mass (kg), height (cm), and body mass index (BMI, kg/m^2) were measured according to the International Society for the Advancement of Kinanthropometry and expressed as the mean ± SEM. Anthropometrics parameters and cardiopulmonary exercise test were performed 3–21 days before marathon race and 3–15 after race using a treadmill protocol. The test was performed in 1% fixed slope and speed began with 8 km/h increasing 1 km/h per minute until maximal exhaustion of the runner. Expired gas analysis was performed in a breath-by-breath system (Ergostik®, Geratherm, Bad Kissingen, Germany). Blood samples (30 ml) and urine (20–50 ml) were collected before; immediately after; and 1, 3, and 15 after the marathon race.

Blood Samples

Blood samples were collected from the antecubital vein in vacuum tubes. Urine, hematological, and iron metabolism analyses were evaluated with routine automated methodology in Clinical Laboratory of Dante Pazzanese Institute of Cardiology immediately after collection. TRIZOL reagent was added in one vacuum tube and stored at −80°C for later genetic analysis.

Genetic analysis of *ACTN3* was performed at the Center for Research and Molecular Diagnosis of Genetic Diseases at Federal University of São Paulo.

Hematological Markers and Iron Metabolism

Plasma iron, creatinine, and bilirubin analyses were performed by colorimetric method; hematological markers hemoglobin (Hb), hematocrit (Ht), red blood cell distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were measured by cytochemical/isovolumetric method; and levels of ferritin and myoglobin were evaluated by chemiluminescence assay, erythropoietin by immunochemiluminometric assay, and transferrin by immunoturbidimetry. Hematuria and leukocyturia were analyzed by flow cytometry.

ACTN3 Analyses

ACTN3 genotypes were determined by allelic discrimination assay (TaqMan® SNP Genotyping Assays, Applied Biosystems, Foster City, CA, USA) using Applied Biosystems™ 7,500 Real-Time PCR Systems equipment (Applied Biosystems, Foster City, CA, USA).

Statistical Analyses

Statistical analyses were performed using ordinary one-way ANOVA and Holm-Sidak's multiple comparisons *post hoc* test for comparison before and after the race (immediately after, 1 day after, 3 days after, and 15 days after the race) in different genotypes (RR, RX, and XX). Statistical significance was set at $p < 0.05$ (GraphPad Prism version 6.01 software, CA, USA). The values are presented by mean ± standard error mean of 16 XX runners, 43 RX runners, and 22 RR runners and in percentage of runners in RR, RX, and XX genotypes.

RESULTS

The general and training characteristics of amateur runners are summarized as follows: age, 39.0 ± 1.0 years; height, 1.74 ± 0.1 m; body mass, 74.0 ± 1.0 kg; % of fat mass, 20.0 ± 1.0%; body mass index, 25.0 ± 0.3 kg/m^2; average training race, 56.0 ± 2.0 km/week; training experience, 6.0 ± 0.5; frequency of training, 4.4 ± 0.7 times/week; time on 10 km race, 46.0 ± 0.7 min. The genotype frequency distribution was 27.2% (n = 22) RR, 53.1% (n = 43) RX, and 19.8% (n = 16) XX genotypes, consistent with the Hardy-Weinberg equilibrium. No significant differences were observed in general and training characteristics among the genotypes (**Table 1**). During the cardiopulmonary exercise test, speed and oxygen consumption in the anaerobic threshold, respiratory compensation point, and peak were similar before and after marathon race, as well as, among the genotypes (**Table 2**).

Immediately after racing, there was an increase of 40-fold in hematuria (p = 4.0e−6), 2% in transferrin (p = 1.3e−5), 1% in MCHC (p = 0.0005), 40% in creatinine (p < 1.0e−15), 23-fold in myoglobin (p < 1.0e−15), and 33% in direct bilirubin (p = 1.2e−5) and a decrease of Ht (p = 0.031) compared to pre-race levels (**Table 3**). One day after the race, there was a further decrease in erythrocytes (by 6%, p = 3.6e−7), Hb (by 6%, p = 6.5e−9), and Ht (by 6%, p = 3.4e−11), which is accompanied by an elevation of iron (by 20%, p = 0.001) and RDW (by 2%, p = 0.035). Indirect bilirubin level was stable at 33% above baseline (p = 0.0014) and transferrin saturation was increased by 23% (p = 1.1e−4) (**Table 3**). Three days after

TABLE 1 | General and training characteristics of marathon runners separated by genotype.

ACTN3 R577X	RR	RX	XX
Age (years)	37 ± 2.1	40 ± 1.2	38 ± 1.9
Body mass (kg)	71.6 ± 8.8	77.4 ± 10	70.1 ± 8.5
Height (cm)	1.71 ± 0.0	1.75 ± 0.1	1.73 ± 0.1
Training experience (years)	6.14 ± 4.3	5.6 ± 4.7	7.15 ± 5.1
Training (km/week)	62.2 ± 13	54.2 ± 22	56.1 ± 19.4
Frequency of training (time/week)	4.4 ± 1.2	4.5 ± 1.4	3.9 ± 1
Time of race (min)	254 ± 11	261 ± 6	257 ± 6
10-km race (min)	44.8 ± 5	46.7 ± 6.8	45.7 ± 5.2

The values presented are the mean ± SEM of 22, 43, and 16 runners in RR, RX, and XX genotypes, respectively.

TABLE 2 | Cardiopulmonary exercise test in the RR, RX, and XX genotypes.

	RR		RX		XX	
	Before	After	Before	After	Before	After
AT speed (km/h)	9.3 ± 0.2	9.3 ± 0.2	9.6 ± 0.3	9.4 ± 0.3	9.2 ± 0.1	9.1 ± 0.1
Respiratory compensation speed (km/h)	17.2 ± 0.5	17.1 ± 0.6	16.5 ± 0.4	16.5 ± 0.4	16.5 ± 0.5	17.1 ± 0.5
Exhaustion speed (km/h)	19.3 ± 0.4	19.3 ± 0.5	18.4 ± 0.4	18.6 ± 0.4	18.2 ± 0.5	18.9 ± 0.6
VO_2 AT (ml kg^{-1} min^{-1})	32.4 ± 0.8	32.5 ± 0.8	32.4 ± 0.7	30.8 ± 0.6	33.6 ± 1.0	31.4 ± 0.8
VO_{2RCP} (ml kg^{-1} min^{-1})	55.1 ± 1.5	53.2 ± 1.4	51.6 ± 0.9	50.7 ± 0.9	54.3 ± 1.9	54.4 ± 1.6
VO_{2peak} (ml kg^{-1} min^{-1})	58.3 ± 1.5	56.5 ± 1.4	56.6 ± 0.9	54.5 ± 0.9	59.2 ± 1.9	57.0 ± 1.5

The values are presented as mean ± SDM of 22, 43, and 16 runners in RR, RX, and XX genotypes, respectively. AT, anaerobic threshold; RCP, respiratory compensation point; VO_2, oxygen consumption.

TABLE 3 | Hematological changes and iron metabolism after marathon race.

	Before	After	1 day after	3 days after	15 days after
Erythrocytes (10^6/mm^3)	5.3 ± 0.04	5.2 ± 0.04	5.0 ± 0.05[b]	4.9 ± 0.05[b]	5.1 ± 0.05[a]
Hb (g/dl)	15.2 ± 0.1	15.1 ± 0.1	14.3 ± 0.1[b]	14.3 ± 0.1[b]	15.0 ± 0.1
Ht (%)	46.9 ± 0.3	45.9 ± 0.3[a]	44.1 ± 0.3[b]	44.6 ± 0.3[b]	44.7 ± 0.3[b]
MCV (fl)	88.1 ± 0.5	88.0 ± 0.5	88.0 ± 0.6	90.0 ± 0.6	88.1 ± 0.6
MCH (pg)	28.6 ± 0.2	28.9 ± 0.2	28.5 ± 0.2	28.8 ± 0.2	29.6 ± 0.2[a]
MCHC(g/dl)	32.4 ± 0.1	32.8 ± 0.1[b]	32.4 ± 0.1	32.0 ± 0.1[b]	33.5 ± 0.1[b]
RDW (%)	12.5 ± 0.1	12.7 ± 0.1	12.7 ± 0.1[a]	12.8 ± 0.1[a]	12.5 ± 0.1
Iron (μg/dl)	112 ± 4.6	119 ± 4.6	134 ± 4.8[a]	91 ± 3.6[a]	97 ± 3.8[a]
Ferritin (ng/ml)	129 ± 10	162 ± 13	149 ± 10	131 ± 9	124 ± 10
Transferrin (mg/dl)	252 ± 3	274 ± 4[b]	247 ± 3	238 ± 3[a]	246 ± 3
Transferrin sat. (%)	44.7 ± 1.8	43.7 ± 1.6	55.1 ± 2.1[b]	38.6 ± 1.6[a]	39.1 ± 1.6[a]
Erythropoietin (mUI/L)	10.0 ± 0.4	10.8 ± 0.5	12.5 ± 1.2	13.3 ± 0.8[a]	11.5 ± 0.7
Hematuria (number/ml)	1,225 ± 89	51,198 ± 15,427[b]	4,052 ± 1,485	2,789 ± 972	2,395 ± 999
TB (mg/dl)	0.9 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	0.8 ± 0.0	0.8 ± 0.0
DB (mg/dl)	0.3 ± 0.0	0.4 ± 0.0[b]	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
IB (mg/dl)	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.0[a]	0.5 ± 0.0	0.5 ± 0.0
Creatinine (mg/dl)	1.0 ± 0.01	1.4 ± 0.04[b]	1.0 ± 0.02	0.9 ± 0.02	0.9 ± 0.02
Myoglobin (ng/ml)	46 ± 6	1,048 ± 78[b]	184 ± 18[a]	69 ± 12	71 ± 17

Hb, hemoglobin; Ht, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; TB, total bilirubin; DB, direct bilirubin; IB, indirect bilirubin. The values are presented as mean ± SEM of 22, 43, and 16 runners in RR, RX, and XX genotypes, respectively. [a]$p < 0.05$ vs. before the race; [b]$p < 0.001$ vs. before the race.

the race, erythrocytes (p = 1.0e−7), Hb (p = 6.5e−9), and Ht (p = 2.5e−6) remained altered and iron (by 19%, p = 0.0023), transferrin saturation (p = 0.038), transferrin levels (by 6%, p = 0.008) and MCHC (p = 0.0005) decreased and RDW (by 2.4% p = 0.012) and erythropoietin (by 33%, p = 0.0014) increased, suggesting a compensatory response (**Table 3**). Fifteen days after race, erythrocytes (p = 1.610e−4), transferrin saturation (p = 0.046), iron levels (p = 0.03), and Ht (p = 4.9e−6) remained low compared to baseline while MCH and MCHC were still 3–4% higher (p = 0.0017 and p < 1.0e−15, respectively) (**Table 3**).

There were no genotype differences in the effect of marathon race on erythrocytes, Hb, Ht, MCV, MCH, CHCM, and RDW response among RR, RX, and XX genotypes (**Figure 1**). However, 1 day after race, there was a decrease in erythrocytes number and an increase in MCHC in RX genotypes (p = 0.02 and p = 0.018, respectively) (**Figures 1A,E,F**), and a reduction of Hb and Ht in RR (p = 0.006 and p = 0.01, respectively) and RX genotypes (p = 0.0015 and p = 0.0004, respectively) (**Figures 1B,C**), while XX genotypes remained at baseline levels for hematological parameters (**Figure 1**). Three days after race, we observed a decrease in erythrocytes, Hb, and Ht in RR (p = 0.019, p = 0.0018 and p = 0.016, respectively) and RX (p = 0.0031, p = 0.0018 and p = 0.034, respectively) genotypes (**Figures 1B,C**), while they remained at baseline levels in XX genotypes (**Figure 1**). MCHC elevated 15 days after race in all genotypes (**Figure 1E**).

Immediately after race, the results also demonstrated hematuria in RR and RX (p = 0.038 and p = 0.0039), but not significantly in XX participants (p > 0.9999) (**Figure 2A**). There were no genotype differences in the leukocyturia, creatinine, and erythropoietin levels (**Figures 2B–D**).

However, the percentage of runners with hematuria (>10,000 red blood cells/ml) and leukocyturia (>5,000 white blood cells/ml) was higher in RR (40 and 47%, respectively) compared to XX genotype (18 and 25%, respectively) immediately after race (**Figures 3A,B**). Creatinine (>1.3 mg/dl) imbalance was also higher in RR (59%) compared to XX genotype (31%), suggesting renal dysfunction (**Figure 3C**).

Transferrin levels increased 1 day after race in RX genotypes (p = 0.004) and the iron levels decreased 3 days after race in RR genotypes (p = 0.031), while they remained at baseline levels in XX genotypes (**Table 4**). In addition, 3 days after race, the percentage of runners with iron deficiency (<60 mg/dl) was higher in RR (18%, 4/22 runners) compared to XX genotype (6.25%, 1/16 runner) (**Figure 4A**).

The DB concentration was higher in RR genotypes compared to XX genotypes in all periods after the marathon (p = 0.0008, p < 0.0001, p = 0.0167, and p = 0.049). There was no genotype difference in the myoglobin levels (**Table 4**). The percentage of runners with impairment of hemoglobin degradation metabolites, myoglobin (>1,000 ng/ml), TB, and IB (>1.2 μg/ml) was also enhanced in RR (40, 36 and 14%, respectively) compared to XX genotype (19, 19 and 6%, respectively) (**Figures 4B–D**). Similarly, the percentage of runners with suboptimal levels of ferritin (<100 mg/dl) was higher in RR (62.5%) compared to XX (18.8%) genotype.

DISCUSSION

Our findings indicate that marathon running induces hemolysis and hematuria and that the body compensates for this by producing erythropoietin and elevating MCHC and MCH in the recovery period. In spite of the compensatory response, hematological parameters (erythrocytes and Ht) and iron levels remained low 15 days after race. Iron levels and hematological parameters seem to be less affected in the runners carrying XX genotype when compared to RR after long-distance exercise.

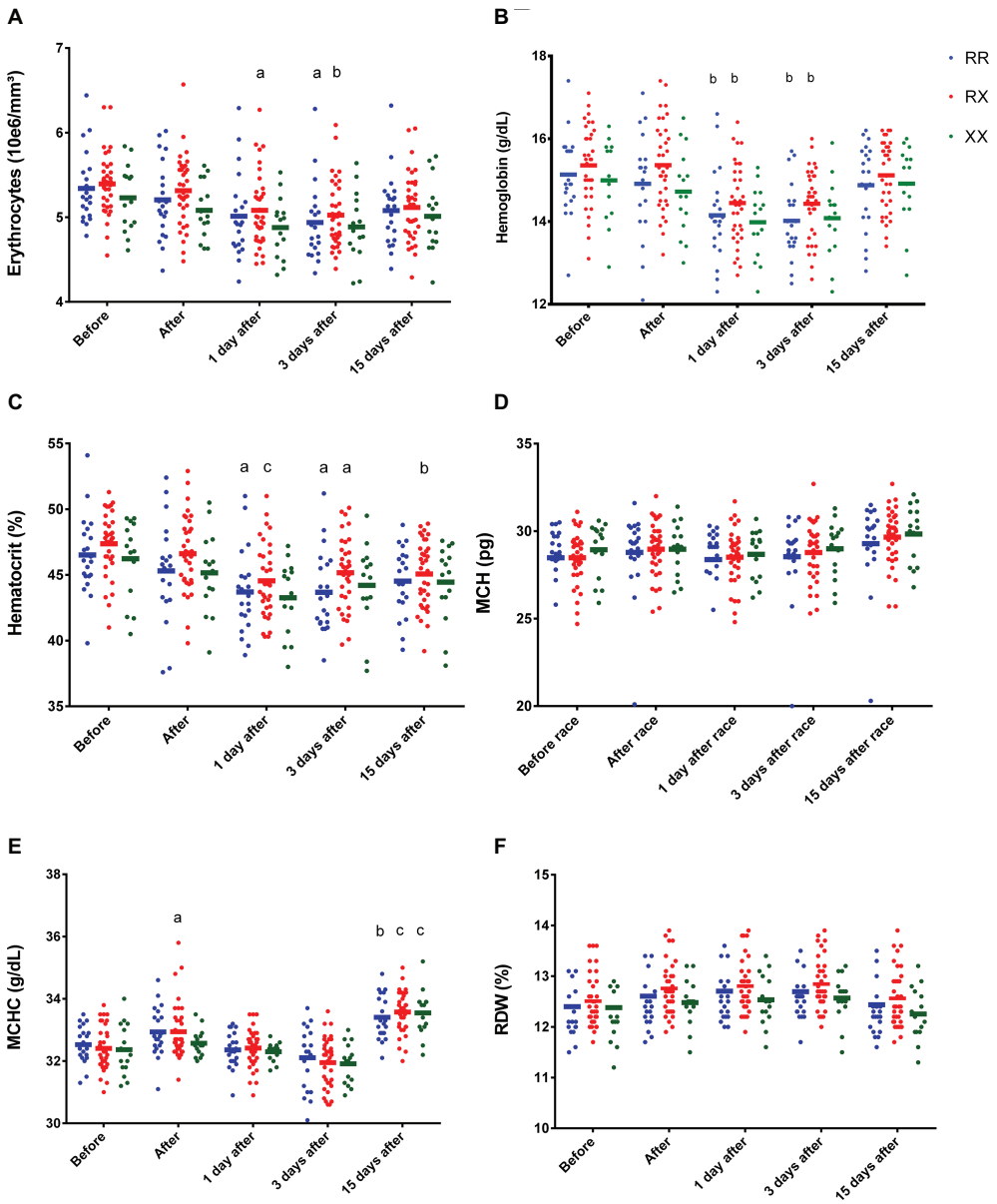

FIGURE 1 | Effect of marathon on hematological markers in RR, RX, and XX genotypes. The hematological markers evaluated were erythrocytes **(A)**; Hb, hemoglobin **(B)**; Ht, hematocrit **(C)**; MCH, mean corpuscular hemoglobin **(D)**; MCHC, mean corpuscular hemoglobin concentration **(E)**; and RDW, red cell distribution width **(F)**. The values are presented as mean ± SEM of 22, 43, and 16 runners in RR, RX, and XX genotypes, respectively. [a]$p < 0.05$ vs. before race, [b]$p < 0.001$ vs. before race, [c]$p < 0.0001$ vs. before race.

We show in our preliminary results that marathoners with the *ACTN3* XX genotype have reduced susceptibility to hematuria and iron deficiency. Despite these findings being based on a small sample, they raise the possibility of an important role for ACTN3 genotype in marathon performance and, therefore, this investigation should be further replicated.

Decreases in iron concentration between days 3 and 6 are caused by hemolysis, hematuria (25%), gastrointestinal blood loss (10%), and by sweating (4.5 micrograms iron/dl sweat) after long-distance running (Magnusson et al., 1984a,b; Selby and Eichner, 1986; Miller et al., 1988; Seiler et al., 1989; Kratz et al., 2002; Robinson et al., 2006). Iron is transported from the bloodstream bonded to transferrin and the excess intracellular iron is stored in ferritin. It was previously suggested that increased erythrocyte turnover could prevent iron deficiency in endurance athletes (Weight et al., 1991; Peeling et al., 2008). In our study, we demonstrated a decrease in erythrocytes, Hb, and Ht, which is accompanied by an elevation of iron, transferrin saturation, and indirect bilirubin 1 day after race. However, this is followed by a reduction in blood iron, transferrin levels, and saturation and an increase in erythropoietin levels in the recovery period

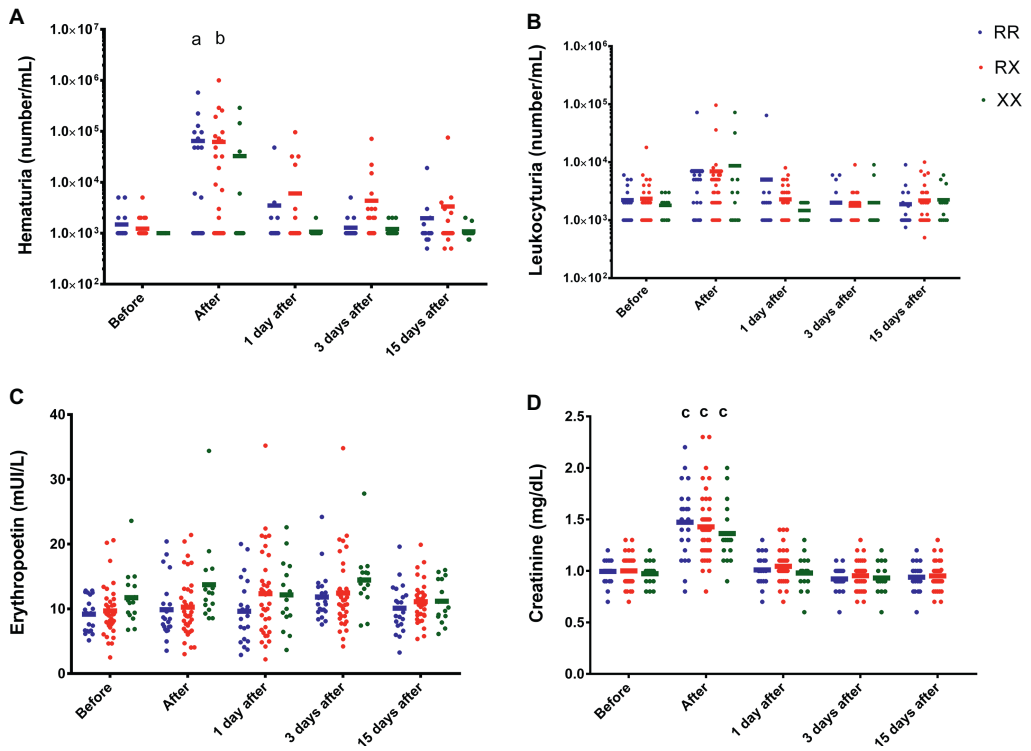

FIGURE 2 | Effect of marathon on hematuria **(A)**, leukocyturia **(B)**, erythropoietin **(C)**, and creatinine levels **(D)** in RR, RX, and XX genotypes. $p < 0.0001$ vs. before race. The values are presented as mean ± SEM of 22, 43, and 16 runners in RR, RX, and XX genotypes, respectively. $^{a}p < 0.05$ vs. before race, $^{b}p < 0.001$ vs. before race, $^{c}p < 0.0001$.

(3 or 15 days after race), consistent with the reported acute iron deficiency in marathon runners during this time period.

Despite the early compensatory increases in erythropoietin, our findings indicate that erythrocytes and Ht remain low compared to baseline 15 days after race, indicating an incomplete hematological system recovery in endurance runners after the conclusion of the race. Peak oxygen consumption is known to decrease after marathon (Sierra et al., 2016) but whether erythrocyte number and Ht contribute to the reduction in oxygen transport and consumption remains unclear. Endurance capacity seems to have high correlation with hemoglobin mass but a low correlation with hematocrit in elite athletes. A systematic review and meta-analysis show that Hb reduction by around 4–7% (after standard blood donation) promotes 7% lower maximal oxygen uptake and 10% lower maximal exercise capacity (Van Remoortel et al., 2017). In our study, Hb returned to baseline levels, while MCH and MCHC increased by 15 days after a marathon that may contribute to improve erythrocyte oxygen release as a compensatory response for low erythrocyte number.

The transferrin receptor activity, electrolytes levels, temperature, and pH interfere with iron release to the cells (He et al., 2000). Iron deficiency with or without anemia may cause a decline in work capacity, reducing delivery of oxygen and aerobic energy system of the working skeletal muscle (Peeling et al., 2008). ACTN3 deficiency seems to be associated with benefits to muscle metabolism in endurance runners (MacArthur and North, 2007). *Actn3* knockout mice have lower muscle mass and strength, IIb fiber diameter, as well as enhanced recovery from fatigue due to "slowing" of the fast muscle fiber profile, with increased aerobic enzymes activity, glycogen accumulation, calcineurin activity, calcium release and absorption by sarcoplasmic reticulum (SR), as well as reduced glycogen phosphorylase and lactate dehydrogenase activity (MacArthur et al., 2007; Chan et al., 2011; Lee et al., 2016). Tissue oxygen supply during exercise depends on temperature, pH, and some products of oxidative metabolism such as CO_2; ATP; 2,3-DPG; and Cl^- (Mairbaurl et al., 2013). The association of *ACTN3* polymorphism and parameters of tissue oxygen supply, such as hematological markers and iron metabolism after exercise has not been investigated. We did not observe differences in hematological response between RR, RX, and XX genotypes, but significantly higher hematuria and iron reduction were observed only in RR and/or RX genotypes but not in the XX group. Further studies are needed to determine the association between hematuria and *ACTN3* polymorphism due to low sample size of this study. Moreover, we observed a higher percentage of runners with leukocyturia and hematuria in RR compared to XX genotype. Exhaustive exercise may also enhance serum creatinine to concentrations >0.3 mg/dl or >50% over baseline, indicating acute renal dysfunction (Shephard, 2016).

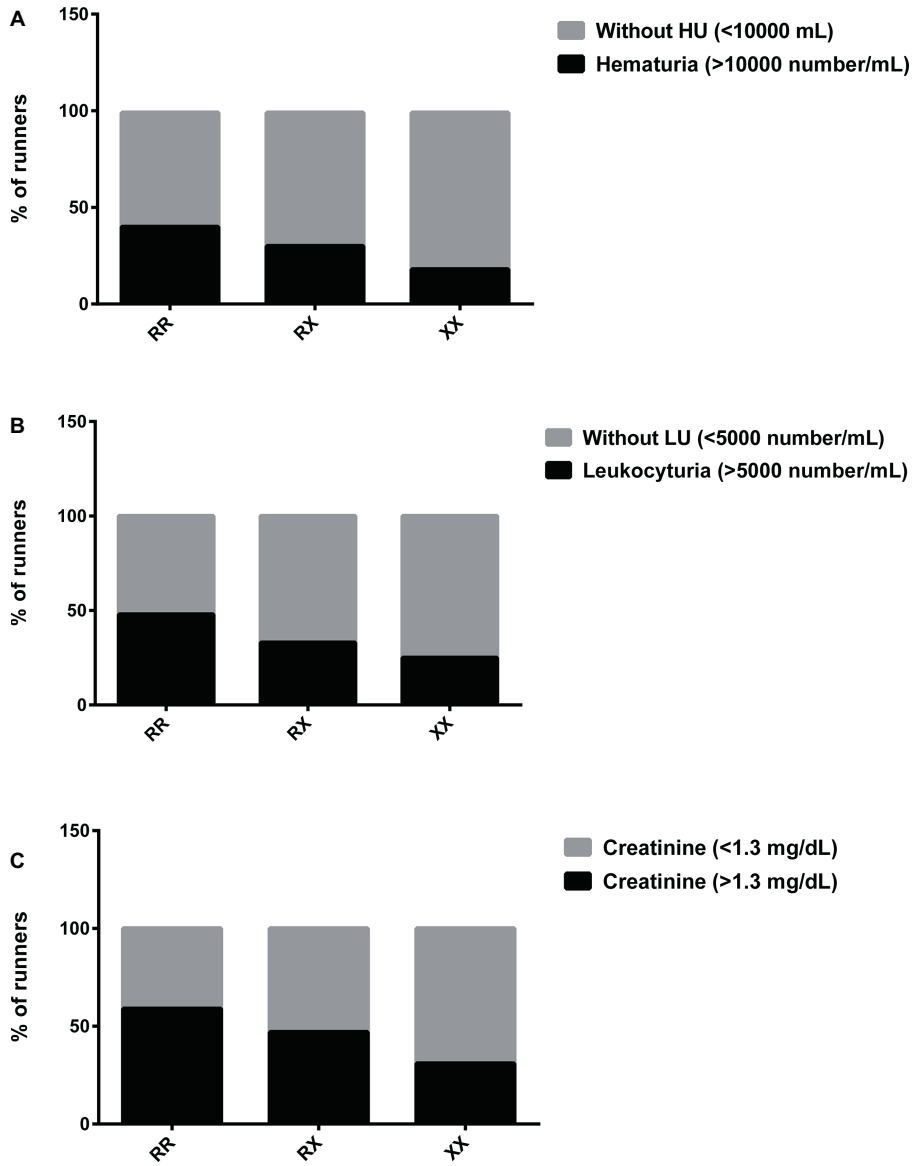

FIGURE 3 | Percentage of runners with hematuria **(A)**, leukocyturia **(B)**, and abnormal creatinine levels **(C)** after marathon race in XX, RX, and RR genotypes. HU, Hematuria; LU, leukocyturia. The values are presented as percentage of 22, 43, and 16 runners in RR, RX, and XX genotypes, respectively.

The potential mechanisms of renal injury, demonstrated by hematuria, leukocyturia, and creatinine levels are tract trauma, hypoxic renal injury and ischemia, release of a hemolysing factor during exercise, dehydration, products of hemolysis, myoglobinuria release, and peroxidation of red blood cells (Bellinghieri et al., 2008; Akiboye and Sharma, 2018). In our study, we also observed a higher percentage of runners with impairment of hemolysis metabolites and myoglobinuria with the RR genotype compared to those with the XX genotype. Hematuria also could be attributed to bladder trauma, foot strike, presence of dysmorphic red blood cells, acidosis, and hypoxia-related glomerular injury (Bellinghieri et al., 2008; Shephard, 2016; Grygorczyk and Orlov, 2017; Akiboye and Sharma, 2018).

We also observed lower percentage of runners with iron deficiency in XX genotype. The main marker of erythrocyte turnover is the hormone erythropoietin, which was higher in XX genotypes, suggesting that the small number of XX runners with iron deficiency were also influenced by increased erythrocyte turnover after race. However, the efficient turnover could contribute to higher prevalence of runners with suboptimal ferritin levels (<100 μg/L) in the XX group, but not ferritin and iron deficiency. A known contributing factor to iron and ferritin status is the level of hepcidin, which is a key regulator of iron homeostasis. Peeling et al. (2014) reported that athletes with ferritin levels <30 μg/L are unlikely to present with increased hepcidin post-exercise. In our study, all the athletes presented with ferritin values above 30 μg/L.

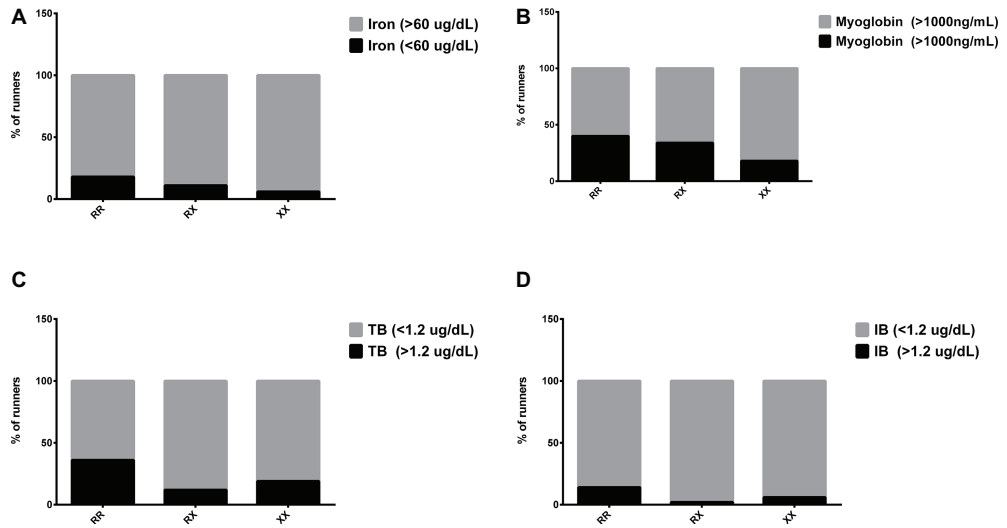

FIGURE 4 | Percentage of runners with abnormal levels of iron **(A)**, myoglobin **(B)**, total bilirubin **(C)**, and indirect bilirubin **(D)** after marathon race in XX, RX, and RR genotypes. The values are presented as percentage of 22, 43, and 16 runners in RR, RX, and XX genotypes, respectively.

TABLE 4 | Iron metabolism after marathon race in RR, RX, and XX genotypes.

		Before	After	1 day after	3 days after	15 days after
Iron (μg/dl)	RR	118 ± 5.2	119 ± 5.4	131 ± 4.8	81 ± 2.7[a]	95 ± 4.1
	RX	109 ± 4.2	116 ± 4.3	132 ± 4.3	89 ± 3.3	94 ± 3.4
	XX	110 ± 4.8	129 ± 4.4	144 ± 5.4	110 ± 4.1	106 ± 4
Ferritin (ng/ml)	RR	153 ± 9.3	183 ± 11	172 ± 9.5	151 ± 7.6	142 ± 7.9
	RX	123 ± 9.7	158 ± 14	146 ± 10.8	127 ± 9.5	122 ± 11
	XX	111 ± 8.8	142 ± 14	124 ± 8.4	112 ± 7.6	102 ± 6.9
Transferrin (mg/dl)	RR	245 ± 3	264 ± 4	239 ± 2	231 ± 3	236 ± 3
	RX	252 ± 3	276 ± 4[b]	248 ± 3	238 ± 3	248 ± 2
	XX	261 ± 3	281 ± 5	255 ± 3	246 ± 3	257 ± 3
Transferrin sat. (%)	RR	48 ± 1.9	45 ± 2.1	56 ± 2.2	36 ± 1.3	40 ± 1.6
	RX	44 ± 1.8	42 ± 1.5	54 ± 2	38 ± 1.6	38 ± 1.5
	XX	41 ± 1.6	45 ± 1.1	57 ± 2	45 ± 1.5	41 ± 1.4
TB (mg/dl)	RR	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.8 ± 0.0	0.9 ± 0.0
	RX	0.8 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.7 ± 0.0	0.8 ± 0.0
	XX	0.7 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	0.8 ± 0.0	0.9 ± 0.0
DB (mg/dl)	RR	0.5 ± 0.05	0.7 ± 0.06[d]	0.6 ± 0.06[d]	0.5 ± 0.05[c]	0.6 ± 0.1[c]
	RX	0.3 ± 0.01	0.4 ± 0.02	0.3 ± 0.01	0.3 ± 0.02	0.4 ± 0.0
	XX	0.3 ± 0.02	0.4 ± 0.03	0.3 ± 0.01	0.3 ± 0.01	0.4 ± 0.0
IB (mg/dl)	RR	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1[c]	0.5 ± 0.0	0.5 ± 0.0
	RX	0.5 ± 0.0	0.6 ± 0.0	0.7 ± 0.0[c]	0.4 ± 0.0[c]	0.4 ± 0.0
	XX	0.4 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
Myoglobin (ng/ml)	RR	59 ± 19	1,046±134[d]	147 ± 26	54 ± 8	68 ± 24
	RX	43 ± 4	1,099 ± 112[d]	195 ± 26	58 ± 5	80 ± 32
	XX	38 ± 3	911 ± 184[d]	206 ± 46	116 ± 54	56 ± 7

TB, total bilirubin; DB, direct bilirubin; IB, indirect bilirubin. The values are presented as mean ± SEM of 22, 43, and 16 runners in RR, RX, and XX genotypes, respectively. [a]p < 0.05 vs. before the race; [b]p < 0.001 vs. before the race; [c]p < 0.05 vs. XX genotypes; [d]p < 0.001 vs. XX genotypes.

CONCLUSION

Hematological changes induced by long-distance running exercise could impair performance and health, increasing the risk of sports anemia and renal failure. In conclusion, our results show that *ACTN3* polymorphism may in part explain different hematological responses in endurance athletes. This is the first study that suggested that RR individuals seem to be more susceptible to hemolytic anemia and hematuria and that supplementation and renal function evaluation should be monitored. These results are based on a small sample and should be further replicated. Strategies to improve erythrocytes turnover in endurance athletes could be proposed.

ETHICS STATEMENT

Subjects were informed of the experimental procedures and possible risks and signed a term of informed consent before participating, approved by the Ethics Committee of Dante Pazzanese Institute of Cardiology, Brazil (Permit Number: 979/2010), in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

AS designed and conducted research, analyzed the data, and performed statistical analysis. RO, ES, GL, and MB analyzed the data, conducted research, and revised the manuscript. NG, CS, MK, and JP designed and conducted research and revised the manuscript. JS revised the manuscript. MC-B designed and conducted research, performed statistical analysis, revised the manuscript, and had primary responsibility for the final content. All authors have read and approved the final version of the manuscript, and agree with the order of presentation of their names.

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Novel Associations Between Interleukin-15 Polymorphisms and Post-training Changes of Body Composition Parameters in Young Nonobese Women

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Based on the important role of interleukin-15 (IL-15) in human metabolism and, in consequence, in body composition modulation, we examined whether rs1589241 and rs1057972 polymorphisms, analyzed individually or in combination, would influence the effects of a training program. Accordingly, we studied the allele and genotype distribution in a group of 163 young nonobese Caucasian women measured for selected body mass and composition, as well as biochemical parameters before and after the completion of a 12-week endurance training program. After a week-long familiarization stage, low-high impact aerobics were conducted three times a week for 60 min, at an increasing intensity from about 50 to 80% of HRmax. With reference to rs1057972 genotypes, there were two significant genotype × training interactions, in which (i) fat mass percentage (FM%) significantly decreased among the AA homozygotes ($p = 0.00002$) and AT heterozygotes ($p = 0.00002$), and (ii) fat free mass (FFM) increased only among the AT heterozygotes ($p = 0.0003$), whereas in the AA homozygotes there was only a borderline significance ($p = 0.065$). No genotype × training interactions were found for the second rs1589241 polymorphism. Moreover, the carriers of the[T;A] haplotype (when compared with reference haplotype) displayed significant decrease in FM% ($p = 0.027$) and increase in FFM ($p = 0.014$) in response to the applied training program. Our data highlight novel associations between specific *IL-15* genotype and different post-training changes of FM% and FFM parameters. The results suggest that harboring the rs1057972 A allele and/or the [T;A] haplotype is favorable for achieving specific positive training-induced body composition changes.

Keywords: Interleukin-15, sport genetics, gene, polymorphisms, physical activity, body composition

INTRODUCTION

Interleukin-15 (IL-15) is in the four-helix bundle cytokine family that was singled out due to its ability to stimulate T and B cell proliferation, contribute to maintaining $CD8^+$ T cell memory, and activate natural killer (NK) cells (Carson et al., 1994; Grabstein et al., 1994; Armitage et al., 1995; Lügering et al., 1999; Klebanoff et al., 2004). Despite being most commonly known as an immunomodulatory cytokine, this IL-15 has also been demonstrated that its effects are not limited to the immune system (Horseman and Yu-Lee, 1994; Barra et al., 2010). The expression of IL-15 has been observed in various non-lymphoid tissues, particularly in skeletal muscle (Grabstein et al., 1994), where it influences processes ranging from angiogenesis to skeletal muscle hypertrophy (Quinn and Anderson, 2011).

As a result of IL-15's high expression in skeletal muscle and indications that suggest other cytokines are released from muscle after physical activity, some authors have theorized that IL-15 may function as a myokine modulating body composition by means of an endocrine mechanism (Carbó et al., 2001; Riechman et al., 2004; Quinn et al., 2005; Quinn and Anderson, 2011). In a study of young participants divided into two groups, those with no training and those who completed a 10-week-training program, Riechman et al. (2004) showed IL-15 level increases in plasma following resistance exercise. However, some studies have not replicated this association (Nielsen et al., 2007). Laboratory animal studies reported IL-15 to induce adipose tissue mass, especially visceral fat mass, without reducing muscle mass. Additionally, when IL-15 was administered, white fat consequently decreased, circulating triacylglycerols (TG), which correlated with lower rates of hepatic lipogenesis rate and very low density lipoprotein (VLDL) triacylglycerol content. In humans, plasma IL-15 level is inversely related to total fat mass, trunk fat mass, and percent fat mass (Carbó et al., 2001; Nielsen et al., 2007). Based on these results, it is plausible to infer IL-15's involvement in adipose tissue regulation and association with obesity (Ye, 2015).

The *IL-15* gene is located in human chromosome 4q31 (Anderson et al., 1995). In a genome-wide study of large and rare copy-number variations associated with obesity, a deletion of a region was revealed which included *IL-15* and mitochondrial uncoupling protein gene (*UPC1*) (Wang et al., 2010). Since *UPC1*'s role in regulating energy homeostasis has already been characterized (Kozak and Anunciado-Koza, 2008), the possible impact of *IL-15* loss due to this mutation was not given additional consideration. Data from cell culture, animal model, human genetic, and human subject studies all suggest IL-15 may also exert positive effects on total body mass as well as bodily composition (Carbó et al., 2001; Riechman et al., 2004; Pistilli et al., 2008; Barra et al., 2010; Quinn and Anderson, 2011). A number of single nucleotide polymorphisms (SNPs) have been isolated within the human *IL-15* gene and studied for associations with obesity and skeletal muscle response to resistance exercise (Pistilli et al., 2008). Recently, two polymorphisms (rs1589241 and rs1057972) have been clearly linked to body mass index (BMI), muscle strength, and predictors of various metabolic syndromes (Riechman et al., 2004; Pistilli et al., 2008). These SNPs, found in the 5′ and 3′ untranslated regions (UTRs) of the *IL-15* gene, seem to regulate *IL-15* expression (Quinn and Anderson, 2011).

On the basis of the aforementioned roles of IL-15 cytokine, we set out to determine whether rs1589241 and rs1057972 polymorphisms, analyzed in isolation or collectively, would impact the effectiveness of an exercise training program. Accordingly, we examined the allele and genotype distribution in young Caucasian women whose body mass and composition met certain criteria, as well as biochemical parameters before and after the completion of a 12-week aerobic training program to investigate potential interaction between genotype and fitness outcomes.

MATERIALS AND METHODS

Ethics Statement

All the procedures followed in the study were approved by the Ethics Committee of the Regional Medical Chamber in Szczecin (Approval number 09/KB/IV/2011) and were conducted ethically according to the principles of the World Medical Association Declaration of Helsinki and ethical standards in sport and exercise science research. Furthermore, the experimental procedures were conducted in accordance with the set of guiding principles for reporting the results of genetic association studies defined by the Strengthening the Reporting of Genetic Association studies (STREGA) Statement. All participants were given a consent form and a written information sheet concerning the study, providing all pertinent information (purpose, procedures, risks, and benefits of participation). The potential participant had time to read the information sheet and the consent form. After ensuring that the participant had understood the information, every participant gave written informed consent (signed consent form) to genotyping with the understanding that it was anonymous and that the obtained results would be confidential.

Participants

One hundred sixty three Polish Caucasian women (age 21 ± 1 years, height 168 ± 2 cm, body mass 61 ± 2 kg) were included in the study. None of these individuals had engaged in regular physical activity in the previous 6 months. They had no history of any metabolic or cardiovascular diseases. Participants were non-smokers and refrained from taking any medications or supplements known to affect metabolism. Participants were included in a dietary program and on the basis of an individual dietary plan, were asked to keep a balanced diet. The nutritional appointment included a prescription of an adequate diet for individual energy needs and nutritional status, with a food replacement list, in addition to orientation on a healthy diet. The share of macronutrients in the energy pool of the average food rations was as follows: 45–65% of total calories coming from carbohydrates, 10–20% from protein, and 20–35% from fat (increase the intake of unsaturated fats and decrease the intake of saturated fats). Daily

cholesterol intake <300 mg and dietary fiber intake >25 g were recommended. The participants kept a food diary every day. Weekly consultations were held during which the quality and quantity of meals were analyzed and, if necessary, minor adjustments were made.

Body Composition Measurements

Before and after the completion of a 12-week training period, all participants were measured for selected body mass and body composition variables, which were assessed with the bioimpedance method using a Tanita TBF 300 M electronic scale (Arlington Heights, Illinois, United States). The device was plugged in and calibrated to account for the weight of clothing (0.2 kg). Afterward, data regarding age, body height, and sex of the subject were inserted. The subjects then stood on the scale with their bare feet on the designated markers. The device analyzes body composition based on the differences of the ability to conduct electrical current by body tissues (different resistance) due to different water content. Body mass and body composition measurements taken with the use of the "Tanita" electronic scale are as follows: total body mass (kg), fat free mass (FFM, kg), fat mass (FM, kg), fat mass percentage (FM%, %), BMI (kg/m^2), total body water (TBW, kg), and basal metabolic rate (BMR, kJ, or kcal) (Leońska-Duniec et al., 2018b).

Biochemical and Hematological Analyses

Fasting blood samples were obtained in the morning from the antecubital vein. Blood samples from each participant were collected in two tubes. For biochemical analyses, a 4.9 mL S-Monovette tube with ethylenediaminetetraacetic acid (K 3 EDTA; 1.6 mg EDTA/mL blood) and separating gel (SARSTEDT AG & Co., Nümbrecht, Germany) were used. For complete blood count, a 2.6 mL S-Monovette tube with K 3 EDTA (1.6 mg EDTA/mL blood) (SARSTEDT AG & Co.) was used. Blood samples for biochemical analyses were centrifuged 300 × *g* for 15 min at room temperature in order to receive blood plasma. Biochemical and hematological analyses were performed before the start of the aerobic fitness training program and repeated at the 12th week of this training program (after the 36th training unit). The analyses were performed immediately after the blood collection. Blood count, including white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and total platelet level (PLT) were obtained using Sysmex K-4500 Hematology Analyzer (TOA SYSMEX, Kobe, Japan). All biochemical analyses were conducted using Random Access Automatic Biochemical Analyzer for Clinical Chemistry and Turbidimetry A15 (BIOSYSTEMS S.A., Barcelona, Spain). Blood plasma was used to determine lipid profile: TG, total cholesterol (TC), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) concentrations. Plasma TG and TC concentrations were determined using diagnostic colorimetric enzymatic method according to the manufacturer's protocol (BioMaxima S.A., Lublin, Poland). The manufacturer's stated intra-assay coefficients of variation (CV) of the method were <2.5% and <1.5% for the TG and TC determinations, respectively. HDL plasma concentration was determined using human anti-ß-lipoprotein antibody and colorimetric enzymatic method according to the manufacturer's protocol (BioMaxima S.A.). The manufacturer's declared intra-assay CV of the method was <1.5%. Plasma concentrations of LDL were determined using a direct method according to the manufacturer's protocol (PZ Cormay S.A., Łomianki, Poland). The manufacturer's declared intra-assay CV of the method was 4.97%. All analysis procedures were verified with the use of multiparametric control serum (BIOLABO S.A.S, Maizy, France), as well as control serum of normal level (BioNormL) and high level (BioPathL) lipid profiles (BioMaxima S.A.) (Leonska-Duniec et al., 2018a).

Training Phase

Before the training program, the maximum heart rate (HRmax) of each participant was evaluated on the basis of a continuous graded exercise test on an electronically braked cycle ergometer (Oxycon Pro, Erich JAEGER GmbH, Höchberg, Germany) according to the previously described protocol (Kostrzewa-Nowak et al., 2015). Individual heart rate (HR) of all participants was also monitored with HR monitors to control the intensity during the class. All participants were instructed to maintain a HR or relative value of HRmax within pre-designated ranges. The training stage was preceded by a week-long familiarization stage, when the examined women exercised three times a week for 30 min, at an intensity of about 50% of HRmax. After the week-long familiarization stage, the proper training started. Each training unit consisted of a warm-up routine (10 min), the main aerobic routine (43 min), and cool-down phase (stretching and breathing exercise for 7 min). The main aerobic routine was a combination of two alternating styles – low and high impact. Low impact style is comprised of movements with at least one foot on the floor at all times, whereas high impact styles include running, hopping, and jumping with a variety of flight phases. Music of variable rhythm intensity (tempo) was incorporated into both styles. A 12-week program of low-high impact aerobics was divided as follows: (i) 3 weeks (9 training units), 60 min each, at about 50–60% of HRmax, tempo 135–140 beats per minute (BPM), (ii) 3 weeks (9 training units), 60 min each, at 60–70% of HRmax, tempo 140–152 BPM, (iii) 3 weeks (9 training units), 60 min with the intensity of 65–75% of HRmax, tempo 145–158 BPM, and (iv) 3 weeks (9 training units), 60 min with an intensity of 65–80% of HRmax, tempo 145–160 BPM. All 36 training units were administered and supervised by the same instructor (Leonska-Duniec et al., 2018a).

Genetic Analyses

Buccal swabs were collected with two Copan FLOQSwabs, (Interpath, Heidelberg West, Australia), using methods that involved a member of the research team rubbing swabs up and down against the inside of each participant's check twenty times, then in the maxillary and mandibular buccal

sulcus (the upper and lower furrows between the gingiva and the inner cheek) for ten seconds per side. DNA was extracted using a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, Germany) according to the manufacturer's protocol. All samples were genotyped in duplicate using an allelic discrimination assay on a C1000 Touch Thermal Cycler (Bio-Rad, Germany) instrument with TaqMan® probes. To discriminate *IL-15* rs1589241 and rs1057972 alleles, TaqMan® Pre-Designed SNP Genotyping Assays were used (Applied Biosystems, United States) (assay ID: C__8865652_10 and C__8976918_10, respectively), including primers and fluorescently labeled (FAM and VIC) MGBTM probes to detect alleles.

Statistical Analyses

Allele frequencies were determined by gene counting. An χ^2 test was used to test the Hardy-Weinberg equilibrium. To test the influence of *IL-15* rs1589241 and rs1057972 polymorphisms on training response, the mixed 2 × 2 ANOVA with one between-subject factor (genotype) and one within-subject factor (time: before training versus after training) was employed. Additionally, a normality Kolmogorov–Smirnov test was used to check for data normality, and a *post hoc* Tukey test was applied when interaction was significant and was used to perform pair-wise comparisons. Haplotype analysis was conducted with R[1] using haplo.stats package and haplo.glm regression function. Percentage change over training was used as the dependent variable, while the IL-15 haplotypes were used as the independent variables. The level of statistical significance was set at $p < 0.05$.

RESULTS

The genotype frequencies of both *IL15* polymorphisms, rs1589241 (NM_000585.4:c.−222+7188T > A) and rs1057972 (NM_000585.4:c.*431A > T), did not differ from their Hardy–Weinberg equilibrium expectations ($p = 0.655$, respectively).

No genotype × training interactions were found for the rs1589241 polymorphism (**Table 1**). Training adaptation stratified by genotype of the rs1057972 polymorphism is shown in **Table 2**. We observed a genotype x training interaction for FM% (all models) and FFM (general and dominant model). FM% decreased significantly in the AA homozygotes (24.3 ± 6.2 vs 22.3 ± 7.0, SMD = −0.77, Tukey's HSD test $p = 0.00002$) and AT heterozygotes (24.7 ± 4.7 vs 23.2 ± 4.9, SMD = −0.65, Tukey's HSD test $p = 0.00002$). FFM increased significantly only among the AT heterozygotes (45.5 ± 2.7 vs 46.1 ± 2.8, SMD = 0.44, Tukey's HSD test $p = 0.0003$), whereas among the AA homozygotes there was only a borderline significance (46.1 ± 3.7 vs 46.7 ± 3.7, SMD = 0.43, Tukey's HSD test $p = 0.065$).

Table 3 presents the results of haplotype-based analysis. Four rs1589241-rs1057972 haplotypes were inferred: [A;T], [T;A], [A:A], and [T;T] with frequencies of 51.8, 24.2, 21.2, and 2.8%, respectively. Haplotype [T;A] (recessive model) was associated with a decrease in FM% with response to training (coefficient −1.64, $p = 0.027$) compared with individuals homozygous for the reference®[A;T] haplotype. The same haplotype was associated with an increase in FFM under the recessive model (coefficient 1.02, $p = 0.014$) compared with individuals homozygous for the reference [A;T] haplotype (**Table 3**).

DISCUSSION

In an effort to ascertain whether common *IL-15* polymorphisms have an impact on selected mass and body composition parameters, as well as TC, TG, HDL, and LDL levels, we analyzed the distribution of genotypes, alleles, and haplotypes. The describedrs1589241 and rs1057972 polymorphisms were examined in a group of young nonobese women who took part in a 12-week aerobic training program. The results support the claim that *IL-15* plays an important role in body composition modulation.

The results of our statistical analyses indicated that the presence of a specific *IL-15* genotype might correlate with differing outcomes in response to training. Among rs1057972 genotypes, two significant genotype × training interactions were observed in which (i) FM% significantly decreased in the AA homozygotes and AT heterozygotes, and (ii) FFM increased only in the AT heterozygotes, whereas in the AA homozygotes there was only borderline significance. The results suggest that when specific changes in body composition in response to training are considered, carrying the AA or AT genotype might impart an advantage. In contrast, the TT genotype seems to be an unfavorable factor with regards to the effectiveness of training in yielding body composition changes. Provided muscle-derived IL-15 is capable of lipolysis production, it could be the case that the presence of the A allele modifies plasma IL-15 levels, resulting in lipolysis reduction and, in turn, a FM% decrease and FFM increase. Interactions of genotype × training were not found for the second rs1589241 polymorphism.

One novel finding emerged when a complex haplotype analysis was performed using the data from this study, specifically that harboring the [T;A] haplotype (when compared with reference haplotype) displayed significant FM% reduction and elevated FFM in response to the applied training program. The next significant insight from the current study suggests that carriers of this specific [T;A] haplotype have more favorable outcomes with regards to obtaining improved body composition. These associations are intriguing with respect to muscle-derived IL-15's potential role in modulating lipolysis and glycogenolysis. However, the mediating role of the molecular mechanisms of this cytokine and their effect on adipose tissue have yet to be fully explained. Almendro et al. (2009) suggested that IL-15 upregulates calcineurin mRNA expression, thus hindering adipose cell differentiation (Quinn and Anderson, 2011).

[1]https://cran.r-project.org, version 3.1.0

TABLE 1 | Training adaptation with respect to rs1589241 polymorphism (ANOVA with repeated measures).

Parameter	AA (*n* = 88)		AT (*n* = 62)		TT (*n* = 13)		Genotype × training interaction		
	Pre	Post	Pre	Post	Pre	Post	General	Dom†	Rec‡
body mass (kg)	60.6 ± 8.2	59.9 ± 7.9	61.6 ± 6.8	60.8 ± 6.8	58.0 ± 7.6	57.8 ± 8.3	0.460	0.800	0.216
BMI (kg/m^2)	21.5 ± 2.5	21.3 ± 2.4	22.0 ± 2.4	21.8 ± 2.3	20.8 ± 2.3	20.7 ± 2.4	0.513	0.534	0.261
BMR (kcal)	1448 ± 83	1444 ± 83	1457 ± 70	1450 ± 70	1424 ± 81	1429 ± 81	0.115	0.908	0.054
FM% (%)	23.5 ± 5.6	22.3 ± 5.7	25.0 ± 5.0	23.7 ± 4.9	22.3 ± 6.2	20.0 ± 8.0	0.302	0.588	0.122
FM (kg)	14.6 ± 5.3	13.7 ± 5.3	15.7 ± 4.6	14.7 ± 4.6	13.4 ± 5.2	12.2 ± 6.2	0.874	0.682	0.665
FFM (kg)	45.9 ± 3.4	46.3 ± 3.5	45.8 ± 3.0	46.1 ± 3.0	44.7 ± 2.9	45.8 ± 2.8	0.153	0.973	0.068
TBW (kg)	33.5 ± 2.7	33.9 ± 2.6	33.7 ± 2.5	33.8 ± 2.2	32.8 ± 2.1	33.6 ± 2.0	0.132	0.284	0.205
TC (mg/dL)	170 ± 25	171 ± 28	170 ± 25	167 ± 27	165 ± 26	161 ± 24	0.275	0.107	0.577
TG (mg/dL)*	4.33 ± 0.37	4.37 ± 0.34	4.29 ± 0.33	4.34 ± 0.38	4.22 ± 0.39	4.31 ± 0.37	0.870	0.710	0.634
HDL (mg/dL)	66.0 ± 13.6	62.6 ± 13.4	63.3 ± 12.5	58.7 ± 11.5	66.4 ± 10.2	60.1 ± 13.3	0.627	0.409	0.460
LDL (mg/dL)	87.0 ± 20.8	91.5 ± 24.3	91.7 ± 23.0	91.2 ± 23.2	84.3 ± 24.9	85.0 ± 24.1	0.315	0.131	0.766

†*Dominant model: TT+AT vs AA;* ‡*Recessive model: TT vs AT+AA; *natural logarithm of TG; BMI, body mass index; BMR, basal metabolic rate; FM%, fat mass percentage; FM, fat mass; FFM, fat free mass; TBW, total body water; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.*

TABLE 2 | Training adaptation with respect to rs1057972 polymorphism (ANOVA with repeated measures.

Parameter	TT (*n* = 50)		AT (*n* = 78)		AA (*n* = 35)		Genotype × training interaction		
	Pre	Post	Pre	Post	Pre	Post	General	Dom†	Rec‡
body mass (kg)	60.0 ± 8.5	59.3 ± 8.4	60.9 ± 6.2	60.1 ± 6.0	61.7 ± 9.2	60.8 ± 9.1	0.791	0.695	0.507
BMI (kg/m^2)	21.3 ± 2.5	21.1 ± 2.5	21.7 ± 2.2	21.5 ± 2.1	22.0 ± 2.8	21.7 ± 2.7	0.740	0.802	0.438
BMR (kcal)	1442 ± 89	1436 ± 88	1450 ± 61	1446 ± 63	1461 ± 95	1454 ± 93	0.862	0.826	0.694
FM% (%)	22.7 ± 5.8	21.9 ± 5.7	24.7 ± 4.7	23.2 ± 4.9	24.3 ± 6.2	22.3 ± 7.0	0.027	0.019	0.042
FM (kg)	14.0 ± 5.6	13.4 ± 5.5	15.2 ± 4.1	14.3 ± 4.3	15.5 ± 6.0	14.1 ± 6.3	0.087	0.069	0.071
FFM (kg)	45.9 ± 3.5	46.0 ± 3.5	45.5 ± 2.7	46.1 ± 2.8	46.1 ± 3.7	46.7 ± 3.7	0.045	0.013	0.429
TBW (kg)	33.4 ± 3.0	33.7 ± 2.7	33.5 ± 2.2	33.8 ± 2.1	33.8 ± 2.7	34.2 ± 2.7	0.877	0.737	0.631
TC (mg/dL)	172 ± 24	174 ± 24	168 ± 25	165 ± 28	169 ± 25	169 ± 31	0.223	0.134	0.770
TG (mg/dL)*	4.41 ± 0.42	4.42 ± 0.36	4.28 ± 0.31	4.32 ± 0.36	4.24 ± 0.34	4.34 ± 0.38	0.473	0.383	0.267
HDL (mg/dL)	67.9 ± 14.8	64.6 ± 13.3	63.6 ± 12.6	58.7 ± 12.8	64.1 ± 10.6	60.6 ± 10.9	0.633	0.506	0.680
LDL (mg/dL)	85.7 ± 18.7	92.0 ± 20.6	89.7 ± 23.6	89.8 ± 24.2	90.2 ± 22.9	91.4 ± 27.3	0.222	0.085	0.710

†*Dominant model: AA+AT vs TT;* ‡*Recessive model: AA vs AT+TT; *natural logarithm of TG; BMI, body mass index; BMR, basal metabolic rate; FM%, fat mass percentage; FM, fat mass; FFM, fat free mass; TBW, total body water; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.*

Numerous cytokines are activated within skeletal muscle tissue after physical exertion, affecting the plasma myokine levels. Nieman et al. (2003) showed that in skeletal muscle, IL-15 was the mostly highly expressed cytokine measured at the level of mRNA (Quinn and Anderson, 2011). As both IL-15 and exercise yield beneficial outcomes in terms of improving body mass and composition parameters, it was hypothesized that previous training or state of physical fitness might mediate the supposed release of IL-15 within skeletal muscle tissue in response to exercise (Pedersen, 2009). In a study of 153 participants who completed a 10-week resistance exercise training program, Riechman et al. (2004) showed increased levels of IL-15 concentration in plasma immediately after resistance exercise, speculating that the cytokine was released through physical activity by means of micro tears in muscle fibers (Quinn and Anderson, 2011). Contrary to these findings, a similar study using healthy, young male volunteers who performed high intensity resistance exercise involving only the quadriceps muscle, no elevation of muscle or circulating IL-15 levels were revealed at intervals from 6 to 48 h afterward; however, heightened IL-15 mRNA expression was seen in the quadriceps muscle 24 h after exercise (Nielsen et al., 2007; Banitalebi et al., 2019). There is a lack of consistency in the findings as to whether a training program influences IL-15 expression in skeletal muscle and/or increasing IL-15 levels in blood, however, the inverse relationship between plasma IL-15 concentration and body mass and composition parameters is well confirmed. Barra et al. described obese participants with lower plasma IL-15 levels than lean people (Barra et al., 2010; Pedersen, 2013). This cytokine was also considered to be a local factor implicated in muscle hypertrophy and inhibiting lipogenesis.

TABLE 3 | Training adaptation with respect to rs1589241 – rs1057972 haplotypes.

Parameter	Additive				Dominant				Recessive			
	Intercept	[A;A]	[T;A]	[T;T]	Intercept	[A;A]	[T;A]	[T;T]	Intercept	[A;A]	[T;A]	[T;T]
body mass (kg)	1.29	−0.28 (0.201)	0.08 (0.705)	−0.15 (0.754)	1.37	−0.36 (0.185)	0.007 (0.979)	−0.06 (0.934)	1.13	−0.36 (0.509)	0.66 (0.209)	−0.66 (0.555)
BMI (kg/m^2)	0.66	−0.11 (0.125)	0.05 (0.463)	−0.11 (0.475)	0.69	−0.11 (0.227)	0.05 (0.562)	−0.09 (0.703)	0.60	−0.29 (0.097)	0.22 (0.194)	−0.35 (0.328)
BMR (kcal)	39.1	−1.93 (0.488)	1.69 (0.531)	−2.51 (0.671)	40.8	−2.84 (0.404)	−0.68 (0.836)	−2.81 (0.734)	31.0	−3.55 (0.599)	14.7 (0.022)	−7.92 (0.000)
FM% (%)	0.03	−0.62 (0.049)	−0.59 (0.053)	0.29 (0.662)	−0.14	−0.64 (0.098)	−0.41 (0.273)	0.62 (0.509)	0.07	−0.68 (0.372)	−1.64 (0.027)	0.64 (0.687)
FM (kg)	−0.11	−0.45 (0.052)	−0.28 (0.204)	0.10 (0.830)	−0.17	−0.46 (0.098)	−0.25 (0.364)	0.28 (0.685)	−0.25	−0.67 (0.237)	−0.46 (0.294)	0.22 (0.850)
FFM (kg)	3.23	0.27 (0.130)	0.26 (0.126)	−0.40 (0.282)	3.43	0.31 (0.147)	0.12 (0.550)	−0.55 (0.304)	2.90	0.11 (0.793)	1.02 (0.014)	−1.09 (0.218)
TBW (kg)	7.66	0.15 (0.420)	0.02 (0.889)	−0.36 (0.436)	7.71	0.20 (0.408)	−0.12 (0.600)	−0.53 (0.522)	7.39	0.04 (0.937)	0.58 (0.176)	−0.67 (0.471)
TC (mg/dL)	41.2	0.13 (0.964)	−4.42 (0.106)	−1.09 (0.859)	41.0	0.08 (0.982)	−5.19 (0.119)	−1.20 (0.859)	39.3	3.69 (0.593)	−6.47 (0.326)	−0.33 (0.982)
TG (mg/dL)	3.29	0.01 (0.757)	−0.05 (0.283)	0.13 (0.228)	3.27	0.03 (0.594)	−0.05 (0.361)	0.22 (0.169)	3.30	0.02 (0.843)	−0.12 (0.262)	0.22 (0.356)
HDL (mg/dL)	21.3	−1.01 (0.462)	−1.54 (0.250)	−4.65 (0.122)	22.4	−1.98 (0.238)	−2.59 (0.113)	−6.41 (0.137)	19.1	2.47 (0.466)	1.41 (0.662)	−8.40 (0.232)
LDL (mg/dL)	31.9	−0.44 (0.866)	−3.55 (0.164)	0.99 (0.862)	31.4	−0.07 (0.981)	−3.68 (0.237)	1.70 (0.838)	31.2	0.27 (0.966)	−6.77 (0.269)	3.64 (0.785)

BMI, body mass index; BMR, basal metabolic rate; FM%, fat mass percentage; FM, fat mass; FFM, fat free mass; TBW, total body water; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

Nielsen et al. demonstrated that a high IL-15 level correlates with lower BMI, total fat mass, trunk fat mass, and limb fat mass (Nielsen et al., 2007). These findings were supported by Tamura et al. who suggested that IL-15 might be involved in mediating systemic and local benefits as a result of endurance exercise, specifically insulin sensitizing and limiting adipogenesis as well as anabolic processes in skeletal muscle (Tamura et al., 2011). These studies seem to indirectly support the described association between *IL-15* polymorphisms and FFM changes induced by endurance training. However, the mechanism of these reactions is still unknown and further investigation is needed.

Two polymorphisms (rs1589241 and rs1057972) suggested to modulate IL-15 expression, were associated with TC, LDL, glucose levels, BMI, and muscle strength (Riechman et al., 2004; Pistilli et al., 2008). Our findings did not reveal any correlation between the polymorphism variants and biochemical profile. One possible explanation relies on population-specific characteristics, which include generally high PA levels and comparatively small body mass in the studied population. Another possible factor includes a lack of sufficient statistical power given the limited number of participants and low occurrence of the minor alleles in the studied cohort. Finally, the SNPs selected for study may also play a role. However, our results confirmed that the rs1057972 A allele and the [T;A] haplotype are associated with body composition parameters, such as FM% and FFM, however, it is inconsistent with other authors' findings. Pistilli et al. (2008) showed that the presence of the rs1589241 T allele correlated with higher BMI levels and homeostatic model assessment (HOMA) in male participants, as well as greater TC and LDL levels in women. Additionally, the presence of the rs1057972 A allele correlated with higher fasting glucose values and greater BMI in men (Pistilli et al., 2008). There could be numerous reasons for discrepancies between these studies: (i) heterogeneity between study populations, (ii) varying diets, (iii) discrepancy in training program and methods of physical activity measurement, and (iv), relatively small sizes of study groups which might lack the statistical power necessary to conduct meaningful analysis and interpretation (Leonska-Duniec et al., 2016). What is more, Pistilli et al. (2008) showed that numerous *IL-15* genotype × training interactions were gender-specific. Unfortunately, our study was focused only on women, because men did not report to the experiment, so we could not observe these dependences.

In summary, our findings indicate previously unidentified associations between specific *IL-15* genotypes and different post-training changes of FM% and FFM parameters. The results suggest that harboring the rs1057972 A allele and/or the [T;A] haplotype is favorable for achieving the desired body composition changes as a result of training. These associations are intriguing with respect to muscle-derived IL-15's potential to affect lipolysis and glycogenolysis. One of the primary objectives of exercise genomics is to identify molecular markers which independently, or in conjunction with other biological factors, could be used to project the potential success of training programs. Broadening

our knowledge of the genetic background of physiological processes should greatly enhance the ability of those in the field of exercise science to individualize exercise so that it is more efficient and safer, as well as improve recovery support, traumatology, medical care, diet, supplementation, and many other areas (Leonska-Duniec et al., 2016).

ETHICS STATEMENT

The study was approved by the Ethics Committee of the Regional Medical Chamber in Szczecin (Approval Number 09/KB/IV/2011).

AUTHOR CONTRIBUTIONS

AL-D and PC conceived and designed the experiments. AL-D, KF, and KL performed the experiments. MK performed the statistical analysis. AL-D and PC analyzed and interpreted the data. KF and AL-D wrote or provided the critical revision on the manuscript.

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Independent and Combined Effects of All-Out Sprint and Low-Intensity Continuous Exercise on Plasma Oxidative Stress Biomarkers in Trained Judokas

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The purpose of this study was to assess oxidative stress biomarkers prior to and following different forms of exercise. Ten elite male judokas (age: 18.1 ± 1.7 years, athletic experience: 6 years with a training frequency of 6 Judo-sessions/week) performed three cycle ergometry sessions comprising a 30 s Wingate test (MAX), 30 min at 60% maximal-aerobic-power-output (LOW) or these two exercise protocols combined (COMBINED) in a repeated-measures design. Venous blood-samples were collected before, and 0(P0), 5(P5), 10(P10) and 20(P20) min after each exercise protocol and assessed for malondialdehyde concentration ([MDA]), glutathione peroxidase (GPX), superoxide dismutase (SOD) and glutathione reductase (GR) content, and total-antioxidant-status (TAS). Plasma [MDA] was found to be increased above baseline at P0 and P5 in the MAX, LOW and COMBINED conditions ($p < 0.05$), but was greater at P10 and P20 in the LOW condition compared to MAX and COMBINED conditions ($p < 0.05$). Blood GPX and SOD content increased above baseline at P0 in MAX and COMBINED and at P5 in LOW ($p < 0.05$), with GR content being similar between groups at P0 and P5 ($p > 0.05$). 20 min post-exercise, GPX, SOD, GR content and TAS were lower in the MAX compared to the LOW and COMBINED conditions ($p < 0.05$). In conclusion, the findings from this study reveal that redox-related biomarkers exhibited divergent response dynamics following different forms of exercise, which might have implications for understanding the mechanisms of exercise-induced skeletal muscle fatigue and adaptive remodeling.

Keywords: redox biomarkers, physical exercise, cycle ergometer, physiological responses, response to exercise

INTRODUCTION

Reactive oxygen species (ROS) are free radical molecules that can oxidatively modify and damage cellular constituents including lipids, proteins and DNA (Leeuwenburgh and Heinecke, 2001; Bloomer et al., 2007). In healthy adults at rest, ROS-mediated oxidative damage is mitigated by a variety of enzymatic [e.g., superoxide dismutase (SOD), catalase and glutathione peroxidase

(GPX)] and non-enzymatic (e.g., glutathione, ascorbic acid, and α-tocopherol) antioxidants (Matés et al., 1999). However, in certain disease conditions (Bloomer and Goldfarb, 2004), and during exercise of a sufficient duration and intensity (Alessio et al., 1988; Kayatekin et al., 2002; Baker et al., 2004; Ammar et al., 2015a, 2016a,b), increased ROS production can outweigh antioxidant systems leading to the development of oxidative stress (Finaud et al., 2006; Powers and Jackson, 2008; Ammar et al., 2015a, 2016a, 2017b). Although increased ROS production and the ensuing development of oxidative stress have historically been considered noxious processes that impair human health and function, their prevailing effect on human health and performance remains controversial (Fisher-Wellman and Bloomer, 2009; Powers et al., 2016).

Although, the development of exercise-induced oxidative stress is well documented during different types of exercise, including low-intensity continuous (Wadley et al., 2016), high-intensity interval (Child et al., 1998; Wadley et al., 2016; Parker et al., 2018) and all-out sprint (Marzatico et al., 1997; Parker et al., 2018) exercise, it is presently unclear which type of exercise elicits the greatest oxidative stress response. Indeed, the few studies that have directly compared the effects of short-duration high-intensity exercise bouts and continuous low-intensity exercise bouts on redox balance have yielded disparate findings (Marzatico et al., 1997; Bloomer et al., 2005; Wadley et al., 2016; Parker et al., 2018). Importantly, it appears that when short-duration ≤30 s maximal intensity "all-out" exercise is compared to longer duration lower-intensity continuous exercise, some prooxidant and antioxidant markers are increased to a greater extent in the former (Parker et al., 2018). However, when high-intensity intervals are of a submaximal intensity, post-exercise oxidative stress biomarkers are similar compared to lower-intensity continuous exercise (Wadley et al., 2016). Therefore, exercise intensity appears to be an important stimulus for regulating post-exercise oxidative stress biomarkers. However, the effect of combining short-duration high-intensity and continuous low-intensity exercise bouts in a single exercise session has yet to be investigated. Resolving which type of exercise elicits the greatest oxidative stress response is important as this might reveal the exercise settings with the greatest potential to exhibit improved performance following antioxidant supplementation (Ammar et al., 2016b, 2018), and to elicit the fastest or greatest improvement in physiological and performance adaptations following a chronic exercise training intervention (Ammar et al., 2017a).

Although Parker et al. (2018) recently showed that maximal-intensity sprint interval exercise elicited a greater post-exercise hydrogen peroxide production and CAT activity compared to continuous moderate-intensity exercise, Marzatico et al. (1997) and Inal et al. (2001) demonstrated that the activities of enzymatic antioxidants increased by a similar magnitude following low- and high-intensity exercise. These discrepancies could be attributable to the inter-study differences in the exercise training status of the participants (Ammar et al., 2015a, 2016a, 2017a). Indeed, it is well documented that chronic exercise training augments the antioxidant systems by increasing the production of endogenous antioxidants (GPX, CAT, SOD, and GR; Powers and Jackson, 2008). Consistent with this, recent evidence suggests that high-intensity resistance training can suppress or prevent the increase in [MDA] after short-duration high-intensity exercise (Bloomer et al., 2005; Bloomer et al., 2007; Ammar et al., 2015a, 2016a, 2017a). Therefore, it is important to determine the effect of different forms of exercise on the development of oxidative stress in well-trained subjects as these participants might exhibit divergent responses in post-exercise oxidative stress biomarkers compared to their lesser trained counterparts. As a result, elite Judokas were recruited as participants in the current study as they are accustomed to performing different types of exercise (including continuous low-intensity exercise, short-duration maximal exercise and the combinations of these types of exercise) in their regular training program.

The purpose of the current study was to compare the effects of three different acute exercise sessions, consisting of a 30 s all-out sprint (MAX), 30 min of low-intensity continuous exercise (LOW) and these two exercise protocols combined (COMBINED), on post-exercise oxidative stress biomarkers in elite Judokas. Given that there is a lack of consensus on the most accurate oxidative stress biomarker, the current study assessed exercise-induced changes in a variety of oxidative stress biomarkers to facilitate a more robust evaluation of exercise-induced oxidative stress, in line with previous recommendations (Powers and Jackson, 2008; Ammar et al., 2017a, 2018; Cobley et al., 2017). Specifically, [MDA] was assessed as a marker of lipid peroxidation (Bloomer, 2008), the content of the antioxidant enzymes, GPX, SOD, and GR, were assessed as they are increased in response to elevated ROS production in an attempt to mitigate oxidative stress development (Matés et al., 1999), and the plasma concentration of the non-enzymatic antioxidant, α-tocopherol, was assessed as it declines in conditions of increased ROS production (Groussard et al., 2003; Ammar et al., 2015a, 2017b). Since some oxidative stress biomarkers have been reported to be altered to a greater extent following all-out sprint exercise compared to continuous submaximal exercise (Parker et al., 2018), we hypothesized that the series of oxidative stress biomarkers employed in the current study would be perturbed to a greater extent post-exercise in the MAX condition compared to the LOW condition. In addition, and on the basis that ROS production has been reported to increase in an intensity and duration-dependent way during exercise (Reid, 2016), we hypothesized that a greater degree of oxidative stress would be manifest post-exercise in the COMBINED compared to the MAX and LOW conditions.

MATERIALS AND METHODS

Participants

Ten elite male judokas participating in a regional Judo team and competing at an international standard [mean ± SD age: 18.1 ± 1.7 years, body mass: 77.2 ± 11.7 kg, height: 1.76 ± 0.05 m, peak oxygen uptake ($\dot{V}O_{2peak}$): 51.2 ± 8.4 mL·kg^{-1}·min^{-1}] volunteered to participate in this study. The participants were

recruited on the basis that they are aged between 18 and 24 years old, had a BMI less than 25 kg/m^2, were not regular creatine users and had more than 6 years judo experience with a frequency of six training sessions (1.5–2 h) per week. Typically, the training sessions consisted of a 20-min warm-up, 25 min Uchi-Komi, 10 min Nage-Komi, 20 min Randoris (Ne waza), 15 min Randoris and 15 min strength and stretching exercises. Additionally, participants were required to avoid consuming foods rich in antioxidants and polyphenols during the experimental period and the preceding 3 weeks and to avoid strenuous exercise during the experimental period. Participants provided written informed consent to participate in the study. The study was conducted according to the declaration of Helsinki with the protocol fully approved by the Sfax University Ethics Committee (ID: 8/12) before the commencement of the study.

Experimental Design

One week before the start of the experimental period, $\dot{V}O_{2peak}$ and maximal aerobic power output (MAP) were determined for each participant from an incremental laboratory cycling test (González-Haro et al., 2007). After a 10-min warm-up at 100 W, the test began at an initial power output of 200 W. Subsequently, power output was increased by 30 W every 4 min until RER≥1. Thereafter, power output was increased by 10 W/min until exhaustion. During the test, $\dot{V}O_2$ was measured breath by breath using an indirect calorimetry system (Quark PFT, Cosmed, Rome, Italy) (González-Haro et al., 2007). MAP was calculated using the equation proposed by Kuipers et al. (1985). The $\dot{V}O_{2peak}$ was determined from the mean $\dot{V}O_2$ over the last 30 s of the test.

As part of a repeated-measures, cross-over experimental design, participants performed three randomized test sessions interspersed by a recovery period of 72 h to allow sufficient recovery of oxidative stress biomarkers (Ammar et al., 2015a). Additionally, to avoid any time of day effects, all sessions were conducted in the early evening hours, as suggested by Ammar et al. (2015b, 2017b). The testing sessions required the completion of either MAX, LOW, or COMBINED exercise protocols.

Upon arrival for their first test session, each participant's body mass (Tanita, Tokyo, Japan) and height were recorded. Before completing the experimental testing sessions, a standardized 5 min cycling warm-up was completed at 75 W. The MAX protocol comprised a single standard 30 s Wingate test on an electronically braked cycle ergometer (Excalibur Sport, Lode B.V, Medical Technology, Groningen, Netherlands) connected to a computer with diagnostic software (Ergocard®, Medisoft, Dinant, Belgium). Following the warm-up, participants were instructed to pedal as fast as possible during a 6 s acceleration phase to attain peak cadence. Immediately following the acceleration phase, the load was electronically applied to the flywheel and subjects pedaled "all-out" for the entirety of 30 s. The LOW protocol consisted of pedaling on the same cycle ergometer at an intensity equal to 60% of MAP for a duration of 30 min at a cadence of 60 rpm. The COMBINED protocol involved the completion of the MAX protocol followed by the LOW protocol with 3 min passive recovery between these protocols. Immediately prior to (rest) and following (P0) each testing session, as well as 5 min (P5), 10 min (P10) and 20 min (P20) following each exercise session, blood samples were collected from a forearm vein through an intravenous cannula. Additionally, ratings of perceived exertion (RPE) were measured immediately following each test session.

Ratings of Perceived Exertion Scale

The RPE scale allows participants to give a subjective exertion rating for a physical task (Borg, 1982). The participants were familiarized to the use of the RPE scale. The scale presents a 15-point scale ranging from 6 (very very light) to 20 (very very hard). The RPE scale is a reliable indicator of physical discomfort, has sound psychometric properties, and is strongly correlated with several other physiological measures of exertion (Chtourou et al., 2012).

Dietary Records

To assess the adequacy and consistency of nutrient intake, a daily dietary record was completed over 7 days. All participants received detailed verbal and written instructions on the process of recording their diet. Participants were asked to continue with their usual dietary habits during the period of dietary recording and to be as accurate as possible in recording the amounts and types of food and fluid consumed. A list of common household measures (e.g., tablespoons, cups), and specific information about the quantity in each measurement (grams, etc.) was given to each participant. The individuals diet was evaluated using the Bilnu 4 software (SCDA Nutrisoft, Cerelles, France) and the food composition tables published by the Tunisian National Institute of Statistics in 1978. Estimated nutrient intakes were compared to reference dietary intakes for physically active people and the daily nutriment data showed that total calorie, macronutrient, and micronutrient intakes were within the reference dietary intakes for healthy Tunisian adults with no significant differences between the three test sessions (**Table 1**).

TABLE 1 | Dietary record of the subjects (mean ± SD).

Variables	Mean ± SD		
	Session 1	Session 2	Session 3
Kilocalorie (day^{-1})	3271 ± 485	3244 ± 510	3303 ± 590
Carbohydrates (%)	53.03 ± 5.9	51.54 ± 5.1	52.26 ± 5.3
Protein (%)	11.93 ± 1.2	12.67 ± 1.5	12.11 ± 1.7
Fat (%)	27.09 ± 4.0	28.28 ± 4.8	27.46 ± 3.9
Cholesterol (mg·day^{-1})	324.1 ± 98	310.9 ± 76	350.7 ± 81
Vit C (mg·day^{-1})	45.33 ± 11	43.34 ± 09	44.97 ± 13
Vit E (mg·day^{-1})	4.11 ± 0, 9	4.04 ± 0, 7	4.17 ± 1, 1
Vit A (ER)	1300 ± 252	1270 ± 198	1320 ± 203
Folate (μg·day^{-1})	338.8 ± 63	329.6 ± 54	345.4 ± 71
Vit B12 (μg·day^{-1})	7.2 ± 2.1	6.9 ± 1.8	7.4 ± 2.4

Blood Analysis

Blood samples were taken from an antecubital vein into a vacutainer via venipuncture. Samples were immediately centrifuged for 5 min at 4,000 rpm at 4°C to obtain plasma. To eliminate inter-assay variance, all samples were analyzed in duplicate, in the same assay run, and in the same laboratory. SOD, GPX and GR content, and TAS were measured using standard colorimetric assays (Randox Laboratories Limited, 55 Diamond Road, Crumlin, County Antrim, BT29 4QY, United Kingdom) as described below.

SOD

After recovery of plasma, packed red blood cells were washed 4 times with 3 ml of 9% NaCl and centrifuged for 10 min at 3,000 rpm after each wash. Cell lysis was performed by adding 2 ml of cold double-distilled water. After the red blood cells rested for 15 min at 4°C, the hemolysate was diluted 1:50 with a 0.01 M phosphate buffer, pH 7. The reaction was carried out at 37°C, and the optical density was read at 505 nm. A first reading was performed 30 s after the start of the reaction, and a second reading was performed after 3 min 30 s. The results were calculated using the formulas described by El Abed et al. (2011). A standard curve was constructed on semilogarithmic paper by increasing the percentage of inhibition of standards for subsequent calculation of SOD content (units SOD per milliliter). Intra- and inter-assay coefficient of variation for the SOD were 0.8% and 0.9%.

GPX

A 0.05 mL sample of heparinized whole blood was prediluted with 1 mL of diluent supplied in the kit to convert oxidized glutathione (GSSG) to its reduced form (GSH). After 5 min of incubation, the sample was diluted with 1 mL of Drabkin's reagent to inhibit interference from other peroxidases present in the sample. The samples were assessed within 20 min of the addition of Drabkin's reagent. The decrease in absorbance was measured at 340 nm. Three readings were recorded 1, 2, and 3 min from the start of the reaction. The GPX concentration was calculated from the formula described by El Abed et al. (2011). Intra- and inter-assay coefficient of variation for the GPX were 0.9 and 1.0%.

GR

After recovery of plasma, red blood cells were washed 3 times with 3 mL of NaCl 9% and centrifuged for 5 min at 2,000 rpm after each wash. Cell lysis was performed by adding 0.5 mL of cold double distilled water. After 10 min incubation at 4°C, the hemolysate was centrifuged for 5 min at 2,000 rpm to remove the stroma. Subsequently, 100 mL of hemolysate was diluted with 1.9 mL of 9% saline. The absorbance was read at 340 nm 1, 2, 3, 4, and 5 min from the beginning of the reaction. GR activity was calculated from the formula described by El Abed et al. (2011). Intra- and inter-assay coefficient of variation for the GR were 0.7 and 0.8%.

TAS

After incubation of the reaction mixture consisting of the sample, standard or a blank and a chromogen at 37°C, the initial absorbance was read at 600 nm. The second reading was made exactly 3 min after adding the hydrogen peroxide (H_2O_2) substrate. TAS was calculated as described by El Abed et al. (2011). Intra- and inter-assay coefficient of variation for the TAS were 0.6 and 0.7%.

α-Tocopherol

α-tocopherol was extracted with hexane from human plasma and then measured via high performance liquid chromatography (HPLC) as described by Siluk et al. (2007). For specimen preparation, 100 μL of internal standard and 100 μL of plasma were mixed for 5 s. Subsequently, 200 μL of ethanol was added and mixed for 30 s, followed by 500 μL of hexane which was mixed for 1 min. The mixture was centrifuged at 4000 rpm and 4°C for 8 min with 450 μL of the supernatant removed after centrifugation and evaporated to dryness under a stream of nitrogen at room temperature. Solids were taken by the addition of 250 μL of methanol, vortexing for 30 s followed by centrifugation (4000 rpm and 4°C for 8 min) before finally analyzing using the HPLC method described by Siluk et al. (2007). Intra- and inter-assay coefficient of variation for the α-tocopherol were 1.1 and 1.2%.

MDA

MDA was assessed as a marker of lipid peroxidation using a colorimetric reaction, which uses 1-methyl-2-phenylindole as a chromogen (McCusker et al., 1993). Condensation of one molecule of MDA with 2 molecules of 1-methyl-2-phenylindole under acidic conditions results in the formation of a chromophore with an absorbance maximum at 586 nm. A 7.6 mM solution of 1-methyl-2-phenylindole (MPI) was prepared immediately prior to use, in 33% methanol in acetonitrile. A 650 μL aliquot of MPI was placed in each test tube, followed by the addition of 200 μL of plasma. The tubes were mixed well, and 150 μL of 10 M HCl was added. After mixing once more, tubes were sealed, and incubated for 60 min at 45°C. After incubation, tubes were chilled on ice and centrifuged at 10,000 × *g* for 5 min to remove debris. The absorbance at 586 nm was subsequently measured and subtracted from the blank value, obtained by replacing plasma with water. A calibration plot was prepared using 4, 8, 16, and 20 μmol/L of 1,1,3,3-tetramethoxypropane in 20 mM Tris–HCl, buffer, pH 7.4 (McCusker et al., 1993). Intra- and inter-assay coefficient of variation for the MDA were 1.6 and 1.7%.

Statistical Analysis

All statistical analyses were performed using STATISTICA 10.0 Software. Normality of the data distribution was confirmed using the Shapiro-Wilk's test. To analyze the acute effect of exercise type on oxidative stress biomarkers a two-way repeated-measures ANOVA exercise type (3 levels: MAX, LOW and COMBINED) × time (5 levels: rest, P0, P5, P10, and P20) was utilized. *Post hoc* tests were conducted using Fisher's least significant difference (LSD). Effect sizes (ES) were calculated using partial eta-squared (ηp^2) and magnitudes were interpreted

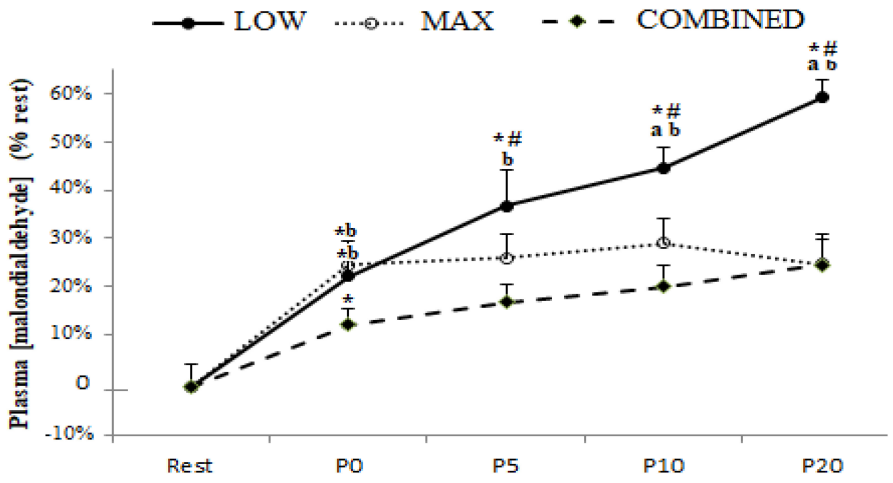

FIGURE 1 | Plasma malondialdehyde concentration before (Rest), immediately after (P0), and 5 (P5), 10 (P10) and 20 (P20) minutes after maximal-intensity, low-intensity and maximal- and low-intensity exercise combined. Data are expressed as the % change from pre-exercise resting concentrations. LOW: low-intensity exercise; MAX: maximum-intensity exercise; COMBINED: combined maximum-intensity and low-intensity exercise; *: significant difference compared to pre-test values; # significant difference compered to P0, a: significant difference compared to the MAX exercise;b: significant difference compared to the COMBINED exercise.

using the thresholds: ES $<$ 0.2 was considered small, ES around 0.5 was considered medium and ES $>$ 0.8 was considered large (Hopkins, 2012). Statistical significance was set at $P < 0.05$ and data are presented as mean ± SE (**Figures 1–3** and **Supplementary Table S1**).

RESULTS

Plasma [MDA] pre- and post-test for the MAX, LOW and COMBINED conditions is presented in **Figure 1**. There was a significant exercise-type × time interaction effect for plasma [MDA] [$F_{(8,72)} = 3.51$, $p = 0.0017$ and $\eta_p^2 = 0.28$]. Plasma [MDA] increased immediately (P0) after the test session with a higher rate of increase during the MAX (25.6 ± 7.8%) and LOW (23.2 ± 5.7%) conditions compared to the COMBINED (10.8 ± 3.6%) condition ($p < 0.05$). Higher MDA concentrations ($p < 0.05$) were registered at P5, P10 and P20 compared to P0 only following the LOW exercise. Moreover, [MDA] was higher at P10 and P20 in the LOW condition compared to the MAX and COMBINED conditions ($p < 0.05$; **Figure 1**).

Blood antioxidant enzyme content in response to the LOW, MAX and COMBINED protocols are presented in **Figure 2**. A significant exercise-type × time interaction was registered for GPX [$F_{(8.72)} = 5.79$, $p = 0.0004$, $\eta p^2 = 0.40$], SOD [$F_{(8.72)} = 3.16$, $p = 0.004$, $\eta p^2 = 0.26$] and GR [$F_{(8.72)} = 2.99$, $p = 0.006$, $\eta p^2 = 0.25$] content. Regardless of the exercise type, blood GR content increased immediately after exercise ($p < 0.05$). The blood content of GPX and SOD, both increased immediately post-exercise in the MAX and COMBINED conditions compared to the resting baseline ($p < 0.05$). However, GPX and SOD content was not increased above the resting baseline in the LOW condition until 5 min post-exercise ($p < 0.05$). Compared to the MAX condition, LOW and COMBINED exercise resulted in greater GPX and GR content at P10 ($p < 0.05$) and P20 ($p < 0.01$), and a greater SOD content at P20 ($p < 0.05$). Following MAX exercise, GPX content had returned to baseline values at P10 ($p < 0.05$), whereas SOD and GR content did not return to baseline values until the P20 sampling point ($p > 0.05$). Blood GPX, SOD and GR content remained elevated above the resting baseline in the LOW and COMBINED conditions ($p < 0.05$).

Plasma TAS and [α-tocopherol] following the MAX, LOW and COMBINED exercise protocols are shown in **Figure 3**. There was a significant main effect for time for TAS [$F_{(4,36)} = 3.47$,$p = 0.017$,$\eta_p^2 = 0.29$] and [α-tocopherol] [$F_{(4,36)} = 7.86$, $p = 0.0001$, $\eta p^2 = 0.46$]. Compared to pre-exercise values, an increase in TAS was only registered following the LOW exercise protocol at P5 ($p < 0.05$). Plasma [α-tocopherol] concentration was lower at P0 ($p < 0.05$) following only MAX exercise and at P5, P10 and P20 following the three types of exercise ($p < 0.05$). Both TAS and [α-tocopherol] were lower following MAX exercise compared to LOW and COMBINED exercise at both P10 ($p < 0.05$) and P20 ($p < 0.01$). TAS levels had returned to baseline values at P20 only following MAX exercise ($P > 0.05$).

There was no significant effect of exercise type on post-exercise RPE (15.7 ± 0.9 for LOW, 15.9 ± 1.1 for MAX and 16.3 ± 0.7 for COMBINED; $p > 0.05$).

DISCUSSION

The present study assessed a series of oxidative stress biomarkers prior to and following MAX, LOW and COMBINED exercise. Plasma [MDA] and TAS, and blood GPX, SOD and GR increased, and plasma [α-tocopherol] declined, post-exercise in all exercise protocols, consistent with the development of exercise-induced oxidative stress. However, the principal original findings of the

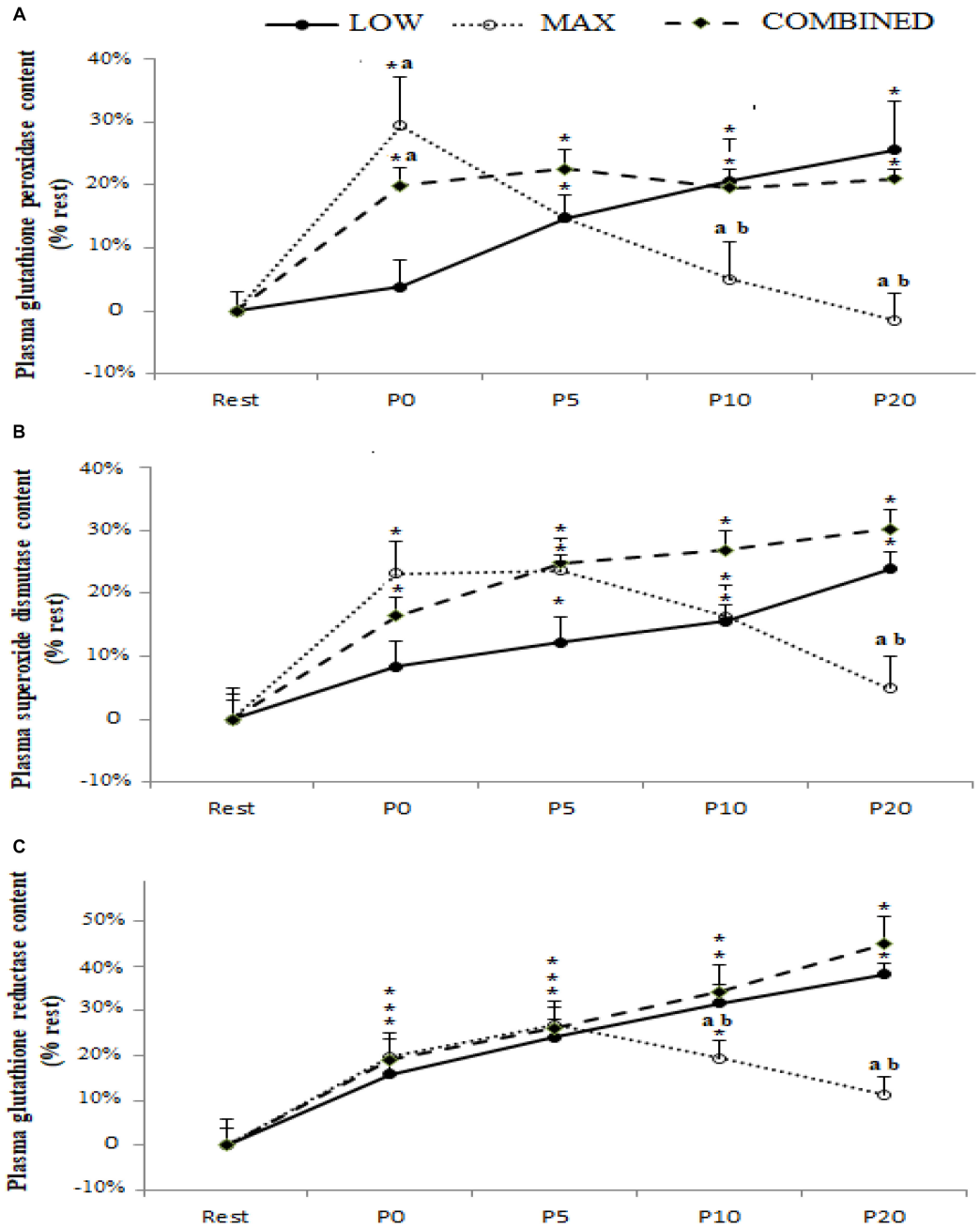

FIGURE 2 | Plasma glutathione peroxidase **(A)**, superoxide dismutase **(B)** and glutathione reductase **(C)** content before (Rest), immediately after (P0), and 5 (P5), 10 (P10) and 20 (P20) minutes after maximal-intensity, low-intensity and maximal- and low-intensity exercise combined. Data are expressed as the % change from pre-exercise resting concentrations. LOW: low-intensity exercise; MAX: maximum-intensity exercise; COMBINED: combined maximum-intensity and low-intensity exercise; *: significant difference compared to pre-test values; a: significant difference compared to the LOW exercise, b: significant difference compared to the COMBINED exercise.

current study were: (1) blood GPX and SOD content increased above baseline immediately post-exercise in the MAX and COMBINED protocols, but not the LOW protocol; (2) blood GPX, SOD, GR and plasma TAS were lower 20 min post-exercise in the MAX compared to the LOW and COMBINED protocols; and (3) plasma [MDA] was highest 20 min following the LOW protocol and plasma [α-tocopherol] was lowest 20 min following the MAX protocol. These findings offer insight into exercise-type-specific oxidative stress development and might have implications for improving understanding of skeletal muscle fatigue, recovery and adaptive remodeling in response to discrete exercise protocols.

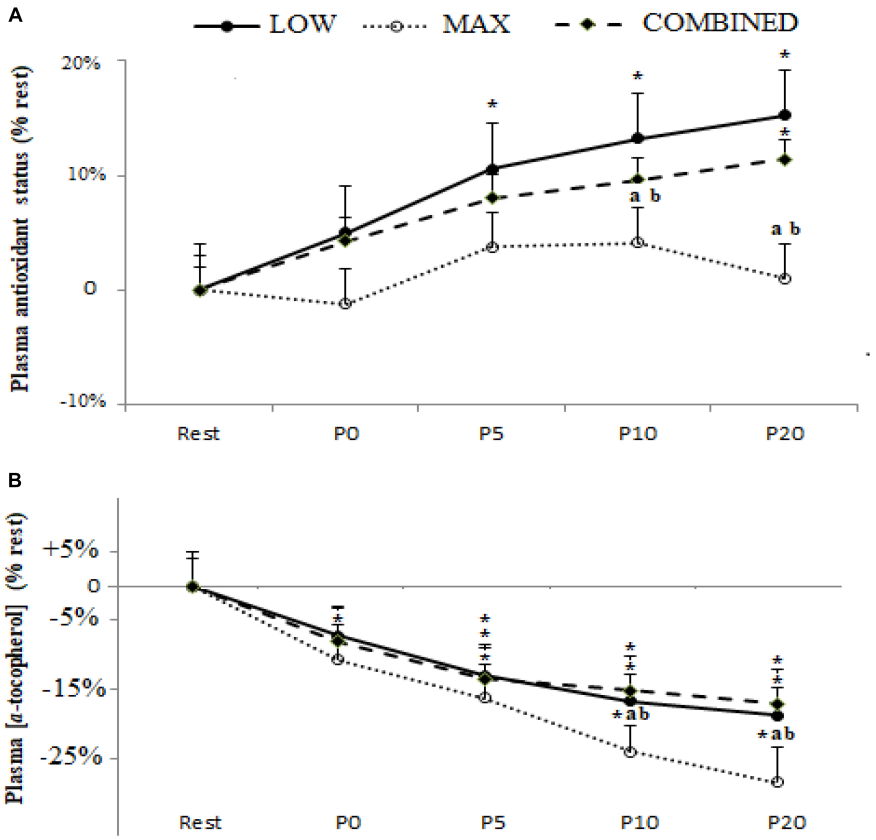

FIGURE 3 | Plasma antioxidant status **(A)** and α-tocopherol concentration **(B)** before (Rest), immediately after (P0), and 5 (P5), 10 (P10) and 20 (P20) min after maximal-intensity, low-intensity and maximal- and low-intensity exercise combined. Data are expressed as the % change from pre-exercise resting concentrations. LOW: low-intensity exercise; MAX: maximum-intensity exercise; COMBINED: combined maximum-intensity and low-intensity exercise; *: significant difference compared to pre-test values; a: significant difference compared to the LOW exercise, b: significant difference compared to the COMBINED exercise.

In the present study, plasma [MDA], GPX, SOD, GR and TAS increased, and [α-tocopherol] declined, post-exercise in the MAX, LOW and COMBINED exercise protocols. These observations are consistent with the development of exercise-induced oxidative stress, and in line with numerous previous studies reporting increased oxidative stress biomarkers after different types of exercise (Ammar et al., 2015a, 2016a, 2017b; Wadley et al., 2016; Parker et al., 2018).

While there is strong evidence to support the development of exercise-induced oxidative stress, it is less clear how different types of exercise influence the degree of oxidative stress. Indeed, existing studies assessing the effects of different exercise protocols on biomarkers of oxidative stress development have yielded equivocal findings (Marzatico et al., 1997; Bloomer et al., 2005; Parker et al., 2018). Baker et al. (2004) and Child et al. (1998) showed a significant increase in oxidative stress responses following low-intensity aerobic exercise, while other studies (Niess et al., 1996; Margaritis et al., 1997) have reported no pre- to post-exercise changes in [MDA]. Similarly, high-intensity anaerobic (Baker et al., 2004) and combined low- and high-intensity exercise (Thompson et al., 2003; Bloomer et al., 2006; Ascensão et al., 2008) have been reported to either increase pre- to post-exercise [MDA] or have no significant effect on pre- to post-exercise [MDA] (Svensson et al., 1999; Bloomer et al., 2006). In part, these discrepancies might be linked to inter-study differences in participant characteristics, exercise protocols and the series of biomarkers employed to assess oxidative stress. To limit the influence of these confounding variables, the present study assessed the effects of three different exercise protocols on the development of oxidative stress, inferred from the same set of oxidative stress biomarkers, in the same participants. In the present study, plasma [MDA] was increased above the pre-exercise baseline in the MAX, LOW and COMBINED exercise protocols immediately post-exercise with low post-exercise values during COMBINED protocol and no differences between the LOW and MAX protocols. These observations are consistent with some (Ascensão et al., 2008; Ammar et al., 2015a, 2016a), but not all (Svensson et al., 1999; Bloomer et al., 2006) previous studies reporting an increased [MDA] immediately post-exercise, with no difference between aerobic and anaerobic based exercise (Bloomer et al., 2005). However, in the present study plasma [MDA] was higher in the LOW protocol compared to the MAX and COMBINED protocols 10- and 20-min post-exercise.

Concerning antioxidant defense biomarkers, it was previously suggested that in response to an increased production of free radicals, concentrations of antioxidant enzymes may increase to counteract the elevated radical production and thereby minimize oxidative damage (Bloomer and Goldfarb, 2004; Ammar et al., 2015a). The present findings confirm this suggestion and showed that the blood content of the antioxidant enzymes, GPX, SOD and GR, as well as TAS, increased post-exercise in the MAX, LOW and COMBINED exercise protocols. These responses have been attributed to an increased production of ROS and a resultant increase in content of antioxidant enzymes to attenuate the development of exercise-induced oxidative stress (Bloomer, 2008; Ammar et al., 2015a, 2016a). The findings of the current study are in line with some previous studies reporting increased enzymatic antioxidant activities immediately following short-duration high-intensity exercise, such as 100 m swimming (Inal et al., 2001) and 6 × 150 m sprints (Marzatico et al., 1997), and longer duration low-intensity running (Ji, 1993) or swimming (Inal et al., 2001) exercise, and extend these observations by revealing an increase in the content of key antioxidant enzymes in the blood following the COMBINED exercise protocol. However, our observations conflict with some previous studies which reported no increase in antioxidant enzyme content following a Wingate test (Groussard et al., 2003). These inter-study discrepancies may reflect the higher training status of the elite athletes assessed in the present study. Indeed, it has been reported that increased activation of redox-sensitive transcription factors (e.g., NF-κB) in well-trained individuals can improve the production of endogenous antioxidants in response to physical exercise (Cuevas et al., 2005).

An important novel observation from the current study was the divergent response dynamics of the antioxidant biomarkers assessed across the different exercise protocols administered. Indeed, the content of SOD and GPX, but not GR and TAS, was higher immediately post-exercise in the MAX and COMBINED protocols compared to the LOW protocol. However, at the P20 time point, SOD, GPX, GR and TAS were lower in the MAX compared to the LOW and COMBINED protocols. Collectively, these plasma biomarkers pertaining to antioxidant status suggest that exercise incorporating short-duration maximal exercise can expedite the increase in some systemic antioxidant processes compared to low-intensity longer duration exercise. Conversely, 20 min following the cessation of exercise, antioxidant biomarkers were elevated during exercise incorporating 30 min of low-intensity exercise compared to shorter duration maximal intensity exercise. These observations are consistent with some evidence that post-exercise oxidative stress biomarkers are impacted by exercise intensity and duration (Ji, 1993; Parker et al., 2014), with the findings of the current study suggesting that maximal duration exercise elicits a more rapid but transient increase in antioxidant processes compared to a slower responding but longer lasting change in antioxidant processes following longer duration and lower intensity.

With regard to the interplay between the markers pertaining to antioxidant status (SOD, GPX, GR and TAS) and ROS-mediated oxidation (plasma [MDA] and [α-tocopherol]), it is possible that the greater increase in SOD and GPX content immediately post-exercise in the MAX compared to the LOW protocol could be linked to enhanced ROS-mediated oxidation in the former. Indeed, plasma [α-tocopherol] declined to a greater extent immediately following the MAX compared to the LOW protocol, which may have resulted in a compensatory up-regulation in antioxidant processes, including blood SOD and GPX content, to limit exercise-induced oxidative stress development. On the other hand, plasma [MDA] was not different immediately following the MAX and LOW protocols. Conversely, 20 min following the completion of the MAX protocol, SOD, GPX, GR and TAS were lower than the LOW protocol concomitant with a lower plasma [MDA]. Therefore, lowering in antioxidant processes 20 min post the MAX protocol could reflect a lowering in ROS-mediated oxidation and, by extension, a lesser requirement to increase antioxidant processes to maintain an optimal cellular redox balance. However, plasma [α-tocopherol] was lower 20 min following the MAX compared to the LOW protocol. Therefore, further research is required to resolve the mechanisms for the changes in, and interplay between, post-exercise prooxidant and antioxidant biomarkers and how these markers relate to exercise performance, recovery and adaptation. Moreover, since oxidative stress biomarkers were only assessed for up to 20 min post-completion of the exercise protocols administered in the current study, further research is required to assess oxidative stress biomarkers over a longer period until all biomarkers have returned to baseline to provide a more complete picture of exercise-induced oxidative stress.

Experimental Considerations

To the best of the authors' knowledge, this is the first study to provide information regarding the effect of different forms of exercise on oxidative stress responses in judo athletes. However, while the findings of the present study indicate exercise intensity might be a key determinant of the redox perturbation evoked by exercise, a limitation of the current study is that the three exercise tests administered were not work-matched. Accordingly, and despite reporting a similar post-exercise RPE, the design of present study did not allow us to separate the effects of exercise intensity from exercise duration when the same amount of work was completed. Additionally, the current study is based on a small sample of participants which is not enough to allow for generalization. Therefore, further research investigating a larger sample of participants is required to improve understanding of the influence of exercise intensity, duration and their interaction on exercise-induced redox perturbations.

CONCLUSION

In conclusion, compared to baseline values [MDA], GPX, SOD and GR and TAS increased, and [α-tocopherol] declined, following the completion of the MAX, LOW and COMBINED exercise protocols administered in the current study. While these observations are consistent with the development of exercise-induced oxidative stress, the principal original findings from this study pertain to the characterization of exercise-type-specific oxidative stress development. Specifically, SOD and

GPX content were increased immediately post-exercise, and GPX, SOD and GR and TAS were lower 20 min post-exercise, in the MAX compared to the LOW and COMBINED exercise protocols. These findings suggest that some antioxidant defense processes increase and subsequently return to baseline more rapidly after MAX exercise. However, the exercise-type-specific effect on prooxidant biomarkers is complicated by our observations that the increase in plasma [MDA] was greatest following LOW exercise and the largest decline in plasma [α-tocopherol] occurred following MAX exercise. These original findings provide insight into exercise-type-specific oxidative stress development which might have implications for exercise performance, recovery and adaptation.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Participants provided written informed consent to participate in the study. The study was conducted according to the declaration of Helsinki with the protocol fully approved by the Sfax University Ethics Committee before the commencement of the study.

AUTHOR CONTRIBUTIONS

KEA, AA, and AH conceived and performed the experiment. KEA, AA, OB, LM, SB, AH, and NB drafted and critically revised the manuscript. All authors approved the final version.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00842/full#supplementary-material

TABLE S1 | Plasma levels of all tested parameters (mean ± SE) before (Rest), immediately after (P0), and 5 (P5), 10 (P10), and 20 (P20) min after maximal-intensity, low-intensity, and maximal- and low-intensity exercise combined.

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Physiological and Biochemical Effects of Intrinsically High and Low Exercise Capacities Through Multiomics Approaches

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Regular exercise prevents lipid abnormalities and conditions such as diabetes mellitus, hypertension, and obesity; it considerably benefits sedentary individuals. However, individuals exhibit highly variable responses to exercise, probably due to genetic variations. Animal models are typically used to investigate the relationship of intrinsic exercise capacity with physiological, pathological, psychological, behavioral, and metabolic disorders. In the present study, we investigated differential physiological adaptations caused by intrinsic exercise capacity and explored the regulatory molecules or mechanisms through multiomics approaches. Outbred ICR mice (n = 100) performed an exhaustive swimming test and were ranked based on the exhaustive swimming time to distinguish intrinsically high- and low-capacity groups. Exercise performance, exercise fatigue indexes, glucose tolerance, and body compositions were assessed during the experimental processes. Furthermore, the gut microbiota, transcriptome, and proteome of soleus muscle with intrinsically high exercise capacity (HEC) and low exercise capacity (LEC) were further analyzed to reveal the most influential factors associated with differential exercise capacities. HEC mice outperformed LEC mice in physical activities (exhaustive swimming and forelimb grip strength tests) and exhibited higher glucose tolerance than LEC mice. Exercise-induced peripheral fatigue and the level of injury biomarkers (lactate, ammonia, creatine kinase, and aspartate aminotransferase) were also significantly lower in HEC mice than in LEC mice. Furthermore, the gut of the HEC mice contained significantly more *Butyricicoccus* than that of the LEC mice. In addition, transcriptome data of the soleus muscle revealed that the expression of microRNAs that are strongly associated with exercise performance-related physiological and metabolic functions (i.e., miR-383, miR-107, miR-30b, miR-669m, miR-191, miR-218, and miR-224) was higher in HEC mice than in LEC mice. The functional proteome data of soleus muscle indicated that the levels of key proteins related to muscle function and carbohydrate metabolism were also significantly

higher in HEC mice than in LEC mice. Our study demonstrated that the mice with various intrinsic exercise capacities have different gut microbiome as well as transcriptome and proteome of soleus muscle by using multiomics approaches. The specific bacteria and regulatory factors, including miRNA and functional proteins, may be highly correlated with the adaptation of physiological functions and exercise capacity.

Keywords: intrinsic exercise capacity, physical activities, gut microbiota, transcriptome, proteome

INTRODUCTION

Unhealthy diet and lack of exercise cause more than 300,000 deaths per year. A sedentary lifestyle is considered an independent risk factor for cardiovascular disease (Blair et al., 1989; McGinnis and Foege, 1993). Regular physical activity can reduce both morbidity and all-cause mortality, including preventing dyslipidemia, diabetes, hypertension, and obesity development (Jennings et al., 1989; Blair et al., 1995). Individuals exhibit variable responses to exercise, which may be primarily mediated through genetic variations (Bouchard et al., 1999). Furthermore, genes determine intrinsic exercise capacity in an untrained state (Braith et al., 1994; Krushkal et al., 1999). High intrinsic exercise capacity is related to genes that determine adaptive responses to exercise (Braith et al., 1994; Bouchard et al., 2000). In addition, aerobic endurance is affected by genetic and environmental factors (Falconer and Mackay, 1996). Researchers have also demonstrated an increased prevalence of interrelated chronic metabolic diseases among individuals with low intrinsic exercise capacity, including insulin resistance, type 2 diabetes mellitus, obesity, and coronary heart disease (Schwarzer et al., 2010; Pekkala et al., 2017; Koch and Britton, 2018). Therefore, aerobic capacity may be closely related to multiple chronic diseases.

Endurance exercise includes running, cross-country skiing, cycling, swimming, and other aerobic exercises, and it can be defined as cardiovascular exercise (Joyner and Coyle, 2008). The physiological and biochemical requirements of endurance exercise elicit muscle- and system-based responses. The main adaptations caused by endurance exercise are improved mechanical, metabolic, neuromuscular, and contractile function in muscles; rebalanced electrolytes (Russell et al., 2013); reduced glycogen stores (Munoz et al., 2010); and increased mitochondrial biogenesis in muscle (Snow et al., 1981). In addition, endurance exercise may cause an increase in oxidative stress, intestinal permeability, muscle damage, systemic inflammation, and immune responses (Davies et al., 1981; Mach and Fuster-Botella, 2017).

The human intestine contains numerous microorganisms that significantly affect host nutrition, metabolic function, intestinal development, immune system function, and epithelial cell maturation (Hooper and Gordon, 2001). The gut microbiota promotes food digestion and absorption (Hsu et al., 2015), and the microbiota digests and subsequently ferments complex carbohydrates in the colon into short-chain fatty acids, such as *n*-butyrate, acetate, and propionate. A study found that elite male rugby players had lower levels of Bacteroidetes and higher levels of Firmicutes than healthy non-athletic individuals (Clarke et al., 2014). Regardless of diet, exercise increased the percentage of Bacteroides and reduced the percentage of Firmicutes (Evans et al., 2014). In ovariectomized female high-capacity runner (HCR) and low-capacity runner (LCR) breed rats, exercise intervention caused differential effects on host metabolism and gut microbial communities. The abundance of Firmicutes, Proteobacteria, and Cyanobacteria caused shifts in ovariectomized LCR, but Christensenellaceae was significantly higher in HCR rats than in LCR rats (Liu et al., 2015). Another similar ovariectomized model also validated that the microbial diversity and number of the Bacteroidetes phylum were significantly increased in LCR rats but unchanged in HCR rats without exercise intervention (Cox-York et al., 2015).

In traditional analysis methods, reductionist approaches are insufficient to comprehensively portray connections and regulation of complicated biological responses. The term *omics* implies a comprehensive (or global) assessment of a set of molecules. Several omics technologies exist, including genomics, epigenomics, transcriptomics, proteomics, metabolomics, and microbiomics, which are all well-developed and widely used in different fields (Hasin et al., 2017). Compared with a single omics type, multiomics can provide different perspectives on the flow of information underlying phenotypes, physiological responses, and disease development.

In this study, we examined the effects of intrinsic high exercise capacity (HEC) and low exercise capacity (LEC) on physical activity performance and on physiological and biochemical data using a multiomics approach. Our results could provide alternative perspectives on regulatory mechanisms for physiological adaptations and even on critical factors affecting health.

MATERIALS AND METHODS

Animals and Group Design

Male outbred ICR mice (7 weeks old) were purchased from a supplier accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (BioLASCO, Yi-Lan, Taiwan). Experimental animals were maintained in the animal room at the National Taiwan Sport University (NTSU), and all procedures were approved by the Institutional Animal Care and Use Committee of the NTSU (IACUC-10103). All animals were maintained at 23 ± 2°C (room temperature) and 60 ± 5% humidity and were fed a standard laboratory diet (Laboratory Rodent Diet #5001, PMI Nutrition International, Brentwood, MO, United States) and distilled water *ad libitum*. Mice were

acclimated to the environment for 1 week before experiments were conducted. Then, all 100 mice, without artificial selection population as F0 generation, performed an exhaustive swimming test, with 5% body weight (BW) loading on the tail. After the test, the mice were categorized into three groups on the basis of the exhaustive swimming time: LEC (15 lowest capacity mice), medium exercise capacity (MEC; 15 medium capacity mice), and HEC (15 highest capacity mice) (**Figure 1**).

Exhaustive Swimming Exercise

Mice were individually placed in a columnar swimming pool (65 cm in height and 20 cm in diameter) with a 40-cm water depth maintained at 27 ± 1°C. A weight equivalent to 5% of the mouse's BW was attached to the base of the tail, and for each mouse, endurance was measured as the swimming time recorded from the beginning of the time in the pool to exhaustion. The swimming period was defined as time spent floating, struggling, and making necessary movements until exhaustion – as indicated by evident uncoordinated movements and failure to swim to the water surface within 7 s (Huang et al., 2012).

Forelimb Grip Strength

Forelimb grip strength was assessed 1 week after the exhaustive swimming test. A low-force testing system (Model-RX-5, Aikoh Engineering, Nagoya, Japan) was used to measure the absolute forelimb grip strength of mice, as previously described (Huang et al., 2012). Tensile force was measured using a force transducer equipped with a metal bar (2 mm in diameter and 7.5 cm in length) for each mouse. The maximal force (g) recorded was used as an indicator of absolute grip strength.

Fatigue-Associated Biochemical Indices

Fatigue-associated biochemical indices were assessed 1 week after the forelimb grip strength test. Blood samples were collected immediately after mice swam for 15 min. After centrifugation at 1,500 × *g* for 10 min at 4°C, serum was analyzed on an automatic analyzer (Hitachi 7060, Hitachi, Tokyo, Japan). Biochemical variables, namely lactate, ammonia, blood urea nitrogen (BUN), glucose, lactic dehydrogenase (LDH), creatinine kinase (CK), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), were evaluated as indices of exercise fatigue, as detailed previously (Huang et al., 2012; Wang et al., 2012; Wu et al., 2013).

Oral Glucose Tolerance Test

An oral glucose tolerance test (OGTT) was conducted 1 week after assessment of fatigue-associated biochemical indices. Mice were fasted for 14 h before the OGTT. An oral gavage of glucose (2.5 g/kg BW) was administered, and blood samples were collected at 0, 15, 30, 60, and 120 min and analyzed using a glucometer, Roche Accu-Chek® Comfort Curve (Roche Diagnostics, Indianapolis, IN, United States).

Blood Biochemical Assessments

At the end of the final experiment, all mice were killed by 95% CO_2 asphyxiation at an optimal flow rate for CO_2 displacement of 15% chamber volume per minute; then, their blood was withdrawn through a cardiac puncture after an 8-h fas. The blood was centrifuged at 1,500 × *g* for 10 min at 4°C, and the serum was used to assess the levels of AST, ALT, LDH, albumin, total protein (TP), BUN, alkaline phosphatase (Alk-P), creatinine, CK, uric acid (UA), total cholesterol (TC), triacylglycerol (TG), and glucose using the autoanalyzer (Hitachi 7060, Tokyo, Japan).

Tissue Sample Preparation

At the end of the study, the key tissues and organs – the liver, muscles (gastrocnemius and soleus muscles), kidney, heart, lung, epididymal fat pad (EFP), and brown adipose tissue (BAT) – were carefully removed and weighed. The total weight and specific tissue weights relative to individual BW (%) were recorded. Then, these tissues were immediately stored in liquid nitrogen, and the soleus muscle was collected separately for further proteomics and transcriptomics analyses.

Bacterial DNA Extraction and 16S rRNA Sequencing

Fecal samples were collected 1 week after the OGTT. Mice in each group were housed in a metabolic cage with food and water calculated to produce individual feces. The feces were immediately stored at −80°C for bacterial DNA extraction. Feces (500 mg) were homogenized (MagNA Lyser System; Roche, Basel, Switzerland) in ASL buffer, and DNA was extracted directly using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted DNA was stored at −80°C before 16S rRNA sequencing. The hypervariable V3–V5 region of the bacterial 16S rRNA gene was amplified through polymerase chain reaction (PCR) with barcoded universal primers V3-357F (forward primer; 5′-CCTA TCCCCTGTGTGCCTTGGCAGTCTCAG**CCTACGGGAGGCAGCAG**-3′) and V5-926R (reverse primer; 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNN**CCGTCAATTCMTTTRAGT**-3′). The underlined sequences are the 454 FLX sequencing primers, and the bold letters denote the universal 16S rRNA primers. The barcode within the primer is denoted by Ns. The regions of the 16S rRNA gene were amplified using FastStart HiFi Polymerase (Roche, Basel, Switzerland). The sequencing reaction was performed in a 9700 thermal cycler (Applied Biosystems, Foster, CA, United States) at 94°C for 4 min, followed by 40 cycles at 94°C for 15 s, 50°C for 45 s, and 72°C for 1 min, then held at 72°C for 8 min, and finally maintained at 4°C until use. The presence of amplicons was confirmed through 1.5% agarose gel electrophoresis. PCR amplicons were purified using the Agencourt AMPure XP Reagent (Beckman Coulter™, Pasadena, CA, United States) and quantified using Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States). Equimolar amounts of PCR amplicons were mixed in a single tube. The purified amplicon mixtures were sequenced on the GS Junior System (454 Life Sciences, a Roche Company, Branford, CT, United States) according to the protocols recommended by the manufacturer. The sequences were further analyzed using a Ribosomal Database Project Naive Bayesian rRNA Classifier (version 2.5) to categorize the

microbiota and investigate the relative proportions of microbiota existing in an indicated sample.

Soleus Muscle MicroRNA TaqMan Low-Density Array Analysis

Total RNA was isolated using the TRI reagent (Thermo Scientific, San Jose, CA, United States) for further miRNA expression profiling. Total RNA was quantified using NanoDrop ND-1000 (Thermo Scientific, San Jose, CA, United States), and RNA integrity (RNA integrity number $\geq$ 6) was verified using the RNA 6000 Nano kit (Agilent Technologies, Palo Alto, CA, United States). The miRNA expression profile in the soleus muscle of mice in each capacity group was analyzed using the TaqMan Low-Density Array (TLDA) Rodent MicroRNA Cards (version 3A and B; Applied Biosystems, Foster, CA, United States). Each card contains 375 precoated rodent miRNA targets, all cataloged in the miRBase database, with three endogenous controls: Mamm U6, U87, and Y1. In this study, U87 was used as the endogenous normalizer. Total RNA (100 ng) was reverse-transcribed using Megaplex RT primer Pools A and B (381 stem-looped primers per pool) and the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster, CA, United States). The preamplified product was diluted with the TaqMan Universal PCR master mix (Applied Biosystems, Foster, CA, United States) and deionized distilled water, and it was loaded into one of the eight fill ports on the TLDA microfluidic card. The card was centrifuged for 1 min at 331 $\times$ g to distribute samples to multiple wells connected to the fill ports and was then sealed to prevent well-to-well contamination. All cards were processed and analyzed on a 7900 HT Real-Time PCR System (Applied Biosystems, Foster, CA, United States). Real-time RT-PCR data analysis was performed using the RQ Manager (version 1.2.1; Applied Biosystems, Foster, CA, United States) and Partek Genomic Suite (version 6.6). The expression level was calculated using the comparative Ct ($\Delta\Delta$Ct) method and was further analyzed by comparing the fold change relative to HEC and LEC. The results of the TLDA analysis were converted into a graphic display heat map based on hierarchical clustering using DataAssist (version 2.0).

Soleus Muscle Proteomics Analysis

Soleus muscle protein samples (50 μg) were applied to the gel in triplicate, and the sizes of proteins were visualized by staining with Coomassie Brilliant Blue G-250 (Bio-Rad, Hercules, CA, United States). After electrophoresis gel lanes were split into 10 equal fractions, the slices were destained through repeated washing in a solution of 25 mM ammonium bicarbonate and 50% (V/V) acetonitrile (1:1) until the protein bands were invisible. After completely drying them on Speed-Vac (Thermo Electron, Waltham, MA, United States), the proteins in the gel fragments were then subjected to reduction and cysteine alkylation reactions to irreversibly break the disulfide bridges in the proteins. For reduction, each gel piece was rehydrated with 2% (V/V) β-mercaptoethanol in 25 mM ammonium bicarbonate and incubated at room temperature for 20 min in the dark. Cysteine alkylation was performed by adding an equal volume of 10% (V/V) 4-vinylpyridine in 25 mM ammonium bicarbonate and 50% (V/V) acetonitrile for 20 min. The samples were then washed by soaking them in 1 mL of 25 mM ammonium bicarbonate for 10 min. After drying on Speed-Vac for 20 min, in-gel trypsin digestion was performed by incubating the samples with 100 ng of modified trypsin (Promega, Mannheim, Germany) in 25 mM ammonium bicarbonate at 37°C overnight. The supernatant of the tryptic digest was transferred to an Eppendorf tube. For extraction of the remaining peptides from the gel, 25 mM ammonium bicarbonate and 50% (V/V) acetonitrile were added, the samples were incubated for 10 min, and then the solution was collected. The resulting digests were dried in Speed-Vac and stored at −20°C. Each cryo-stored tryptic digest was resuspended in 30 μL of 0.1% (V/V) formic acid and analyzed using an online nanoAcquity ultra-performance liquid chromatography system (Waters, Manchester, United Kingdom) coupled to a hybrid linear ion trap Orbitrap (LTQ-Orbitrap Discovery) mass spectrometer with a nanoelectrospray ionization source (Thermo Scientific, San Jose, CA, United States). The eluted peptides were ionized with a spray of 2.33 kV and placed in the mass spectrometer. Mass spectrometric data were obtained using the data-dependent acquisition method, in which one full mass spectrometry survey scan (m/z 200–1,500) at a high resolution of 30,000 full width at half maximum width was followed by tandem MS (m/z 200–1,500) of the six most intense multiple-charged ions. A protein was identified when at least two unique peptides were matched with an Xcorr score of >2.5 for each peptide. The false discovery rate (≤1%) obtained from the search was compared against the decoy database to identify the protein.

Statistical Analysis

Statistical analyses were performed using SPSS (version 18.0; SPSS, Chicago, IL, United States). Data are expressed as mean ± standard deviation (SD). Significant differences in weight, diet, body composition, biochemical data, and physical activity performance among HEC, MEC, and LEC mice were calculated using one-way analysis of variance (ANOVA) and Duncan's test, and $p < 0.05$ was considered significant. In addition, an independent t-test was used to compare the intestinal microbiota, transcriptome, and proteome of soleus muscle between HEC and LEC mice, and $p < 0.05$ (*) was considered statistically significant.

RESULTS

Intrinsic Aerobic Endurance and Grip Strength Performance

Mice (n = 100) performed an exhaustive swimming test under 5% BW loading. Intrinsic aerobic endurance was ranked according to the exhaustive swimming exercise performance of mice (**Figure 1**). Most mice were exhausted within 100 min, but a few mice demonstrated extreme endurance capacity and swam for >200 min. On the basis of their performance, mice were divided into three experimental groups: LEC (bottom 0–15%

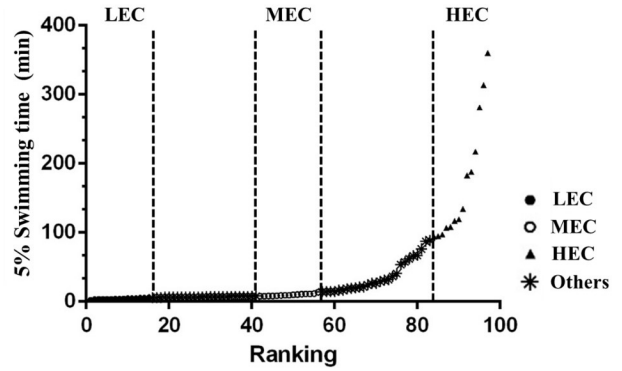

FIGURE 1 | Capacity ranking from low to high for male mice (n = 100). The 15 lowest ranked, 15 medium ranked, and 15 highest ranked mice were categorized as low exercise capacity (LEC), medium exercise capacity (MEC), and high exercise capacity (HEC) mice for study experiments.

performers), MEC (intermediate 42.5–57.5% performers), and HEC (top 85–100% performers) groups. Each group contained 15 mice, all of whom underwent additional physical tests. The exhaustive swimming times of LEC, MEC, and HEC mice were 4.35 ± 0.23, 9.72 ± 0.42, and 172.4 ± 23.79 min, respectively (**Figure 2A**). Thus, the exhaustive swimming exercise performance of HEC mice significantly exceeded that of LEC mice by 38.6-fold ($p < 0.001$). In addition, the grip strength of HEC mice (139.2 ± 5.1 g) was higher than that of LEC (124.9 ± 3.2 g) and MEC (129.5 ± 3.2 g) mice ($p = 0.032$ and $p = 0.038$, respectively; **Figure 2B**).

BW, Food Uptake, and Tissue Weight

Table 1 lists the BW, daily food intake, and tissue weight of LEC, MEC, and HEC mice. BW, diet, and water intake did not significantly differ among the groups. Therefore, differences in exercise performance were not attributable to nutritional supplementation but to other adaptive physiological factors. In addition, only the muscle weight (particularly the weight of gastrocnemius and soleus muscles) was significantly different between LEC and HEC mice (**Table 1**): It was 7.7% higher in HEC mice than in LEC mice ($p = 0.042$).

Clinical Biochemistry

We assessed fatigue-related (lactate, ammonia, BUN, and glucose) and injury-related (LDH, CK, AST, and ALT) biomarkers after the acute exercise challenge and evaluated biochemical data at the end of the study. Lactate and ammonia levels in HEC and MEC mice were significantly lower than those in LEC mice ($p < 0.05$; **Figure 3**). Regarding injury-related biomarkers, HEC and MEC mice had significantly lower CK and AST levels than LEC mice ($p < 0.05$; **Figure 4**). The remaining indicators, namely BUN, glucose, LDH, and ALT, did not differ significantly among the three groups ($p > 0.05$).

Table 2 presents the biochemical data assessed at the end of the study. Compared with LEC mice, HEC and MEC mice demonstrated significantly higher serum albumin levels but significantly lower BUN levels (both $p < 0.05$). Although ALT, LDH, and Alk-P levels were significantly different between the three groups, no significant difference was noted between HEC and LEC mice ($p > 0.05$). Similarly, AST, TP, creatinine, CK, UA, TC, TG, and Glu levels did not differ significantly among the three groups ($p > 0.05$).

OGTT

After the administration of glucose (2.5 g/kg) through oral gavage, blood was individually collected for glucose measurement at 0, 15, 30, 60, and 120 min (**Figure 5**). At the beginning (0 min), glucose levels did not differ significantly among the three groups ($p > 0.05$). However, at 15 and 30 min, glucose levels were significantly higher in LEC mice than in MEC and HEC mice ($p = 0.024$ and $p < 0.001$, respectively). At 60 min, LEC and MEC mice did not exhibit significantly different glucose levels; however, HEC mice had significantly lower glucose levels than did LEC and MEC mice ($p = 0.004$ and $p = 0.012$, respectively). Finally, at 120 min, LEC mice exhibited 2.01-fold higher glucose levels than HEC mice ($p < 0.001$).

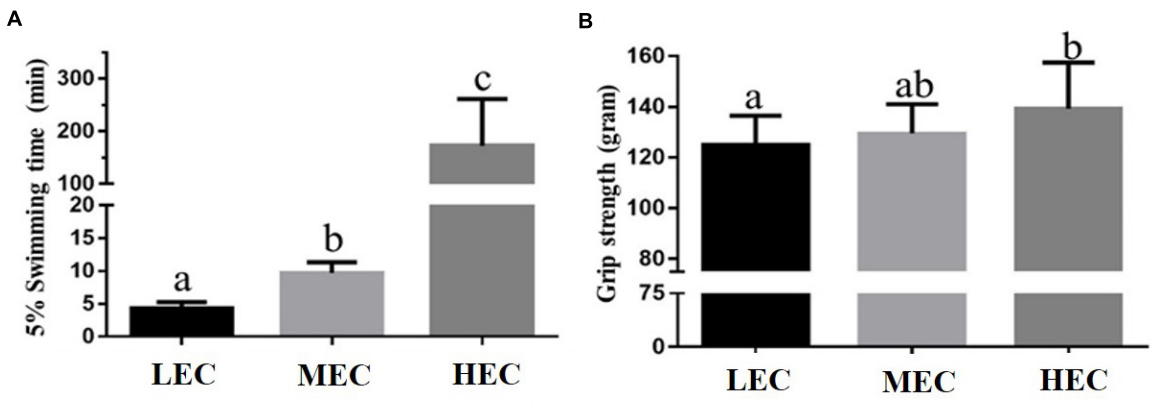

FIGURE 2 | Effect of exercise capacity on **(A)** exhaustive swimming time and **(B)** forelimb grip strength. Data are presented as mean ± standard deviation for 15 mice per group. Different letters (a,b,c) indicate a significant difference at $p < 0.05$ using one-way ANOVA.

TABLE 1 | Effect of different exercise capacities on general characteristics.

Characteristic	LEC	MEC	HEC	Trend analysis
Initial BW (g)	35.3 ± 0.7	34.8 ± 0.7	34.4 ± 0.6	0.9655
Final BW (g)	39.5 ± 0.7	40.1 ± 0.9	39.6 ± 0.5	0.2490
Food intake (g/day)	7.9 ± 0.4	7.5 ± 0.3	7.8 ± 0.3	0.9344
Water intake (mL/day)	11.8 ± 0.4	10.9 ± 0.4	10.9 ± 0.2	0.2374
Liver (g)	2.27 ± 0.07	2.22 ± 0.05	2.25 ± 0.07	0.8609
Muscle (g)	0.39 ± 0.02^{a}	0.40 ± 0.01^{ab}	0.42 ± 0.01^{b}	0.0504
Kidney (g)	0.71 ± 0.02	0.70 ± 0.01	0.70 ± 0.02	0.8611
Heart (g)	0.26 ± 0.01	0.25 ± 0.01	0.25 ± 0.01	0.9842
Lung (g)	0.21 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	0.9729
EFP (g)	0.29 ± 0.03	0.26 ± 0.02	0.27 ± 0.01	0.9388
BAT (g)	0.09 ± 0.001^{a}	0.11 ± 0.006^{b}	0.09 ± 0.002^{a}	0.3432

Muscle mass includes both gastrocnemius and soleus muscles in the back part of the lower legs. LEC, low exercise capacity; MEC, medium exercise capacity; HEC, high exercise capacity; BW, body weight; EFP, epididymal fat pad; BAT, brown adipocyte tissue. Data are the mean ± standard error measurement for n = 10 mice in each group. Values in the same row with different superscript letters (a, b) differ significantly, $p < 0.05$, by one-way ANOVA.

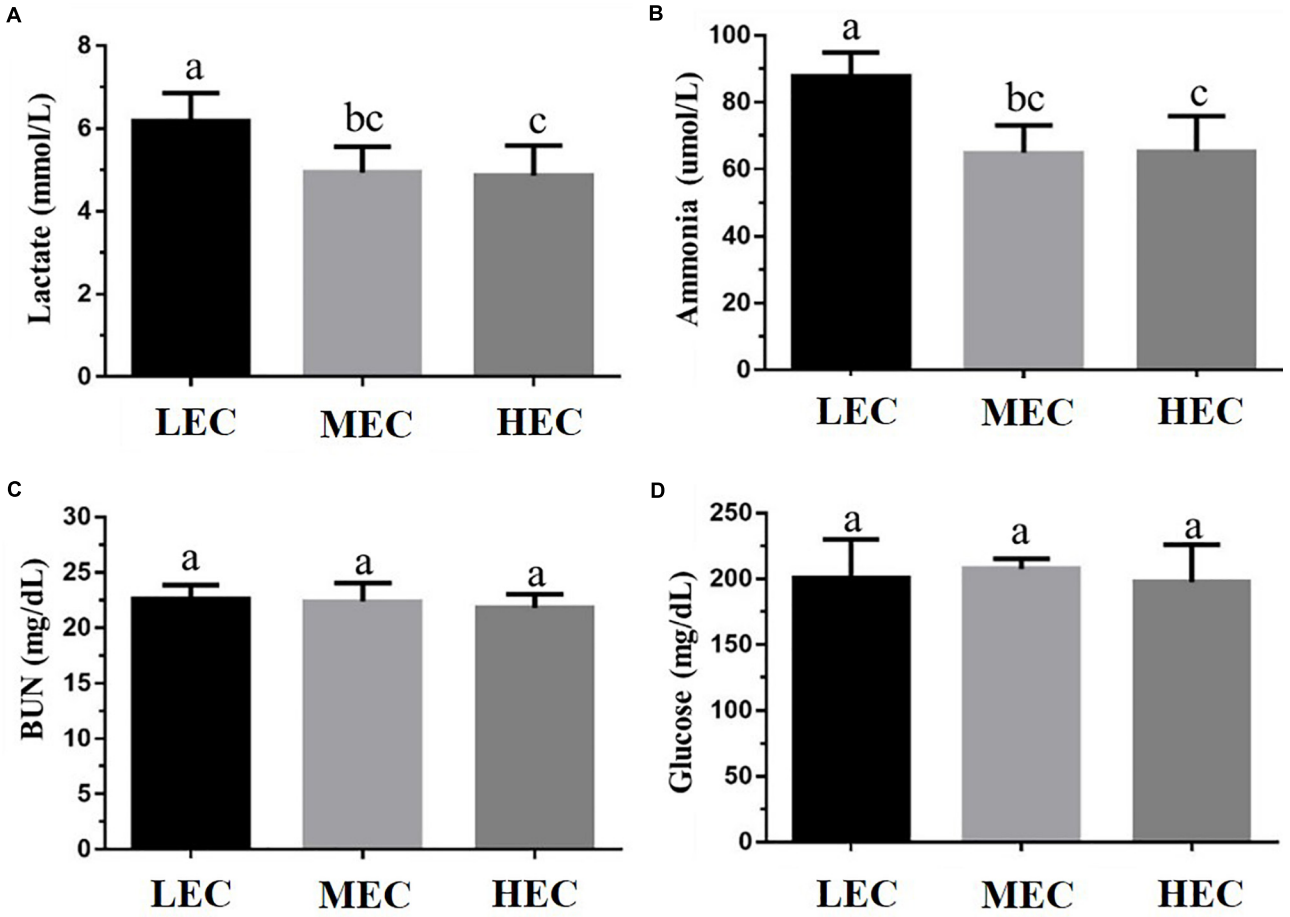

FIGURE 3 | Effect of exercise capacity on fatigue-related biomarkers, namely serum **(A)** lactate, **(B)** ammonia, **(C)** blood urea nitrogen (BUN), and **(D)** glucose level, after the acute exercise challenge. Data are presented as mean ± standard deviation for 15 mice in each group. Different letters (a,b,c) indicate a significant difference at $p < 0.05$ using one-way ANOVA.

Gut Microbiota

A partial least squares discriminant analysis showed that mice clustered into relatively distinct groups based on LEC, MEC, and HEC mice (**Figure 6A**). Alpha-diversity indexes of richness (Observed and Chao1) were slightly increased in HEC mice compared with LEC mice (**Figure 6B**). The gut microbiota

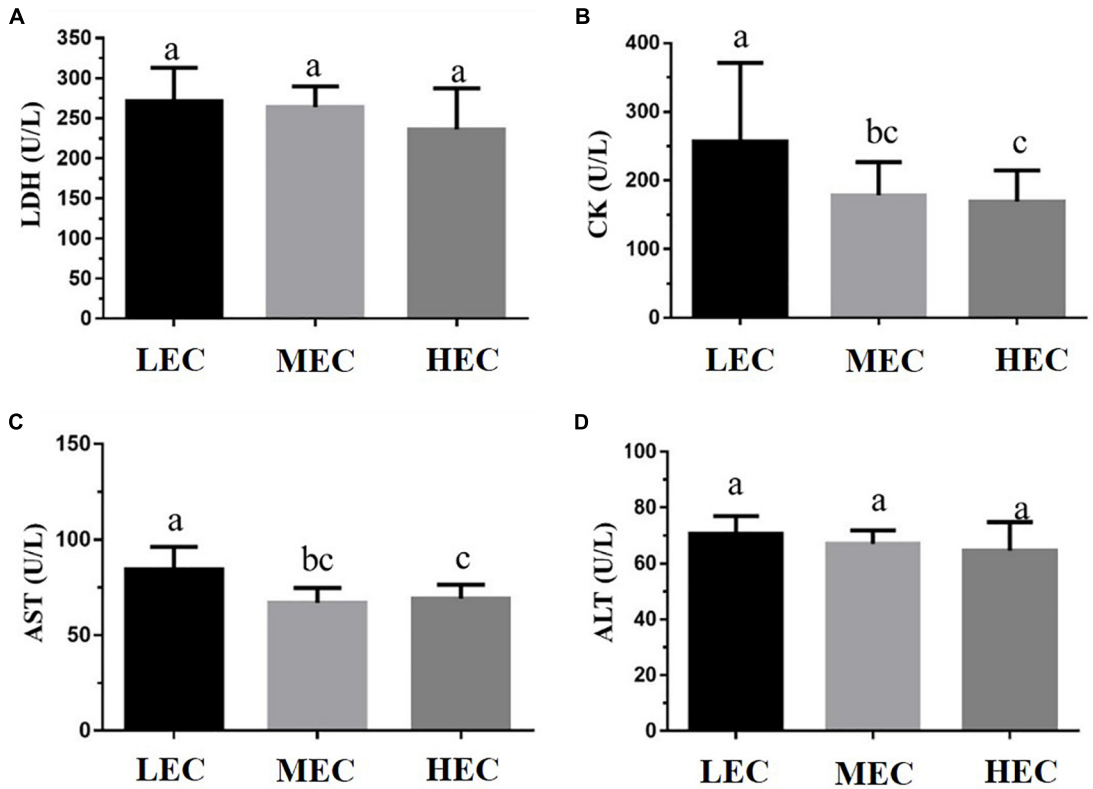

FIGURE 4 | Effect of exercise capacity on injury-related markers, namely serum **(A)** lactic dehydrogenase (LDH), **(B)** creatinine kinase (CK), **(C)** aspartate aminotransferase (AST), and **(D)** alanine transaminase (ALT) levels, after the acute exercise challenge. Data are presented as mean ± standard deviation for 15 mice in each group. Different letters (a,b,c) indicate a significant difference at $p < 0.05$ using one-way ANOVA.

TABLE 2 | Effect of different exercise capacity on biochemical analysis at the end of treatment.

Parameter	LEC	MEC	HEC	Trend analysis
AST (U/L)	70 ± 4	81 ± 4	73 ± 3	0.545
ALT (U/L)	44 ± 3^{a}	61 ± 4^{b}	54 ± 2^{ab}	0.022
LDH (U/L)	246 ± 12^{a}	387 ± 27^{b}	333 ± 24^{ab}	0.034
Albumin (g/dL)	3.2 ± 0.03^{a}	3.3 ± 0.04^{b}	3.4 ± 0.06^{b}	0.046
TP (g/dL)	4.6 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	0.757
BUN (mg/dL)	23.5 ± 0.7^{b}	20.4 ± 0.7^{a}	21.1 ± 0.4^{a}	0.003
Alk-P (U/L)	38.4 ± 1.8^{a}	47.9 ± 3.3^{b}	45.6 ± 2.6^{ab}	0.017
Creatinine (mg/dL)	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.590
CK (U/L)	286 ± 41	307 ± 37	285 ± 46	0.911
UA (mg/dL)	1.5 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	0.646
TC (mg/dL)	126 ± 6	137 ± 5	136 ± 4	0.301
TG (mg/dL)	141 ± 9	151 ± 9	139 ± 9	1.000
Glu (mg/dL)	157 ± 6	152 ± 7	159 ± 5	0.870

Data are mean ± standard error measurement for n = 10 mice in each group. Different superscript letters (a, b) indicate significant difference ($p < 0.05$) by one-way ANOVA.

of HEC and LEC mice altered considerably. Nine phyla were detected: Actinobacteria, Bacteroidetes, Crenarchaeota, Cyanobacteria/Chloroplast, Deferribacteres, Firmicutes, Proteobacteria, Tenericutes, and TM7; the ratios of these phyla did not differ significantly between HEC and LEC mice. Furthermore, 13 classes were noted: Actinobacteria, Alphaproteobacteria, Bacilli, Bacteroidia, Betaproteobacteria, Chloroplast, Clostridia, Deferribacteres, Deltaproteobacteria, Erysipelotrichia, Gammaproteobacteria, Moliciches, and Thermoprotei. The proportion of Betaproteobacteria was significantly lower in HEC mice than in LEC mice ($p = 0.04$). Next, 14 orders were identified: Anaeroplasmatales, Bacillales, Bacteroidales, Burkholderiales, Clostridiales, Coriobacteriale, Deferribacterales, Desulfovibrionales, Enterobacteriales,

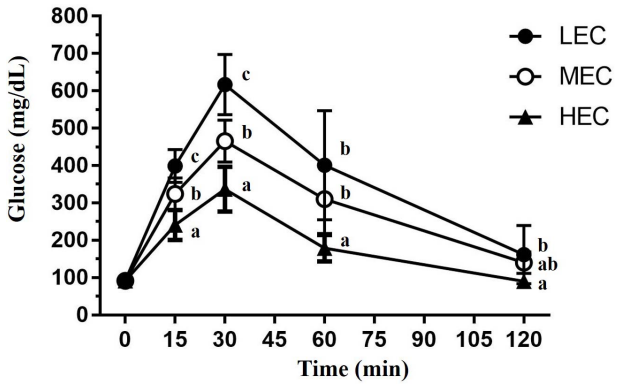

FIGURE 5 | The oral glucose tolerance test was performed in mice with various exercise capacities at the same glucose dosage (2 g/kg) after 14 h of fasting. The indicated sampling times (0, 15, 30, 60, and 120 min) were plotted as the tolerance curve. Data are presented as mean ± standard deviation for 15 mice in each group. Different letters (a,b,c) indicate a significant difference at $p < 0.05$ using one-way ANOVA.

Erysipelotrichales, Lactobacillales, Rhizobiale, Rhodospirillales, and Sphingomonadales. Compared with LEC mice, HEC mice exhibited a significantly lower number of Burkholderiales and Rhizobiales ($p = 0.034$ and 0.017, respectively) and a significantly higher number of Deferribacterales ($p = 0.025$). Of the 21 identified families, the number of Anaeroplasmataceae and Sutterellaceae was significantly lower in HEC mice than in LEC mice ($p = 0.025$ and 0.035). Of the 49 identified genera, the number of *Anaeroplasma*, *Anaerovorax*, *Erysipelotrichia*, *Gemmiger*, and *Parasutterella* was significantly lower in HEC mice than in LEC mice ($p = 0.030$, 0.015, 0.007, 0.018, and 0.04, respectively); however, the number of *Butyricicoccus* was significantly higher in HEC mice than in LEC mice ($p = 0.038$; **Figure 6C**).

Soleus Muscle miRNA Profiles

The 47 miRNA expression profiles of the soleus muscles, revealed using TLDA, varied among the three groups (**Supplementary Figure 1**). Compared with LEC mice, HEC mice exhibited significant changes (27.13–39.44-fold) in several miRNAs (**Table 3**). Seven miRNAs, namely miR-383, miR-107, miR-30b, miR-669m, miR-191, miR-218, and miR-224, were significantly upregulated in HEC mice (**Table 4**). Thus, a set of candidate miRNAs regulated in soleus muscles could be used as potential diagnostic biomarkers of intrinsic exercise capacities.

Soleus Muscle Proteomics Analysis

The soleus muscle protein profiles of LEC and HEC mice were analyzed using LC-MS/MS; next, various soleus muscle proteins were compared between these mice (**Table 5**). We detected 97 soleus proteins in LEC and HEC mice; of these, 8 were common and 89 were significantly regulated proteins in LEC and HEC mice. Among them, the levels of 79 and 10 proteins were significantly increased and decreased, respectively, in HEC mice compared with LEC mice. These major pathways between LEC and HEC mice were generated using ingenuity pathway analysis (IPA), where $p < 0.05$. In **Supplementary Figure 2**, the length of the bar only indicates that the differentially expressed proteins are related to this pathway but not that the pathway is either upregulated or downregulated. Soleus muscle proteins related to biofunctions and physiological metabolic pathways in HEC and LEC mice underwent significant changes related to (a) muscle contraction, (b) cardiovascular disease and organismal injury, (c) skeletal and muscle function, (d) carbohydrate metabolism, (e) muscle cell morphology, and (f) organ morphology.

DISCUSSION

For the 100 mice that performed an exhaustive swimming exercise test under 5% BW loading, their intrinsic endurance was ranked according to exhaustive swimming performance (**Figure 1**). The distribution pattern of exhaustive swimming exercise was similar to that reported by Koch et al. (1998). Koch and Britton (2001) demonstrated that of 96 male mice, the 13 lowest and the 13 highest performers became exhausted after an average of 13.0 ± 0.45 and 31.1 ± 0.81 min of running, respectively; thus, the exercise capacity of HEC mice was 2.4-fold higher than that of LEC mice. By contrast, in this study, of the 100 male mice, the 15 lowest and the 15 highest performers became exhausted after an average of 4.35 ± 0.23 and 172.40 ± 23.79 min of swimming, respectively; thus, the exercise capacity of HEC mice was 39.6-fold higher than that of LEC mice (**Figure 2A**). As illustrated in **Figure 2B**, the results of the grip strength test were similar to those of the exhaustive swimming exercise test. The grip strength of HEC mice (139.2 ± 5.1 g) was higher than that of the LEC (124.9 ± 3.2 g) and MEC (129.5 ± 3.2 g) mice ($p < 0.05$), indicating that the physical performance of HEC mice was significantly higher than that of MEC and LEC mice. Koch and Britton (2001) also detailed that selection for running capacity produced changes in BW and that the BW did not differ between HEC and LEC mice in the first generation; however, after breeding, the LEC- and HEC-line males exhibited significant differences in the fourth and fifth generations, respectively. In this study, BW and the growth curve did not differ significantly among LEC, MEC, and HEC mice (**Table 1**), possibly because these mice were not artificially selected for intrinsic aerobic endurance capacity. Swallow et al. (2010) demonstrated that HCR rats consumed and digested more food than LCR rats. Furthermore, HCR rats exhibited hypertrophy of the heart and kidneys and decreased length of the long intestine; thus, the oxygen utilization of these rats increased because of increased physical activity, resulting in increased food consumption and higher hypertrophy of key organs for O_2 transport compared with LCR rats. Moreover, Novak et al. (2009) demonstrated that HCR rats exhibited increased physical activity, potentially leading to a degree of self-training and resulting in increased energy expenditure, reduced body fat, and other lean characteristics. By contrast, in the present study, no significant differences in the relative weights of organs (liver, kidney, heart, lung, EFP, or BAT) were evident between HEC and LEC mice, probably because our mice were not artificially selected generations. However, the

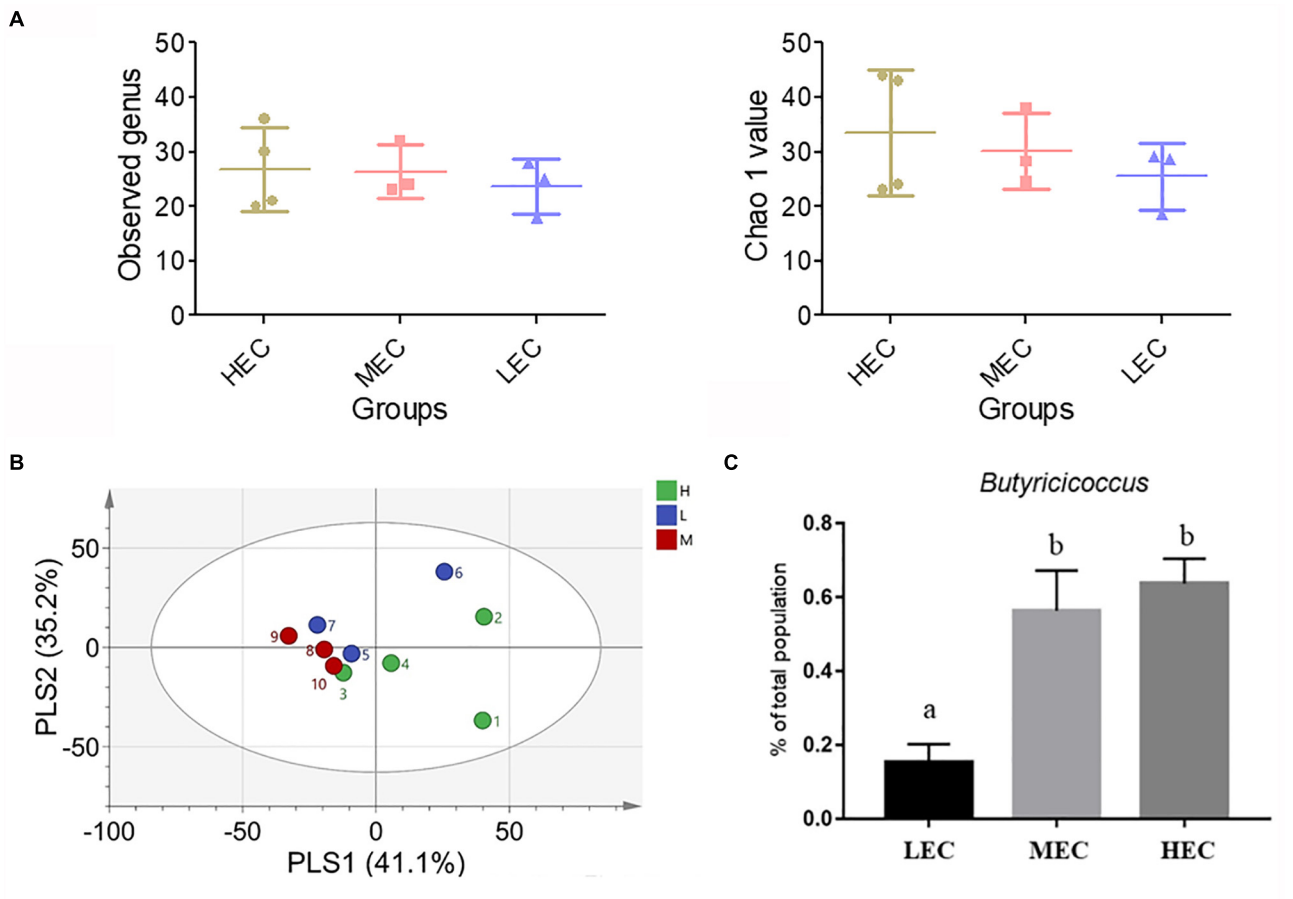

FIGURE 6 | Effect of exercise capacity on **(A)** partial least squares discriminant analysis (PLSDA) of genus level, **(B)** alpha-diversity indexes of the gut microbiota composition, and **(C)** *Butyricicoccus*. Different letters (a,b) indicate a significant difference at $p < 0.05$ using one-way ANOVA.

muscle weight of HEC mice was significantly higher than that of LEC mice, consistent with the results of the physical activity tests (exhaustive swimming exercise and grip strength test); this may explain why HEC mice exhibited more neuromuscular coordination and muscular power improvement than LEC mice.

Exercise fatigue mechanisms can be generally divided into peripheral and central fatigue. Some common blood-based biological indicators can assess the physiological state of peripheral fatigue, including exercise fatigue indicators such as lactate, ammonia, BUN, and glucose and exercise-induced injury indicators (such as AST, ALT, CK, and LDH) (Huang et al., 2015). Although altering the peripheral system at the skeletal muscle level may facilitate the maintenance of endurance capacity, altering neural circuits regulating fatigue may be effective. Foley et al. (2006) demonstrated that increased brain 5-hydroxytryptamine (5-HT) release accelerates exercise fatigue, whereas increased brain dopamine (DA) release can delay it. These results suggest that individual differences in endurance capacity may be attributed to factors influencing the activities of 5-HT and DA systems. HCR rats exhibited higher 5-HT1B autoreceptor mRNA levels in the raphe nucleus and higher DR-D2 autoreceptor mRNA levels in the midbrain and striatum than LCR rats, indicating that central serotonergic and dopaminergic systems may be involved in the delay of exercise fatigue in HCR rats (Foley et al., 2006). In the present study, the levels of the fatigue indicators lactate, ammonia, CK, and AST were significantly lower in HEC mice than in LEC mice, possibly because of the positive effect of exercise on relevant metabolic mechanisms and circulatory systems; thus, the exercise performance of HEC mice was significantly higher than that of LEC mice. Ritchie et al. (2013) demonstrated that low intrinsic aerobic capacity is associated with increased risk factors for cardiovascular disease because of impaired glucose tolerance and elevated plasma insulin. In the present study, glucose levels in LEC mice were higher than those in MEC and HEC mice. Stephenson et al. (2012) demonstrated that the fasting serum insulin concentration in LCR rats was 62% higher than that in HCR rats; therefore, HCR rats had higher glucose tolerance. Ritchie et al. (2013) also determined that LCR rats had impaired glucose tolerance and upregulated gene expression of the glucose transporter GLUT4 compared with HCR rats. Previous studies used the HCR/LCR rat model, which is selected over many generations. However, in the current study, the mice were characterized by their aerobic exercise ability (without artificial

TABLE 3 | Effect of different exercise capacities on miRNA expression.

miR ID	*p*-value	Mean ratio (HEC vs. LEC)	Fold change (HEC vs. LEC)
mmu-miR-878-3p-002541	1.77*E*−02	27.13	27.13
mmu-miR-383-4381093	1.18*E*−03	18.43	18.43
mmu-miR-719-001673	2.40*E*−01	13.50	13.50
mmu-miR-672-4395438	2.52*E*−01	12.22	12.22
mmu-miR-423-5p-4395451	2.54*E*−01	12.11	12.11
hsa-miR-183#-002270	2.76*E*−01	10.80	10.80
mmu-miR-183-4395380	1.51*E*−01	10.19	10.19
mmu-miR-466b-3-3p-002500	6.42*E*−02	10.12	10.12
mmu-miR-543-4395487	1.69*E*−01	9.77	9.77
hsa-miR-338-000548	2.92*E*−01	9.36	9.36
mmu-miR-137-4373301	7.21*E*−02	9.22	9.22
mmu-miR-298-4395728	3.61*E*−02	8.67	8.67
mmu-miR-34c-4373036	1.16*E*−01	7.23	7.23
mmu-miR-466h-4395646	6.52*E*−02	7.00	7.00
mmu-miR-465b-5p-4395615	3.16*E*−01	6.73	6.73
rno-miR-347-4381114	2.75*E*−01	6.49	6.49
mmu-miR-743a-4395599	2.54*E*−01	6.39	6.39
mmu-miR-330-4395341	1.77*E*−01	6.00	6.00
mmu-miR-181A-2#-002687	1.19*E*−01	5.58	5.58
mmu-miR-182-4395729	1.99*E*−01	5.52	5.52
mmu-miR-1962-121173_mat	9.72*E*−02	5.47	5.47
mmu-miR-362-5p-002614	5.55*E*−02	5.25	5.25
mmu-miR-1193-002794	3.78*E*−01	4.83	4.83
mmu-miR-455-4395585	3.82*E*−02	4.74	4.74
mmu-miR-107-4373154	3.68*E*−01	4.55	4.55
mmu-miR-450a-5p-4395414	1.88*E*−01	4.20	4.20
hsa-miR-9#-002231	7.62*E*−02	4.00	4.00
mmu-miR-30b#-002498	2.54*E*−03	3.98	3.98
rno-miR-207-4381096	1.30*E*−01	3.92	3.92
mmu-miR-669m-121190_mat	4.72*E*−02	3.92	3.92
mmu-miR-294-4373326	2.61*E*−01	3.84	3.84
mmu-miR-138#-002554	2.14*E*−01	3.78	3.78
mmu-miR-191#-002576	3.08*E*−01	3.68	3.68
rno-miR-20b-001326	7.61*E*−02	3.60	3.60
mmu-miR-20b-4373263	1.26*E*−01	3.54	3.54
mmu-miR-10a-4373153	1.97*E*−01	3.53	3.53
mmu-miR-433-4373205	1.89*E*−01	3.47	3.47
mmu-miR-1896-121128_mat	7.14*E*−02	3.41	3.41
mmu-miR-218-1#-002552	2.00*E*−01	3.31	3.31
mmu-miR-202-3p-4373311	1.86*E*−02	3.21	3.21
mmu-miR-501-3p-4381069	7.69*E*−02	3.10	3.10
mmu-miR-470-4395718	2.66*E*−01	3.10	3.10
mmu-miR-434-5p-4395711	4.27*E*−01	3.09	3.09
mmu-miR-211-4373315	3.44*E*−01	3.08	3.08
rno-miR-224-4373187	8.47*E*−02	3.04	3.04
rno-miR-1-4395765	2.90E−01	0.22	−4.65
mmu-miR-1188-002866	2.50E−01	0.03	−39.44

The significant difference ($p < 0.05$) for high exercise capacity (HEC) and low exercise capacity (LEC) groups was compared using the t-test.

TABLE 4 | Soleus miRNA molecules with significant difference related to intrinsic exercise capacity.

miRNA molecules	Biological functions	Fold change (HEC/LEC)	*p*-value
miR-383	(1) Testosterone and progesterone regulation, (2) spermatogenesis, and (3) steroidogenesis	18.43	1.18*E*−03
miR-107	Insulin sensitivity	4.55	3.68*E*−01
miR-30b	Role of regeneration	3.98	2.54*E*−03
miR-669m	Adaptation during toxic metabolites accumulation	3.92	4.72*E*−02
miR-191	Adipogenesis inhibition	3.68	3.08*E*−01
miR-218	Vascular organization	3.31	2.00*E*−01
miR-224	Adipogenesis regulation (fatty acid metabolism)	3.04	8.47*E*−02

The significant difference ($p < 0.05$) for high exercise capacity (HEC) and low exercise capacity (LEC) groups was compared by t-test.

population selection as F_0 generation). Additionally, intrinsic LEC is associated with an increase in insulin-related metabolic syndrome, regardless of the intrinsic exercise capacity selected at many generations or at an early point in time (Stephenson et al., 2012; Ritchie et al., 2013).

The gut microbiota is associated with diseases such as obesity, aging, diabetes, allergies, cardiovascular disease, and cancer. Burcelin et al. (2011) discovered that the gut microbiota is involved in host metabolism, including adipose tissue functioning, liver fat storage, skeletal muscle energy metabolism, hepatic lipid metabolism, hepatic steatosis, atherosclerosis and cardiovascular diseases, tissue lipid composition in the retina and lens, periodontitis, behavior and motor activity, and enteroendocrine metabolism. Appukutty et al. (2015) demonstrated that the intervention combining *Lactobacillus plantarum* LAB12 intake and moderate exercise enhances functions and inhibits TNF-α production. Evans et al. (2014) reported that dietary intake and exercise intervention can alter the gut microbiota and produce physiological changes, such as weight control and high glucose tolerance. Hsu et al. (2015) determined that mice free of a specific pathogen exhibit improved exercise compared with completely pathogen-free mice, indicating that the composition of the gut microbiota is essential for exercise performance. In this study, we assessed whether the levels of intrinsic exercise capacity affect gut microbiota. In terms of genera, the numbers of *Anaeroplasma*, *Anaerovorax*, *Erysipelotrichaceae*, *Gemmiger*, and *Parasutterella* were significantly lower in HEC mice than in LEC mice, whereas the numbers of *Butyricicoccus* were significantly higher in LEC mice. Furthermore, *Butyricicoccus pullicaecorum* adapts to the gastrointestinal environment, is potentially probiotic (Geirnaert et al., 2014), and can improve inflammatory bowel disease (Steppe et al., 2014). Overall, our results indicate that HEC mice have a greater abundance and diversity of gut microbiota than LEC mice, which suggests that gut microbiota may be highly correlated with exercise capacity. In addition, the ratio of Firmicutes/Bacteroidetes was significantly higher in HEC mice than in LEC mice (**Supplementary Figure 3**). Exercise in normal rats was associated with higher numbers of Bacteroidetes and lower Firmicutes in fecal matter (Queipo-Ortuño et al., 2013; Evans et al., 2014). By contrast, the opposite was observed in

TABLE 5 | Differential expression for protein molecules in soleus between low exercise capacity (LEC) and high exercise capacity (HEC) groups.

Protein name	Protein-ID	Gene-ID	HEC (mean ± SE)	LEC (mean ± SE)	*p*-value
Myosin, heavy polypeptide 6, cardiac muscle, alpha	Q2TAW4	Q2TAW4	107.87 ± 26.34	10.53 ± 8.55	0.01259
Calsequestrin	Q6P3C3	Q6P3C3	28.3 ± 3.34	16.57 ± 2.29	0.02739
Calsequestrin-1	O09165	CASQ1	28.3 ± 3.34	16.57 ± 2.29	0.02739
Beta-enolase	P21550	ENOB	12.88 ± 1.82	5.19 ± 0.97	0.00971
Cytochrome b–c1 complex subunit 1, mitochondrial	Q9CZ13	QCR1	3.84 ± 0.56	1.62 ± 0.71	0.04955
Putative uncharacterized protein	Q3THM1	Q3THM1	3.84 ± 0.56	1.62 ± 0.71	0.04955
Putative uncharacterized protein	Q3TIC8	Q3TIC8	3.84 ± 0.56	1.62 ± 0.71	0.04955
Putative uncharacterized protein	Q3UIQ2	Q3UIQ2	9.02 ± 1.44	1.77 ± 1.77	0.01918
Putative uncharacterized protein	Q3TX47	Q3TX47	2.18 ± 0.79	0 ± 0	0.03254
Adenylosuccinate synthetase isozyme 1	P28650	PURA1	2.37 ± 0.46	0 ± 0	0.00222
Adenylosuccinate synthetase isozyme 1	J3QN31	J3QN31	2.37 ± 0.46	0 ± 0	0.00222
Glucose-6-phosphate isomerase	P06745	G6PI	10.88 ± 2.67	3.96 ± 0.42	0.04274
Putative uncharacterized protein	Q3U6X6	Q3U6X6	5.1 ± 1.95	0 ± 0	0.03993
Phosphoglucomutase-1	A2CEK3	A2CEK3	5.1 ± 1.95	0 ± 0	0.03993
Myosin light chain 3	P09542	MYL3	1.98 ± 0.75	5.83 ± 1.35	0.04648
Synaptopodin 2	B2RY03	B2RY03	6.48 ± 0.7	5.07 ± 0.92	0.26857
Synaptopodin-2	E9Q1U2	E9Q1U2	6.48 ± 0.7	5.07 ± 0.92	0.26857
Serum deprivation-response protein	Q63918	SDPR	1.19 ± 0.41	0 ± 0	0.02615
2-Oxoglutarate dehydrogenase, mitochondrial	Q60597	ODO1	10.05 ± 1.85	14.69 ± 1.04	0.07145
Moesin	P26041	MOES	0 ± 0	0.37 ± 0.37	0.35592
Nucleoside diphosphate kinase A	P15532	NDKA	0 ± 0	0.37 ± 0.37	0.35592
Nucleoside diphosphate kinase B	Q01768	NDKB	0 ± 0	0.37 ± 0.37	0.35592
Putative uncharacterized protein	Q3TZQ2	Q3TZQ2	0 ± 0	0.37 ± 0.37	0.35592
Radixin	P26043	RADI	0 ± 0	2.23 ± 1.01	0.06861
6-Phosphofructokinase, muscle type	P47857	K6PF	2.05 ± 0.75	0 ± 0	0.03472
Polymerase I and transcript release factor	O54724	PTRF	3.28 ± 0.44	1.2 ± 0.7	0.04571
Triosephosphate isomerase	P17751	TPIS	5.18 ± 1.06	0.97 ± 0.56	0.01249
Synaptopodin 2-like protein	Q8BWB1	SYP2L	3.32 ± 0.21	1.55 ± 0.58	0.02826
Synaptopodin 2-like protein	B2RQK7	B2RQK7	3.32 ± 0.21	1.55 ± 0.58	0.02826
Synaptopodin 2-like protein	D3YU08	D3YU08	3.32 ± 0.21	1.55 ± 0.58	0.02826
Putative uncharacterized protein	Q3UDU4	Q3UDU4	8.93 ± 2.6	2.12 ± 0.71	0.04491
Uncharacterized protein	M0QWZ0	M0QWZ0	17.19 ± 4.16	6.94 ± 0.38	0.04961
PDZ and LIM domain protein 5	Q9CRA2	Q9CRA2	1.74 ± 0.59	0 ± 0	0.02664
Nucleoside diphosphate kinase	E9PZF0	E9PZF0	2.31 ± 0.29	0.62 ± 0.62	0.04843
Aspartate aminotransferase, mitochondrial	P05202	AATM	3.98 ± 1.62	0 ± 0	0.04945
Collagen alpha-1(XV) chain	O35206	COFA1	1.24 ± 0.43	0 ± 0	0.02871
Collagen alpha-1(XV) chain	A2AJY2	A2AJY2	1.24 ± 0.43	0 ± 0	0.02871
Collagen alpha-1(XV) chain	A2AJY7	A2AJY7	1.24 ± 0.43	0 ± 0	0.02871
Ryanodine receptor 1	E9PZQ0	RYR1	1.67 ± 0.59	0 ± 0	0.02968
Ryanodine receptor 1	K3W4M2	K3W4M2	1.67 ± 0.59	0 ± 0	0.02968
Glyceraldehyde-3-phosphate dehydrogenase	P16858	G3P	22.26 ± 1.68	7.88 ± 0.83	0.00026
Very long-chain-specific acyl-CoA dehydrogenase, mitochondrial	P50544	ACADV	3.18 ± 0.55	0 ± 0	0.00119
Protein Mybpc1	D3YU50	D3YU50	23.96 ± 1.47	11.29 ± 3.15	0.01081
Protein Mybpc1	Q6P6L5	Q6P6L5	23.96 ± 1.47	11.29 ± 3.15	0.01081
Troponin T, fast skeletal muscle	A2A6H6	A2A6H6	23.17 ± 1.95	12.68 ± 1.01	0.00309
Troponin T, fast skeletal muscle	A2A6I8	A2A6I8	23.17 ± 1.95	12.68 ± 1.01	0.00309
Troponin T, fast skeletal muscle	A2A6J0	A2A6J0	23.17 ± 1.95	12.68 ± 1.01	0.00309
Troponin T, fast skeletal muscle	A2A6J1	A2A6J1	23.17 ± 1.95	12.68 ± 1.01	0.00309
Troponin T, fast skeletal muscle	J3QP61	J3QP61	23.17 ± 1.95	12.68 ± 1.01	0.00309
Phosphoglucomutase-1	Q9D0F9	PGM1	11.11 ± 1.02	6.84 ± 0.89	0.01973
Actin, alpha skeletal muscle	P68134	ACTS	132.76 ± 8.24	96.79 ± 6.47	0.0139
Alpha-actinin-3	O88990	ACTN3	32.2 ± 3.49	21.89 ± 1.75	0.03854

(Continued)

TABLE 5 | Continued

Protein name	Protein-ID	Gene-ID	HEC (mean ± SE)	LEC (mean ± SE)	*p*-value
Actin, alpha cardiac muscle 1	P68033	ACTC	132.76 ± 8.24	96.79 ± 6.47	0.0139
Putative uncharacterized protein	Q3TG92	Q3TG92	132.76 ± 8.24	96.79 ± 6.47	0.0139
Putative uncharacterized protein	Q9CXK3	Q9CXK3	132.76 ± 8.24	96.79 ± 6.47	0.0139
LIM domain-binding protein 3	E9PYJ9	E9PYJ9	15.02 ± 1.72	9.84 ± 1.22	0.0494
L-Lactate dehydrogenase	G5E8N5	G5E8N5	11.76 ± 0.94	2.12 ± 0.71	0.00018
L-Lactate dehydrogenase A chain	P06151	LDHA	11.76 ± 0.94	2.12 ± 0.71	0.00018
Putative uncharacterized protein	Q3TI99	Q3TI99	11.76 ± 0.94	2.12 ± 0.71	0.00018
Collagen alpha-1(XV) chain	A2AJY5	A2AJY5	1.24 ± 0.43	0 ± 0	0.02871
MCG140784	Q792Z1	Q792Z1	31.39 ± 1.84	25.1 ± 1.58	0.04076
Try10-like trypsinogen	Q7M754	Q7M754	31.39 ± 1.84	25.1 ± 1.58	0.04076
Alpha-enolase	P17182	ENOA	2.11 ± 0.72	0 ± 0	0.02591
Krt6b protein	Q0VDR7	Q0VDR7	25.65 ± 3.96	4.94 ± 1.75	0.00306
60S ribosomal protein L7a	P12970	RL7A	2.63 ± 0.6	0 ± 0	0.00448
MCG18601	D3YVE6	D3YVE6	2.63 ± 0.6	0 ± 0	0.00448
Ribosomal protein L7A	Q5EBG5	Q5EBG5	2.63 ± 0.6	0 ± 0	0.00448
Ribosomal protein L7A	Q6P1A9	Q6P1A9	2.63 ± 0.6	0 ± 0	0.00448
Uncharacterized protein	D3YXT4	D3YXT4	2.63 ± 0.6	0 ± 0	0.00448
Uncharacterized protein	L7N202	L7N202	2.63 ± 0.6	0 ± 0	0.00448
Troponin T, fast skeletal muscle	Q9QZ47	TNNT3	15.56 ± 3.67	5.59 ± 0.51	0.03597
Transitional endoplasmic reticulum ATPase	Q01853	TERA	7.78 ± 1.31	4.11 ± 0.71	0.04918
Uncharacterized protein	D3YU93	D3YU93	1.54 ± 0.54	0 ± 0	0.02921
Uncharacterized protein	F6U2H0	F6U2H0	1.54 ± 0.54	0 ± 0	0.02921
Tropomyosin beta chain	P58774	TPM2	76.85 ± 19.02	15 ± 7.44	0.02316
M-protein	O55124	O55124	13.57 ± 2.5	2.39 ± 1.52	0.00882
Myomesin 2	Q14BI5	Q14BI5	13.57 ± 2.5	2.39 ± 1.52	0.00882
Putative uncharacterized protein	Q3UQS9	Q3UQS9	13.57 ± 2.5	2.39 ± 1.52	0.00882
Carbonic anhydrase 3	P16015	CAH3	9.75 ± 1.1	2.97 ± 1.45	0.00967
Creatine kinase M-type	P07310	KCRM	37.66 ± 4.36	7.89 ± 2.65	0.00112
Putative uncharacterized protein	Q9D6U7	Q9D6U7	37.66 ± 4.36	7.89 ± 2.65	0.00112
Myosin-binding protein C, fast-type	Q5XKE0	MYPC2	27.79 ± 2.85	13.96 ± 3.22	0.01815
6-Phosphofructokinase	Q99K08	Q99K08	2.05 ± 0.75	0 ± 0	0.03472
Pyruvate carboxylase	E9QPD7	E9QPD7	3.33 ± 1.17	0 ± 0	0.02909
Pyruvate carboxylase	G5E8R3	G5E8R3	3.33 ± 1.17	0 ± 0	0.02909
Pyruvate carboxylase	Q3T9S7	Q3T9S7	3.33 ± 1.17	0 ± 0	0.02909
Pyruvate carboxylase	Q3TCQ3	Q3TCQ3	3.33 ± 1.17	0 ± 0	0.02909
Pyruvate carboxylase, mitochondrial	Q05920	PYC	3.33 ± 1.17	0 ± 0	0.02909
Synaptopodin-2	D3YVV9	D3YVV9	1.41 ± 0.49	5.07 ± 0.92	0.01295
Collagen alpha-2(I) chain	Q01149	CO1A2	3.53 ± 0.49	5.77 ± 0.76	0.04821
Putative uncharacterized protein	Q3TU64	Q3TU64	3.53 ± 0.49	5.77 ± 0.76	0.04821
Collagen alpha-1(I) chain	P11087	CO1A1	8.24 ± 0.2	13.2 ± 1.33	0.01023
Microtubule-associated protein	E9PWC0	E9PWC0	0.38 ± 0.38	2.14 ± 0.29	0.01021
Microtubule-associated protein	E9PZ43	E9PZ43	0.38 ± 0.38	2.14 ± 0.29	0.01021
Putative uncharacterized protein	Q8BIZ5	Q8BIZ5	31.15 ± 1.84	48.14 ± 5.33	0.0235
Terminal uridylyltransferase 4	B2RX14	TUT4	31.15 ± 1.84	48.14 ± 5.33	0.0235
Terminal uridylyltransferase 4	A2A8R7	A2A8R7	31.15 ± 1.84	48.14 ± 5.33	0.0235

Data are mean ± standard error (SE) measurement for n = 4 mice per group. The significant difference ($p < 0.05$) for high and low exercise capacity groups was compared using the t-test.

exercised mice exposed to polychlorinated biphenyls (Choi et al., 2013) and also to type 2 diabetic (db/db) and control ($db/^{+}$) mice (Lambert et al., 2015), similar to our own observations in HEC and LEC mice.

miRNAs are a class of highly abundant non-coding RNA molecules that can cause posttranscriptional gene repression; these are involved in various biological processes (Chaveles et al., 2012). In the present study, miR-383, miR-107, miR-30b, miR-669m, miR-191, miR-218, and miR-224 were significantly upregulated in HEC mice (**Table 4**). miR-383 regulates RBMS1 and inhibits downstream c-MYC expression, and along with miR-320, it controls E2F1 and SF1 genes to affect a steroid

hormone. Therefore, miR-383 has therapeutic potential for hormone-related disorders (Yin et al., 2014). Van Caenegem et al. (2015) determined that an increase in testosterone levels can significantly increase muscle, power, and bone growth. miR-107 modulates the expression of the caveolin-1 gene, which encodes an upstream regulatory protein of the insulin receptor, thus improving insulin sensitivity. In this study, the glucose levels in LEC mice were higher than those in MEC and HEC mice; this may be related to the significant upregulation of miR-107 expression in HEC mice. In addition, miR-191 and miR-224 negatively regulate lipogenesis and inhibit lipid synthesis by modulating acyl-CoA synthetase activity (Ji et al., 2014). The proportion of muscle fat to visceral fat in HEC rats was lower than that in LEC rats (Spargo et al., 2007). In the present study, the EFP and BAT did not differ significantly among LEC, MEC, and HEC mice. However, future studies should investigate the influence of miR-191 and miR-224 on lipid regulation in artificially selected generations. Druz et al. (2012) determined that the oxidative stress induced by glucose deprivation caused reactive oxygen species accumulation and reduced glutathione depletion, which together inhibited histone deacetylase (HDAC) activity, reduced protein levels of HDAC2, and increased acetylation in miR-466 and miR-669 promoter regions, causing the activation of miR-466 and miR-669 to regulate physiological functions. The oxidative stress induced by glucose deprivation is similar to the exercise-induced oxidative stress observed in this study. This may explain why the levels of exercise-fatigue-related indicators, such as lactate and ammonia, were higher in LEC mice than in HEC mice. Chaveles et al. (2012) determined that the expression levels of miR-218 and miR-30 in liver tissue were higher after liver regeneration induced by two-thirds partial hepatectomy, suggesting that miR-218 and miR-30 may be crucial for liver regeneration. In this study, the mechanism of recovery from exercise-induced muscle damage was similar to that of liver regeneration. Therefore, the levels of injury-related biomarkers, such as CK, significantly decreased in HEC mice compared with those in LEC mice. These results indicate that these miRNAs related to intrinsic HEC play a major role in the adaptation and regulation of physiological functions.

The soleus muscle proteins exhibiting changes in regulation because of intrinsic exercise capacity were related to six major pathways between HEC and LEC mice, as determined by IPA, with a threshold p of <0.05. The six major biofunctions in the intrinsic exercise capacity-regulated soleus muscle proteins were muscle contraction, cardiovascular disease and injury, skeletal and muscle function, carbohydrate metabolism, muscle cell morphology, and organ morphology. The upregulated proteins associated with muscle contraction in HEC mice were ACTA1, ACTC1, Actin3, MYBPC1, MYBPC2, RYR1, and TNNT3. The muscle contraction was stronger in HEC mice than in LEC mice. Therefore, muscle weight, exhaustive swimming exercise performance, and grip strength were significantly higher in HEC mice than in LEC mice. Furthermore, low aerobic capacity may be associated with high risks of various diseases (Wisløff et al., 2005). Addressing associations and mechanisms concerning the progression of disease development using the respective indexes would be worthwhile. In addition, the results of different protein analysis on the functional network showed that the intrinsic exercise capacity is highly related to organ morphology and skeletomuscular development and function (**Supplementary Figure 4**). Thus, muscle function and carbohydrate metabolism may play an essential role in enhancing exercise performance.

In previous study with early obesity and non-alcoholic fatty liver disease (NAFLD) rat model, the exercise intervention could not only ameliorate HFD-induced body metabolic syndrome and hepatic steatosis, as a result of its lipid metabolism modulatory capacity and the exercise training also increased *Parabacteroides*, *Bacteroides*, and *Flavobacterium* genera, correlating with a beneficial metabolomic profile (Carbajo-Pescador et al., 2019). In our study, we also observed the *Bacteroides* genus is significantly higher proportions in HEC group than LEC group (**Supplementary Figure 3**) but the body composition and metabolic syndrome were not significant difference possible due to several reasons including, F_0 generation, diet, and exercise intervention. There was another study to compare the diet and exercise on metabolic function and gut microbiota in obesity rat. The exercise intervention for obesity rat could reduce the inflammatory markers in all adipose tissue, and improve the BAT mitochondrial function, insulin resistance, and the relative abundance of Streptococcaceae (Welly et al., 2016). The GOTT test also showed that the HEC group exhibited the better glucose tolerance effects (**Figure 5**), which was also consistent with upregulation of miR-107 expression in HEC mice for insulin sensitivity (**Table 4**). In addition, the HEC also showed the higher expression levels in carbohydrate metabolism proteins (**Supplementary Figure 3**) related to aerobic metabolism. However, the diet and exercise training didn't intervene HEC and LEC groups in current study, and the effects of gut microbiota and functional proteins with intrinsic exercise capacity need to be validated by further experimental designs by diet and training conditions.

One limitation of the study was sex of the mice used, as we used only male mice, and sex hormones can influence physiology and physiological adaptations. However, our experimental design focused on possible regulatory factors through a multiomics approach. Therefore, sex differences can be investigated in the future. In addition, ICR mice were subjected to an exhaustive swimming test and were ranked based on the exhaustive swimming time to distinguish intrinsically high- and low-capacity groups in the study. Previous studies have used the exhaustive swimming time method to assess the exercise capacity (Kan et al., 2018; Tung et al., 2019). However, mice are afraid of water, and the exhaustive swimming test causes them high mental stress. The blood biochemical parameters assessed in the present study (lactate, ammonia, BUN, glucose, LDH, CK, AST, and ALT) are not related to mental stress. Nevertheless, future studies should validate the changes in mental stress-related blood biochemical data, including hormones.

CONCLUSION

In this study, exhaustive swimming test was used to determine low and high capacities in mice. In contrast with LEC mice,

glucose tolerance and the number of *Butyricicoccus* were significantly higher in HEC mice, and levels of exercise-induced peripheral fatigue and injury-related biomarkers, including lactic acid, ammonia, CK, and AST, were significantly lower in HEC mice. In addition, differences between the exercise performance of HEC and LEC mice may be attributed to the expression of miR-383, miR-107, miR-30b, miR-669m, miR-191, miR-218, and miR-224, which are strongly associated with physiological and metabolic functions. Moreover, the functional protein profile indicated the effect of key protein levels related to intrinsic exercise capacity on muscle function and carbohydrate metabolism; these proteins may play a major role in the adaptive mechanism of exercise physiology. The mechanisms underlying the influence of these critical factors on disease development warrants further study.

DATA AVAILABILITY

All datasets for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

This study was conducted in accordance with the principles of the Basel Declaration and the recommendations of the Institutional Animal Care and Use Committee (IACUC) of the NTSU. The protocol (IACUC-10103) was approved by the IACUC of the NTSU.

AUTHOR CONTRIBUTIONS

W-CH and C-CH designed the experiments. W-CH, Y-TT, Y-JH, and C-CL conducted the laboratory experiments. C-CH contributed the reagents, materials, and analysis platforms. S-TH analyzed the metagenome result. W-CH, Y-TT, Y-JH, and C-CH analyzed and illustrated the data. Y-TT, W-CH, and C-CH interpreted the results, prepared the figures, and wrote and revised the manuscript.

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SUPPLEMENTARY MATERIAL

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CORRECTION
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Corrigendum: Physiological and Biochemical Effects of Intrinsically High and Low Exercise Capacities Through Multiomics Approaches

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Physiological and Biochemical Effects of Intrinsically High and Low Exercise Capacities Through Multiomics Approaches

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In the original article, there was a mistake in **Figure 5** as published. The incorrect Figure 5 was erroneously uploaded at submission. Furthermore, all data should be represented as mean with SD, however, the original Figure 5 shows mean with SEM. Lastly, the statistical results in the original Figure 5 are correct, however, the way in which it is displayed is incorrect. The statistical letters should start from a, b, and c according to the mean average. The corrected **Figure 5** appears below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

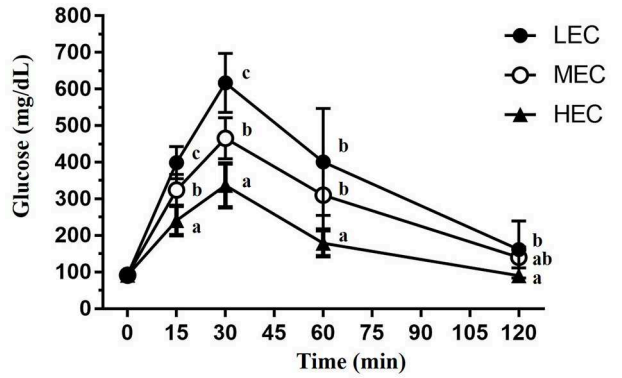

FIGURE 5 | The oral glucose tolerance test was performed in mice with various exercise capacities at the same glucose dosage (2 g/kg) after 14 h of fasting. The indicated sampling times (0, 15, 30, 60, and 120 min) were plotted as the tolerance curve. Data are presented as mean ± standard deviation for 15 mice in each group. Different letters (a,b,c) indicate a significant difference at $p < 0.05$ using one-way ANOVA.

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Artificial Neural Network Correlation and Biostatistics Evaluation of Physiological and Molecular Parameters in Healthy Young Individuals Performing Regular Exercise

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Studies support that regular physical activity (PA) decelerates senescence-related decline of physiological and molecular parameters in the elderly. We have addressed the other end of this spectrum: healthy and young, inactive individuals participated in a 6-month long personal trainer-guided lifestyle program. We have measured physiological and molecular parameters (differentiating high- and low responders) and their correlation with PA (sedentary status). Cluster analysis helped to distinguish individuals with high- or low PA and differentiate high- and low-responders of each parameter. The assessed cardiovascular parameters (heart rate, blood pressure, 6-min walking distance, relative VO_2max), body composition parameters (body fat and muscle mass percentage) metabolic parameters (glucose, insulin, HDL, LDL), immune parameters (cortisol, CRP, lymphocyte counts, hTREC) all showed improvement. Artificial neural network analysis (ANN) showed correlation efficiencies of physiological and molecular parameters using a concept-free approach. ANN analysis appointed PA as the mastermind of molecular level changes. Besides sedentary status, insulin and hTREC showed significant segregation. Biostatistics evaluation also supported the schism of participants for their sedentary status, insulin concentration and hTREC copy number. In the future ANN and biostatistics, may predict individual responses to regular exercise. Our program reveals that high responder individuals of certain parameters may be low responders of others. Our data show that moderate regular PA is essential to counteract senescence in young and healthy individuals, despite individual differences in responsiveness. Such PA may not seem important in the everyday life of young and healthy adults, but shall become the base for healthy aging.

Keywords: aging, physical activity, responsiveness, prediction, prevention

INTRODUCTION

Statistics show that regular physical activity (PA) helps preventing the development of several chronic diseases including cardiovascular events, malignancies (Beaudry et al., 2018), neurodegenerative conditions (Ahlskog, 2018), diabetes (Yanai et al., 2018) and osteoporosis (Senderovich and Kosmopoulos, 2018). Regular exercise also ameliorates their course and outcome, and physicians often recommend a lifestyle change as adjuvant therapy (Dieli-Conwright et al., 2018). Several studies focus on anthropometric, physiological or molecular parameters showing the positive effects of regular PA that aid the treatment of chronic diseases (Beaudry et al., 2018; Yanai et al., 2018). The beneficial effects of regular exercise were first detected among professional endurance athletes (Fitzgerald, 1988). Later the tendency of positive effects was also measured in healthy, young individuals performing habitual (non-professional) sports (Caglar et al., 2009).

Positive response to exercise is often generalized in literature assuming that the group average represents a typical response for all participants (Bouchard and Rankinen, 2001). However, recently it has also become clear that the human population gives diverse responses even for standardized lifestyle programs (Karavirta et al., 2011). This may be due to differences in genetic background and environmental conditions (nutritional status, sleep quality, psychological status etc.). Adherence to study protocol (compliance) is another significant factor that may show variations during a lifestyle program (Mann et al., 2014). Diversity of molecular parameters occurring during lifestyle studies is still challenging to predict. With these in mind we have examined the effect of a 6 month-long, personal trainer-guided lifestyle program in young and healthy, but previously inactive adults. Daily activity was continuously recorded (using Actigraph device), standard physiological parameters were measured, and 6-min walking tests (6MWT) were performed to validate our program. This was followed by the evaluation of molecular parameters of metabolism and immunity (including corticosteroid hormones, lymphocyte count, and thymus function correlated with lymphocyte count). Computer-based artificial neural network analysis (ANN) revealed correlation patterns between physiological and molecular parameters in a concept-free manner. In addition, we have also performed biostatistics evaluation of the most interconnected and deregulated parameters. Our aim was to differentiate high- and low responders of PA and all the other recorded physiological and molecular factors. Our goal was to identify distinct patterns of responsiveness and segregation that may provide basis for future prediction of molecular level gain.

MATERIALS AND METHODS

Study Participants

The lifestyle program was a university-based trial designed to promote PA among physically inactive university students. The trainings were conducted in the Institute of Sport Sciences and Physical Education. The program was executed from November to May, it was approved by the ethics committee of the University of Pécs (ref. no.: 6439/2016). All participants gave written informed consent before starting the lifestyle program in accordance with the Declaration of Helsinki. All measurements involving human subjects and their blood samples were performed with the consent of the Regional and Local Ethics Committee of Clinical Center, University or Pécs according to their guidelines. We recruited 25 participants altogether via advertisements in social media. The inclusion criteria were: no regular PA or controlled diet program in the previous 6 months, no history of disease (metabolic, cardiovascular, hypertension or major injury). Participants were both males (n = 3) and females (n = 22), the mean age was 24.95 years (±4.04). At the end of the program 14 participants (male n = 2 and female n = 12) completed the 6 month-long lifestyle program fulfilling the requirements of attending trainings three times a week.

Combined Endurance and Strength-Training Program

The lifestyle program was created to upscale both endurance and strength combined into a 60-min exercise session, three times a week, for 6 months. Each session was divided into four stages: 10 min warm-up, 30 min strength training, 20 min aerobic training and 10 min stretching and cool-down. The intensity of PA was adjusted to the actual fitness level of participants: ≤65% HRmax for aerobic exercise and ≤85% HRmax for strength training, where HRmax = 220–age (in years). Every other week training was performed wearing Polar Team system heart rate (HR) monitors. Participants were asked to keep their usual diet and normal daily activity level during the 6-month long lifestyle program.

Measurements

Measurements were conducted before starting the training program (0 month), after 3- and 6- months. The 3- and 6-month values were normalized to baseline values and converted to Z-scores. Body Fat% and Muscle mass% were measured using Bioimpedance Analyzer (Omron, HBF-516B, Budapest, Hungary). After 5 min at rest both systolic and diastolic blood pressures were measured (Omron M10-IT).

Six-Minute Walking Test

Physical fitness was assessed by using a 6MWT, slightly different from the original protocol of Enright (2003). VO_2max (L/min) was calculated as described by Laskin et al. (2007) and converted to relative VO_2max (ml/kg/min).

Actigraph

Actigraph GT3X+ (Actigraph, Pensacola, FL, United States) is a tri-axial accelerometer-based method of monitoring activity (John and Freedson, 2012). Actigraph recorded movements in 60-s intervals. Participants were instructed to wear Actigraph continuously day and night for seven consecutive days at study entry, after 3- and 6-months of the program. Due to a limited number of Actigraphs, raw data collection was not performed on the exact same days for all participants, although all recordings were within the appropriate 1-week time period. Data were

excluded from the analysis, if the participants did not wear the device for seven consecutive days. Actigraph recordings were analyzed using ActiLife 6.10 software (Actigraph) and cut-off values were calculated according to previous publications (Freedson et al., 1998).

Heart Rate Monitoring

R-R interval was registered every 2nd week during exercise sessions with a Polar Team System (Polar Electro Oy, Kempele, Finland) following Baynard's method (Baynard et al., 2004). The transmitters were worn by the participants in ventral position under the tip of the xiphoid process in the middle of their chest.

Heart Rate monitoring was accomplished according to recommendations (Baynard et al., 2004). Briefly, subjects were first seated for 5 min to obtain resting HR. Then each subject completed the 30-min resistance exercise followed by the 20-min aerobic exercise with continuous HR monitoring. Finally, HR was continuously recorded for another 5 min in seated position to obtain recovery HR. Results were processed using Polar Pro Trainer 5, and Kubios HRV software.

Blood Sample Collection and Evaluation

Fasting blood samples were taken in the morning. Subjects were instructed to restrain from exercise during the day before their examination. Venous blood samples were collected in suitable vacutainers, tubes containing potassium ethylenediaminetetraacetic acid (K-EDTA) were used for testing cellular blood parameters. Tubes without additives were used to obtain serum for C-reactive protein (CRP) and cortisol tests. After blood collection serum was separated by centrifugation (10 min, room temperature, 1500 rcf). Blood cell parameters were quantified in a multi-parameter automatic hematology analyzer Cell-Dyn 3700 system (Abbott Diagnostics, Abbott Laboratories, Abbott Park, IL, United States). CRP was measured by Cardiac C-Reactive Protein (Latex) High Sensitive turbidimetric immunoassay (Roche Diagnostics) on Cobas 8000 Modular Analyzer (Roche Diagnostics, GmbH, Mannheim, Germany) following the manufacturer's instructions. Cortisol was measured by Cortisol RIA Kit (Beckman Coulter, Cat.: IM1841) on RIA-mat 280 automated analyzer (Stratec Gmbh, Birkenfeld, Germany) following the manufacturers' instructions.

Quantification of hTrec Copy Number Using Digital PCR

Venous blood samples were collected in sodium citrate-containing tubes (BD Vacutainer Blood Collection Tube). Genomic DNA was isolated from 200 μl blood sample using Blood Mini Kit (Qiagen, Hilden, Germany), allowing for sjTRECs (also called hTREC) DNA loops to co-isolate with genomic DNA. Total DNA was quantified using NanoDrop 2000 (Thermo Fisher Scientific, United States). DNA was then diluted to a final concentration of 2000–3000 copies/μl. Digital PCR reactions were prepared according to the QuantStudio^{TM} 3D Digital PCR Reagent Kit (Thermo Fisher Scientific) recommendation. The Digital PCR sample mix was added on each chip and loaded on ProFlex^{TM} 2x Flat PCR System following the manufacturer's instructions. Absolute quantification of hTrec copy number was performed using QuantStudio 3D Digital PCR System (Thermo Fisher Scientific) then analyzed using Analysis SuiteCloud Software.

Artificial Neural Network Correlation of Physiological and Molecular Data

Evaluation of the dataset was carried out by Neurosolutions 6 (Neurodimension Inc.) ANN software of Jordan/Elman type. As being a highly adaptive system, this kind of network extends the basic multilayer perceptron model with context units remembering previous. The network can reveal the hidden relationship between input and output factors of no obvious connection. For network training 14 datasets were used (lifestyle program participants) throughout 10 000 training sessions with an average mean squared error of 0.003. Software. Significance was determined using simple *t*-test.

Biostatistics Evaluation of Physiological and Molecular Data

Euclidean distance matrix was computed based on the acquired data. To detect any pattern in this dataset hierarchical clustering with complete agglomeration method was used. For key ANN parameters (sedentary status, insulin, hTREC) we created dendrograms based on clustering results. In addition to visualize data pattern, 3D plots were also employed using NCSS version 12 statistical software.

Statistical Analyses

To analyze data absolute change was used to measure the difference from baseline since relative change, unlike suggested in literature (Vickers, 2001) prooved statistically inefficient. Then data were converted to Z-scores and shown as means ± SD. Numerous studies have shown that K-means cluster analysis reveals human responsiveness in an unbiased manner (Bamman et al., 2007). With the use of K-means cluster analysis (SPSS version 22 for windows) we have identified two clusters: low responders (LR) and high responders (HiR). Individuals showing biological improvement were termed HiR and individuals with negligible improvement or deterioration were termed LR. After the identification of the two groups (LR and HiR) these were tested by One-Way ANOVA using $p < 0.05$. Power analysis was also performed for confirmation (please refer to **Supplementary Material S3**).

RESULTS

Physiological Parameters

Changes in classical physiological parameters occurring during a lifestyle program are well characterized (Galani and Schneider, 2007). Therefore, to validate our program we confirmed that young and healthy, but previously inactive individuals enrolled in our 6 month-long program show the anticipated gains in these physiological parameters. **Figure 1** summarizes changes in such classical physiological parameters. K-means cluster analysis of absolute scores was performed for all the recorded parameters to differentiate

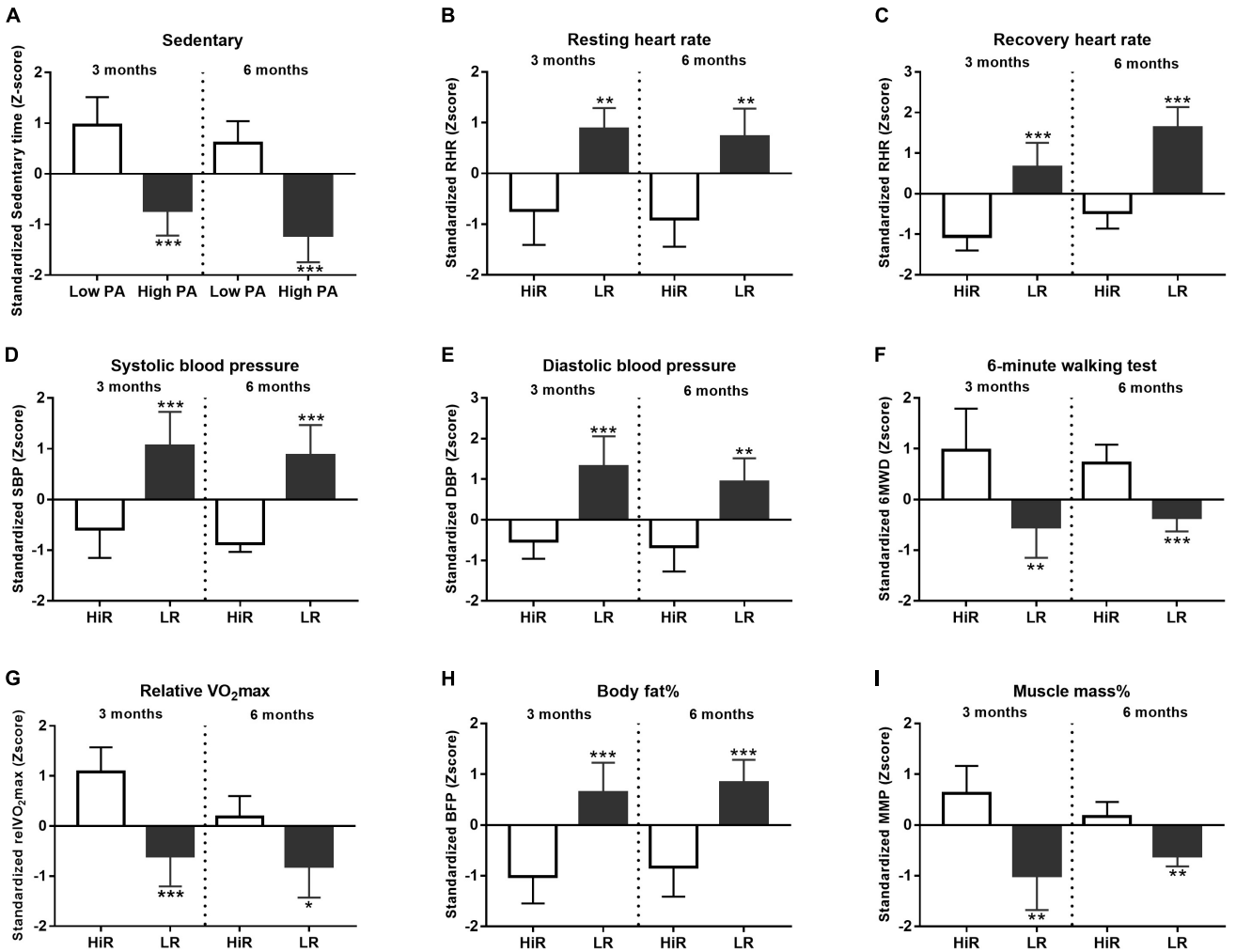

FIGURE 1 | Individual variation of physiological parameters in response to lifestyle program. **(A–I)** K-means cluster analysis was used to differentiate high- (HR) and low (LR) responders ($n = 14$). Bar graph values indicate standardized Z-score values, and standard deviation values. 3- and 6-month values were normalized to baseline values and converted to Z-scores. *Significant difference between HR and LR ($p < 0.05$). **Significant difference between HR and LR ($p < 0.01$). ***Significant difference between HR and LR ($p < 0.001$). Please refer to **Supplementary Material S1** for exact numerical values.

HiR and LR followed by normalization to obtain half time (0–3 months) and final results (0–6 months). We focused on cluster analysis results to differentiate HiR and LR individuals. Exact numerical values of the recorded parameters are listed in **Supplementary Material S1**.

Physical Activity

Physical activity of the recruited participants was recorded and evaluated. By definition inactive individuals spend a significant amount of time in sedentary status, hence increasing PA means that these individuals spend less time in sedentary behavior (Kohl et al., 2012). Z-score values of sedentary status cluster analysis (**Figure 1A**) show that at half time (3 months, left part of **Figure 1A**) high PA (High PA) and low PA (Low PA) clusters segregate significantly ($p < 0.001$). Please, note that both clusters show increasing activity by the end of the program (6 months, right part of **Figure 1A**) without affecting the significance of cluster segregation ($p < 0.001$).

Cardiovascular System

Heart Rate- (**Figures 1B,C**) and blood pressure values (**Figures 1D,E**) were acquired and evaluated as direct cardiovascular parameters. Based on cluster analysis HiR and LR clusters segregate for resting- and recovery HR (**Figures 1B,C**). Resting HR Z-score remains low for HiR individuals during the program, but recovery HR Z-score shows mild increase in comparison of half time and final values. Meanwhile, the statistical power of segregation remains unchanged for both resting- ($p < 0.01$) and recovery- ($p < 0.001$) HR Z-score values. Blood pressure Z-score values obtained by cluster analysis (**Figures 1D,E**) also show the anticipated decrease due to regular PA in HiR individuals. In case of systolic blood pressure, the

significance of HiR and LR segregation remains unaltered during the program ($p < 0.001$) (**Figure 1D**), while diastolic blood pressure Z-score values decrease in the significance of segregation for HiR and LR clusters (mid-term $p < 0.001$, final $p < 0.01$) (**Figure 1E**).

Physical Performance

The enrolled individuals completed a standard 6MWT at program entry, half time and exit. Cluster analysis identifies HiR and LR individuals at half time and program exit as well (**Figure 1F**). HiR individuals show improvement in the 6MWT. However, half time values after 3 months appear to outperform final values at 6 months as Z-score values decrease with time, even though the statistical power of cluster segregation increases ($p < 0.01$ at half time, and $p < 0.001$ at program exit) (**Figure 1F**). Since physical performance correlates with respiratory capacity, relative VO_2max was also recorded and evaluated (**Figure 1G**). As expected VO_2max increases in HiR individuals due to regular PA. Statistical analysis shows that HiR and LR individuals strongly segregate at half time ($p < 0.001$) showing slight decrease by the end of the program ($p < 0.05$). In support VO_2max also shows a moderate decrease with time (**Figure 1G**), similar to the 6MWT results (**Figure 1F**) and the recovery HR (**Figure 1C**) Z-score values above.

Body Composition

Body-mass index (BMI) is calculated based on body mass and height. Regular PA is known to restructure body composition decreasing body fat content and increasing muscle mass (Said et al., 2017). However, since muscle density exceeds fat density BMI values can mask subtle changes of body composition. For this reason body fat percent and muscle mass percent were measured directly (**Figures 1H,I**). It is important to note that participants have not changed their dietary habits during the program. HiR and LR individuals segregate significantly according to cluster analysis. As anticipated, regular PA decreased body fat percent (**Figure 1H**) in an activity-proportional manner as HiR individuals lost fat (left part of **Figure 1H**), while LR individuals gained fat (right part of **Figure 1H**), with unaltered cluster segregation throughout the program ($p < 0.001$). Muscle mass percent shows the opposite tendency, albeit the effect is more profound for HiR individuals at half time (left part of **Figure 1I**) than at exit (right part of **Figure 1I**) despite unaltered significance of cluster segregation ($p < 0.01$).

Molecular Parameters

Changes in classical molecular parameters were also anticipated to occur during a lifestyle program. This section focuses on cluster analysis to differentiate high responders (HiR) and low responders (LR) based on molecular parameters, while the exact numerical values are listed in **Supplementary Material S1**.

Glucose and Lipid Metabolism

Regular PA is known to depend on and gradually reshape energy utilization, both fast-acting glucose and slow-acting lipid metabolism (Yu et al., 2016). For this reason basic relevant metabolic parameters have been recorded and evaluated using cluster analysis. **Figures 2A,B** show glucose and insulin level Z-scores value, at half time and program exit. HiR of glucose metabolism show moderate increase of fasting glucose level, while their insulin show a moderate decrease of fasting insulin level at half time and program exit as a steady-state adaptation to regular PA. Both parameters show significant segregation of HiR and LR individuals. Please, note that there is decreasing glucose and increasing insulin Z-score segregation with time ($p < 0.001$ to $p < 0.01$ for glucose, and $p < 0.01$ to $p < 0.001$ for insulin, comparing half time and final values, respectively). As for lipid metabolism, HDL and LDL cluster analysis results are shown by **Figures 2C,D**. As expected, regular PA increases HDL, but decreases LDL values in HiR individuals. Unlike glucose metabolism above, HiR and LR segregation does not change during the program ($p < 0.01$ for HDL and $p < 0.001$ for LDL).

Immune System

Regular PA is considered to enhance immune responses due to chronic low-level stress also known as hormesis (Pedersen and Hoffman-Goetz, 2000). Since hormesis enhances stress resistance partly due to increased endogenous steroid-response, cortisol level was also recorded (Budde et al., 2015). In order to identify HiR and LR individuals in terms of the immune system, basic parameters including an acute phase protein (CRP) and lymphocyte counts were also evaluated. The endocrine system (via e.g., cortisol) is known to affect both the metabolic system (represented above by glucose and insulin levels) and the immune system (represented here by lymphocyte counts) (Hackney and Lane, 2015).

Cortisol levels increase following regular exercise in HiR individuals, and cluster analysis shows marked segregation of HiR and LR (**Figure 3A**, $p < 0.001$) both at half time and program exit. CRP level Z-score values show the anticipated decrease in HiR individuals and a marked segregation of HiR and LR individuals at half time (**Figure 3B**, $p < 0.001$) that unexpectedly diminishes by program exit (at 6 months $p < 0.05$). Elevated cortisol levels are known to suppress specific leukocyte counts e.g., lymphocyte counts (Pedersen and Hoffman-Goetz, 2000). This is potentially supported by **Figure 3C** as lymphocyte counts decrease in HiR individuals. Cluster analysis shows marked and significant segregation of HiR and LR at both half time and program exit for lymphocyte counts (**Figure 3C**, $p < 0.001$ at mid-term and $p < 0.01$ at program exit). Thymus function (fresh naïve T-cell production) has been reported to improve with regular moderate physical exercise (Duggal et al., 2018). For this reason we have performed digital qPCR measurement to evaluate fresh naïve T-cell production via hTREC measurement (Hazenberg et al., 2001). hTREC is a DNA loop by-product of TcR (T-cell receptor) gene rearrangement produced during thymocyte maturation in the thymus. hTREC co-isolates with genomic DNA during isolation. Its level is proportional with thymus function:

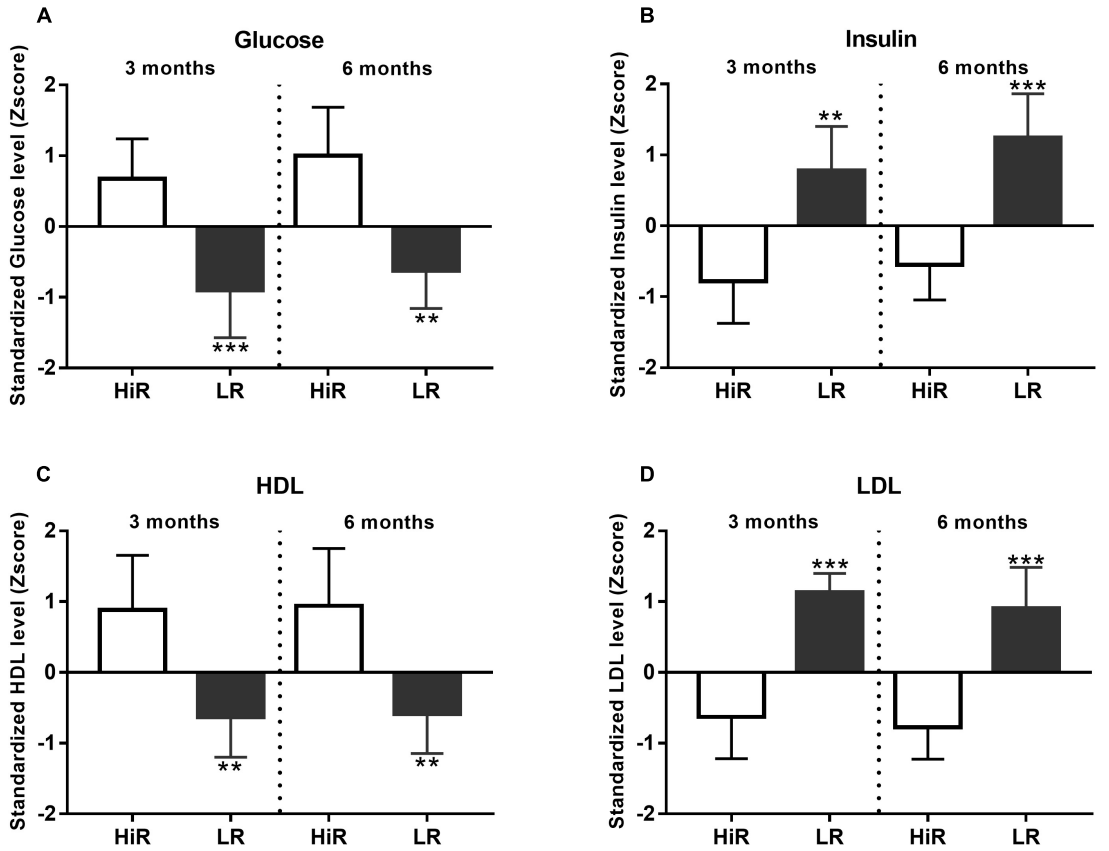

FIGURE 2 | Individual variation of metabolic parameters in response to lifestyle program. **(A–D)** K-means cluster analysis was used to differentiate high- (HR) and low (LR) responders ($n = 14$). Bar graph values indicate standardized Z-score values, and standard deviation values. 3- and 6-month values were normalized to baseline values and converted to Z-scores. *Significant difference between HR and LR ($p < 0.05$). **Significant difference between HR and LR ($p < 0.01$). ***Significant difference between HR and LR ($p < 0.001$). Please refer to **Supplementary Material S1** for exact numerical values.

T-cell production. As anticipated, Z-scores in HiR individuals increased due to PA by half time ($p < 0.01$) that lessened by program exit ($p < 0.001$). Our data support that moderate regular exercise enhances thymus function (fresh naïve T-cell production).

Correlation of Physiological and Molecular Parameters Using ANN

Our lifestyle program yielded a substantial dataset including physiological and molecular parameters. Concept-free ANN analysis was used to find specific correlation patterns among these parameters in a pairwise manner. **Figure 4** shows pairwise correlation efficiency values visualized with a heat map. Physiological and molecular parameters with strong correlation efficiency received high marks (shown by red color), while weak correlation efficiencies received low marks (shown by green color). According to ANN analysis a physiological parameter (activity or sedentary status), a molecular metabolic parameter (insulin concentration) and a molecular immunological parameter (hTREC copy number) showed the strongest correlation with the rest of the recorded parameters appointing them as the most influential mastermind hubs of the current lifestyle program (**Figure 4**).

Evaluation of Physiological and Molecular Parameters Using Biostatistics

Artificial neural network analysis above appointed sedentary status, insulin and hTREC as the most influential, and deregulated parameters. We applied further analysis for these parameters in order to examine the segregation of participants. We have created a concept-free hierarchical clustering with complete agglomeration. **Figure 5** shows the dendrogram representation of participants for sedentary status (**Figure 5A**), insulin (**Figure 5B**), and hTREC (**Figure 5C**). Please, note that all three parameters show an early bifurcation indicating the segregation of low- and high-responders as suggested by K-means statistics. We also show 3D plots for enhanced visualization of sedentary status (**Figure 5D**), insulin concentration (**Figure 5E**) and hTREC copy number (**Figure 5F**) segregation. Please, note the separation of low- and high responder individuals represented by white spheres and gray triangles (view of angle was selected to aid the visualization of separation). *X*-axis shows baseline

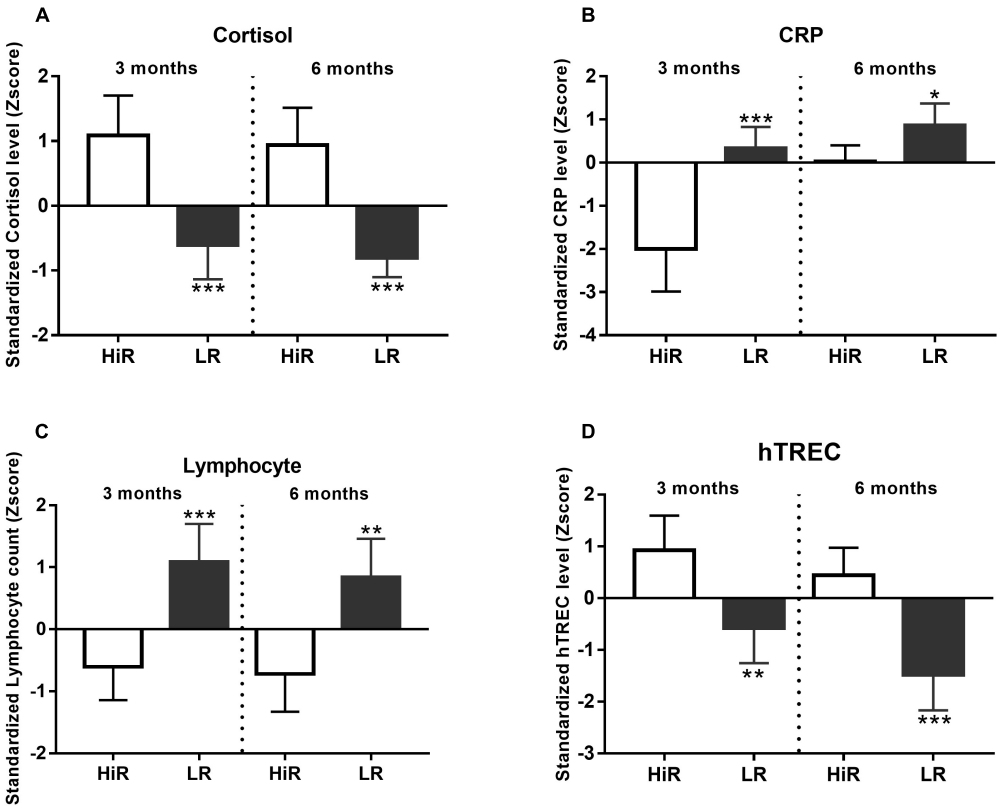

FIGURE 3 | Individual variation of immunological parameters in response to lifestyle program. **(A–D)** K-means cluster analysis was used to differentiate high- (HR) and low (LR) responders ($n = 14$). Bar graph values indicate standardized *Z*-score values, and standard deviation values. 3- and 6-month values were normalized to baseline values and converted to Z-scores. *Significant difference between HR and LR ($p < 0.05$). **Significant difference between HR and LR ($p < 0.01$). ***Significant difference between HR and LR ($p < 0.001$). Please refer to **Supplementary Material S1** for exact numerical values.

(0 month), *Y*-axis shows half time (3 months), and *Z*-axis shows program exit (6 months) values. 2D plots are available for all the assessed physiological and molecular parameters in **Supplementary Material S2**.

DISCUSSION

Physiological System

Physical activity proves to be the major physiological driving force that shows intimate connection with key metabolic (e.g., insulin concentration) and immune (e.g., hTREC copy number) parameters as confirmed by ANN analysis (**Figure 4**). This is in harmony with our working hypothesis suggesting that our lifestyle program achieves molecular level improvement through PA. The degree of PA significantly depends on personal compliance of the participants. Several lifestyle studies report amelioration after 3 months, as also observed in our program (Weston et al., 2014). However, long-term follow-up is advised, as changes in lifestyle are only effective if pursued over an extended period of time. If the major driving force (PA) weakens over time, this may also be reflected by target parameters. In harmony, although 6MWT results showed obvious and significant gain at half time, it diminished by program exit (**Figure 1F**). In support, recovery HR decent (**Figure 1C**) decelerates, VO_2max gain diminishes (**Figure 1G**) and muscle mass percent gain fades (**Figure 1I**) at program exit.

Metabolic System

Cortisol, a family member of corticosteroids, is also acknowledged as a hormone of adaptation that aids acclimatization to chronic low level physical stress triggered by regular exercise also called hormesis (Hackney and Lane, 2015). Cortisol levels showed significant increase by half time that remained sustained during the program in support of aerobic fitness improvement and the overall adaptation process (**Figure 3A**). Regular physical exercise provokes a tight glucose control, but not necessarily a lowered fasting glucose level. Along with elevated cortisol level this resulted in an indicative (not significant) increase of fasting blood glucose level, most noticeable in HiR individuals by program exit (**Figure 2A**). As expected based on glucose physiology, fasting insulin levels showed an anti-parallel, indicative decrease (**Figure 2B**). Regular physical exercise is also known to up-regulate the level of HDL cholesterol. Please, note the indicative changes of HDL level (**Figure 2C**) that show alignment with the extent of PA (**Figure 1A**). The recorded anti-parallel changes of HDL and

Pairwise sensitivity	Actigraph sedentary status		Resting HR		Recovery HR		Systolic BP		Diastolic BP		6 minute WT		relVO2 max		Body Fat%		Muscle Mass%		Glucose		Insulin		HDL		LDL		CRP		Cortisol		Lymphocyte		hTREC		Mean Squared Error
Actigraph sedentary status			0,1867	**	0,1521		0,0537		0,1682	**	0,0557		0,1938		0,0499		0,0298		0,0688		1,1941		0,1173	*	0,1780		0,0890		0,1629		0,1869	**	0,6352		7,14E-05
Resting HR	0,8887	*			0,1308		0,1350	**	0,1498		0,0766		0,1551		0,0463		0,0272		0,0498		0,9310		0,0661		0,2183	**	0,5281	**	0,1954		0,1503		0,9248	***	3,79E-04
Recovery HR	0,9671	***	0,0707				0,1371	**	0,1584	*	0,0516		0,1019		0,0434		0,0506	***	0,0609		0,9269		0,1159		0,2008	*	0,4039		0,2566	****	0,1641	*	0,7763		1,98E-02
Systolic BP	0,7721		0,1880	*	0,1938				0,1112		0,1044	**	0,2285		0,0796	***	0,0523	***	0,0804	**	0,6362		0,1235	**	0,1414		0,4314		0,1783		0,0544		0,7733		5,29E-03
Diastolic BP	0,6977		0,0970		0,1292		0,1279	*			0,0335		0,2378		0,0634		0,0523	***	0,0434		0,7955		0,1110		0,1820		0,4629		0,1995		0,1946	***	0,7576		1,28E-03
6 minute WT	0,5466		0,1998	***	0,1146		0,1243		0,0948				0,2622	**	0,0764	**	0,0338		0,0910	****	1,4057	*	0,0823		0,2200	**	0,4023		0,2153		0,1908	***	0,4932		6,30E-04
relVO2 max	0,4450		0,1631		0,0613		0,1227		0,1142		0,1110	***			0,0741	*	0,0422		0,0716		1,5036	**	0,1119		0,1867		0,6033	***	0,1963		0,1507		0,6683		7,75E-03
Body Fat%	0,7913		0,1634		0,1644		0,0818		0,0854		0,1145	***	0,1747				0,0426		0,0785	*	1,4830		0,1012		0,1358		0,5569	**	0,2220	*	0,1666	*	0,5317		8,37E-04
Muscle Mass%	0,9782	***	0,1674	*	0,1586		0,1374	**	0,1392		0,1132	***	0,2896	****	0,0806	***			0,0765	*	1,5792	***	0,1010		0,2085	*	0,3082		0,2502	****	0,0495		0,8567	*	4,30E-04
Glucose	0,8758		0,1972	***	0,2346	***	0,1234		0,0268		0,0328		0,2550	*	0,0488		0,0412				1,3613		0,1240	**	0,1550		0,1198		0,1960		0,0179		0,9499	****	2,07E-03
Insulin	0,9311	**	0,1218		0,2375	***	0,1329	**	0,1623	**	0,0857		0,1671		0,0415		0,0269		0,0830	**			0,1176	*	0,2154	**	0,5036	*	0,2372	**	0,0588		0,9174	***	6,30E-03
HDL	0,9160	*	0,1994	***	0,2493	****	0,1191		0,1638	**	0,0563		0,2761	***	0,0797	***	0,0417		0,0371		1,4859	**			0,2052	*	0,4111		0,1521		0,1110		0,8015		2,96E-04
LDL	0,5738		0,0866		0,0602		0,1201		0,1010		0,1135	***	0,2967	****	0,0784	***	0,0405		0,0696		1,5130	**	0,0999				0,5308	**	0,2416	***	0,1963	***	0,6304		4,53E-04
CRP	0,7498		0,1563		0,2017		0,0930		0,1306		0,0862		0,2103		0,0644		0,0086		0,0430		0,4625		0,0348		0,0641				0,2159		0,1592		0,7034		3,81E-04
Cortisol	0,9376	**	0,0497		0,2403	***	0,1037		0,1735	***	0,1126	***	0,2400		0,0504		0,0161		0,0934	****	1,5919	***	0,1207	**	0,1800		0,5337	**			0,1351		0,8097		2,10E-04
Lymphocyte	0,9398	**	0,0917		0,2171	**	0,1244		0,1658	**	0,0832		0,1887		0,0684		0,0501	**	0,0727		1,0106		0,0315		0,2260	***	0,4797		0,1996				0,9448	***	8,96E-05
hTREC	0,7625		0,0971		0,1783		0,1163		0,1673	**	0,0455		0,1800		0,0722	*	0,0386		0,0332		0,7803		0,0952		0,0949		0,1979		0,1423		0,0723				2,87E-05
Average	0,7983	****	0,1366		0,1714		0,1199		0,1296		0,0814		0,2176		0,0645		0,0376		0,0656		1,1644	****	0,0958		0,1756		0,4316		0,2065		0,1248		0,7693	****	
SD	0,1631		0,0511		0,0602		0,0226		0,0406		0,0302		0,0540		0,0145		0,0128		0,0191		0,3727		0,0294		0,0468		0,1554		0,0338		0,0596		0,1422		

FIGURE 4 | Artificial neural network (ANN) analysis of physiological and molecular parameters in response to lifestyle program. Heat map shows pairwise correlation efficiencies of physiological and molecular parameters (n = 14). Red color shows high correlation, green color indicates low correlation efficiency between parameters. Significance level of pairwise sensitivity correlation is shown by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p <$ 0. 0001). Significance was determined using simple t-test. Mean squared error in last column indicates model accuracy.

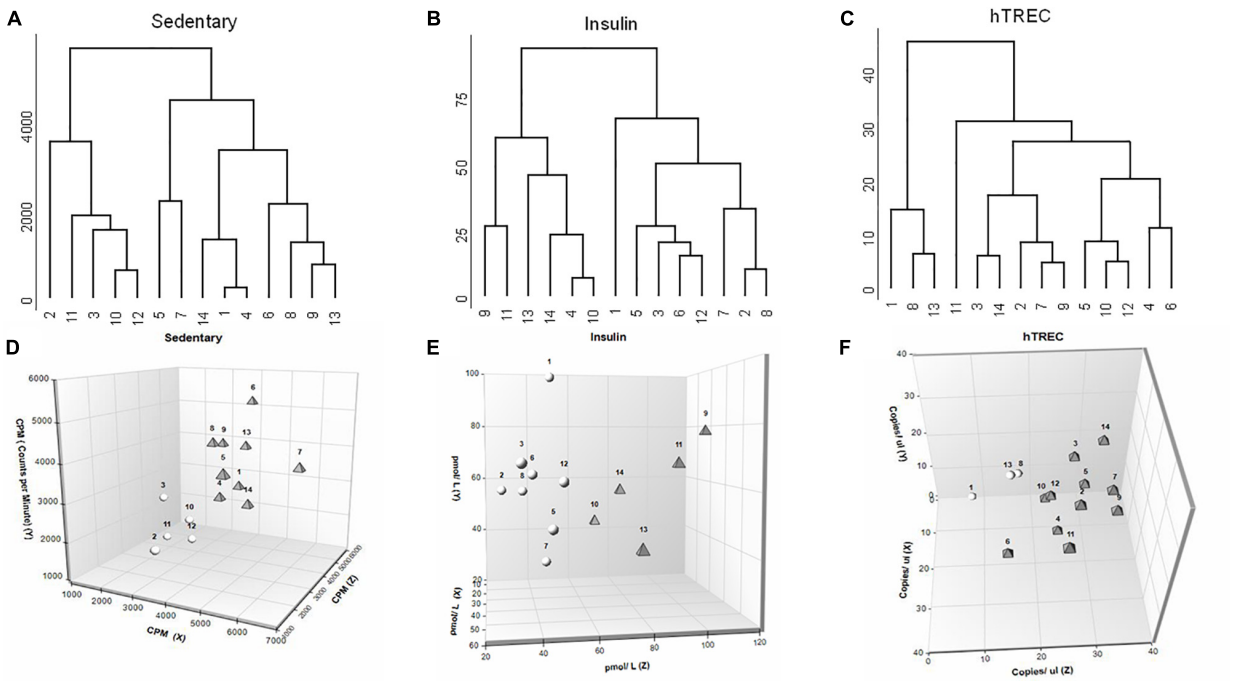

FIGURE 5 | Biostatistics evaluation of physiological and molecular parameters in response to lifestyle program. **(A–C)** Dendrogram representation shows segregation of participants (n = 14) for sedentary status, insulin and hTREC. Please note that all three parameters show an early bifurcation indicating segregation into HR and LR individuals. **(D–F)** 3D plots allow for enhanced visualization of sedentary, insulin and hTREC parameter segregation. Please note the separation of HR and LR individuals (n = 14) represented by white spheres and gray triangles (view of angle was selected to aid the visualization of separation). X-axis shows baseline (0 month), Y-axis shows mid-term (3 months) and Z-axis shows program exit (6 months) values.

LDL cholesterol levels are in harmony with literature data (Hagner-Derengowska et al., 2015).

Immune System

Corticosteroids are potent anti-inflammatory agents (Pedersen and Hoffman-Goetz, 2000; Hackney and Lane, 2015). In our study CRP levels decrease during the program in response to increasing cortisol levels supporting the immune-biology relevance of enhanced cortisol production in the current lifestyle program setting (**Figure 3B**), although the effect is temporary as CRP decrease diminishes in HiR individuals by program exit. Cortisol is also known to suppress multiple pathways of cell activation in several leukocyte subtypes (Hackney and Lane, 2015), a direct target group being the lymphocyte population (Pedersen and Hoffman-Goetz, 2000). In harmony with cortisol level changes lymphocyte counts showed significant decrease in HiR vs. LR individuals (**Figure 3C**). T-cells developing in the thymus (termed thymocytes) are especially sensitive to cortisol and readily undergo apoptosis (Sajdel-Sulkowska et al., 1978). However, thymus function (naïve T-cell production) has been

reported to show improvement in response to moderate regular exercise (Duggal et al., 2018). In order to detect the net effect of cortisol (suppressing T-cell production) and regular exercise (improving T-cell production) we have measured the quantity of a T-cell production by-product (hTREC or human T-cell receptor excision circle) in peripheral blood lymphocytes. As anticipated, hTREC values showed significant increase by half time of the program, emphasizing the net immunological gain achieved by regular moderate exercise (**Figure 3D**).

Personal Responsiveness

It has been reported that personal responsiveness relies on multiple factors, including genetics and environmental factors (physical and mental stress, sleep quality, dietary habits etc.) (Mann et al., 2014). Fixed training programs not aligned with personal requirements yield significant standard deviation, whereas personalized training sessions provide more coherent results (Mann et al., 2014). For this reason subjects enrolled in the current program performed moderate, regular exercise under the guidance of a personal trainer. Despite all possible precautions participating individuals segregate into high-responder (HiR) and low-responder (LR) individuals for all the assessed parameters. Of extreme importance HiR individuals of a particular parameter may prove to be LR individuals of another parameter. The phenomenon is easily observed by comparing the biostatistics dendrograms of our key ANN parameters (sedentary status, insulin, hTREC) as shown by **Figures 5A–C**. Please, note individual responsiveness patterns showing significant person-to-person variation. Our results highlight the diversity of human healthy young population in terms of responsiveness observed during combined endurance and strength-training program. A responsiveness table (please refer to **Supplementary Material S4**) provides further detail for all the assessed parameters in every participant, separately.

CONCLUSION

Our observations provide novel information on individual differences in response to combined endurance and resistance training (Hautala et al., 2006). Previous studies mainly focused on individual response patterns of endurance training alone (Scharhag-Rosenberger et al., 2012; Mann et al., 2014). In most studies the frequency and intensity of training sessions was pre-determined and did not allow positive adaptive changes for the individuals and to fully recover from each session (Bouchard and Rankinen, 2001; Vollaard et al., 2009; Scharhag-Rosenberger et al., 2012). In contrast to such fixed training sessions in our study participants performed moderate, regular exercise individually adjusted to HR monitoring results during the 6 months under the guidance of a personal trainer. Such personalization suits individual needs and abilities, and improves the coherence of results. Our study showed considerable variation in training adaptation for parameters like relVO2max, blood pressure, glucose, insulin, HDL, LDL, and cortisol, all in healthy and young, previously inactive individuals. More importantly, similarly to other studies, our data confirm that high responders for one parameter are not necessarily high responders for others (Vollaard et al., 2009; Mann et al., 2014). Apparently, the more parameters are assessed, the more individual health-improvement patterns are revealed. Such individual patterns of thymic naïve T cells production (through hTrec) are of considerable interest, since immune-senescence is a major trigger of senior frailty causing elevated incidence of infection, cancer and autoimmunity. To our knowledge in the present study we have shown for the first time the correlation of hTrec and regular PA.

A frequent misconception is to generalize data and present mean response alone, however, individual responses typically show remarkable variation, including segregation of high and low responders for each and every parameter. Using concept-free techniques, performing ANN analysis and biostatistics evaluation we have identified numerous molecular changes triggered by regular PA, and show evidence for individual responsiveness at molecular level. Our study proposes that ANN analysis combined with biostatistics evaluation – especially hierarchical cluster analysis – will help in the future to predict individual molecular responsiveness, however, more participants and further studies are required for proof-of-concept.

ETHICS STATEMENT

The program was approved by the ethics committee of the University of Pécs (ref. no.: 6439/2016). All participants gave written informed consent before starting the lifestyle program in accordance with the Declaration of Helsinki. Experiments involving human subjects and their blood samples were performed with the consent of the Regional and Local Ethics Committee of Clinical Centre, University or Pécs according to their guidelines.

AUTHOR CONTRIBUTIONS

KG performed many anthropometric and most molecular measurements, and involved in the manuscript preparation. ZA performed the statistical evaluation of the assessed parameters. RH executed biostatistics evaluation of the assessed parameters. TN designed and orchestrated, while EK executed most physiological measurements. SP performed the ANN analysis. AG planned and supervised the biostatistics evaluation. JP involved in trial design of molecular procedures and manuscript preparation. MW involved in anthropometric and physiological trial design, and manuscript preparation. KK orchestrated the current research, involved in the design of most molecular procedures, and took significant part in the manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01242/full#supplementary-material

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REVIEW
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A Review of Countermovement and Squat Jump Testing Methods in the Context of Public Health Examination in Adolescence: Reliability and Feasibility of Current Testing Procedures

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Background: In the context of a public health physical fitness (PF) examination in adolescence, a countermovement jump (CMJ) and a squat jump (SJ) are two vertical jump (VJ) tests widely used to evaluate lower limb muscle strength and power, respectively. The main criticism of both the CMJ and SJ test is the lack of test standardization. Therefore, the objectives of this review are: (a) to gather information about both jumps; (b) to investigate whether it is possible to identify common procedures referred to in the CMJ and SJ technical execution, and (c) to design standard operating procedures (SOPs) to promote CMJ and SJ standardization in an adolescent population aged 12–18 years.

Methods: The review partially adopted the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Statement (PRISMA). Due to growing attention in monitoring physical health through field tests in recent years, articles were collected using the PubMed, Web of Science, and Scopus databases from January 2009 to July 2019. Original articles in which CMJ or SJ were used to assess the muscular strength in adolescents were eligible for further analysis. Articles written in English was imposed as a limit.

Results: A total of 117 studies met the inclusion criteria. The description of the CMJ and SJ test procedures was different within the literature, with discrepancies in the jump technique, number of jumps, and measurement devices used.

Conclusions: A lack of method standardization for both the CMJ and the SJ test was identified. Based on the literature, SOPs for both VJs were proposed. These are useful in the context of public health PF examination in adolescents, as they facilitate an unbiased comparison of jump performance data between published studies.

Keywords: vertical jump, CMJ, SJ, standardized protocol, adolescent, public health, standard operating procedure, physical fitness

INTRODUCTION

Muscular strength and power, cardiorespiratory endurance, body composition, and flexibility are health-related attributes of physical fitness (PF) (Caspersen et al., 1985) and consequently are considered key health status markers in humans (Catley and Tomkinson, 2013). To prevent pathologies and diseases that occur during adulthood, monitoring PF during adolescence is, therefore, important (Ortega et al., 2008b). In this context, the identification of children who are not developing healthy fitness habits using valid, reliable but also feasible measurement tools is essential (Davis et al., 2008; Faigenbaum et al., 2011; Garber et al., 2011). In the last decade, several research groups have focused their work on assessing the health status of children and adolescents, developing test batteries such as the ALPHA health-related fitness test battery (Ruiz et al., 2011), the ASSO project (Bianco et al., 2015), and the PREFIT battery (Ortega et al., 2015).

Physical fitness can be objectively and accurately measured through laboratory and field tests (Cooper, 1968; Astrand, 1976; Boone et al., 1978; Leger and Lambert, 1982; Inbar et al., 1996; Mayorga-Vega et al., 2014). Laboratory tests are generally more reliable, while field tests are commonly less expensive and more comfortable to administer (Heyward, 1991), and importantly they are characterized by a high level of ecological validity. Therefore, field tests are more suitable in population-based studies, especially in a school or college setting (Artero et al., 2011). Some field tests are subject to a standardized testing procedure, for example, the Cooper test (Cooper, 1968); the 20-m Shuttle Run Test (Leger and Lambert, 1982), or the sit-and-reach test (Wells and Evelyn, 1952). Conversely, to date the vertical jump (VJ) test does not consist of such standardized testing procedures.

Vertical jumping is a multi-joint movement that requires complex motor coordination, and it has been identified as one of the fundamental movement skills (Gallahue, 2002). VJ tests are widely used to evaluate simple and complex tasks (Suchomel et al., 2016), such as sprint acceleration, sprint deceleration, throwing (Manno, 2008; Comfort et al., 2012a,b; Seitz et al., 2014), and change of direction (Nimphius et al., 2010; Spiteri et al., 2014; Suchomel et al., 2016). Furthermore, to evaluate lower limb muscular strength and due to the simplicity and richness in outcome information, VJ tests are widely used by strength and conditioning professionals, coaches, and health care professionals (Liebermann and Katz, 2003; Duthie, 2006).

The countermovement jump and squat jump (CMJ and SJ, respectively) are two examples of VJs which are both derived from the Sargent jump (Sargent, 1921, 1924). Both CMJ and SJ are considered reliable and valid (Markovic et al., 2004) in children (Fernandez-Santos et al., 2015). The CMJ is characterized by an initial countermovement (CM) before the toe-off phase (Bobbert et al., 1996), and the CMJ provides information about the reactive strength of the lower limbs (Young, 1995). In contrast, the SJ starts from a stationary, semi-squatting position and it provides information about leg power performance (Anderson and Pandy, 1993; Young, 1995). In sedentary individuals, as well as in elite athletes, the resultant jump height is correlated with explosive muscle strength (Sargent, 1921, 1924; Bosco and Komi, 1979, 1980; Bosco and Viitasalo, 1982) and with performance components, such as speed (Wisloff et al., 2004), agility (Barnes et al., 2007), and power (Liebermann and Katz, 2003; Markovic et al., 2004; Patterson and Peterson, 2004; Tricoli et al., 2005). The SJ performance is also considered a measure of coordinated activities (Tomioka et al., 2001; Eloranta, 2003; Myer et al., 2005). In this context, Van Hooren and Zolotarjova (2017) in a recent review highlighted the differences between CMJ and SJ performances, emphasizing the need for future research to investigate the exact interaction of the mechanisms that explain the difference between the two jumps.

The lack of robust and consistent testing methods for CMJ and SJ evident in the literature compromises the quality of the research in this area (Eagles et al., 2015). Eagles et al. (2015) in their meta-analysis on VJ tests stated a lack of standardization in jump phase identification (i.e., starting, push-off, toe-off, and apex of the jump phase) which results in notable differences in the duration of the jump phases, the time to reach peak force, and in the rate of force development. Fitzgerald et al. (2018) suggested the use of the SJ to bypass the problem of the identification of the related phases, as this jump comprises of less variables than other kinds of jumps. Van Praagh and Dore (2002) stated that there is a distinct need to create a standardized jump protocol. Furthermore, some researchers advocate the use of standard operating procedures (SOPs) as being superior to the teaching of "laboratory manuals," in that SOPs provide a step-by-step guide to the details related to a process which allows for the exact replication of all steps involved when repeating the process (Angiuoli et al., 2008; Tuck et al., 2009). It is important to note that SOPs are widely adopted in many other areas (Angiuoli et al., 2008), such as biology (Roseti et al., 2015) or medicine, for example in stroke prevention and treatment (Ntaios et al., 2015), critical illness (Sherren et al., 2014), or pre-hospital critical care interventions (Rognas et al., 2013).

To the best of our knowledge, no research to-date has stipulated clear guidelines for the CMJ and SJ tests. In fact, published works used different testing procedures, without specifying some essential parts for the replicability of the work.

Objectives

The first objective of this review was to gather information about testing methods used in research related to the assessment of PF, specifically muscular strength, using CMJ and SJ performance tests in adolescence. The second objective was to investigate if there are standard aspects between these CMJ and SJ testing methods and to identify the most common ones used. If these were not evident, the third objective was to develop SOPs considering: the jump phases; the devices used, and the number of jumps performed. As described by Bobbert et al. (1996), for a better understanding of the differences between the CMJ and the SJ, it is useful to divide both VJs into phases (e.g., starting position, the start of the push-off, the toe-off, and the apex of the jump phase).

Research Question

With a particular view of the adolescent population, the research question addressed whether there are clearly defined SOPs for the CMJ and the SJ test evident in the literature and then, in parallel, to gather information about both VJs.

MATERIALS AND METHODS

Study Design

This review of literature partially adopted the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Moher et al., 2009). The following key points were not used: protocol and registration (5), data items (11), risk of bias in individual studies (12), summary measures (13), synthesis of results (14), risk of bias across studies (15, 22), additional analyses (16, 23), risk of bias within studies (19), results of individual studies (20), and synthesis of results (21).

Participants, Interventions, Comparators

Population, Intervention, Comparison, Outcomes, Study design criteria (PICO-S criteria) described in PRISMA (Moher et al., 2009) were considered, to include and exclude research publications. The population under examination were adolescents. According to Radnor et al. (2018), adolescent females include an age range between 12 and 18 years, and for males this age range is between 14 and 18 years. A population between 12 and 18 years old, of both genders with no particular conditions (i.e., mental disease or physical problems), was considered in the present work, to avoid misunderstandings between the different gender age ranges. Children, adults, the elderly, and elite athletes (due to the possible adaptation of the VJs for the sport characteristic) were excluded. As the authors wanted to examine the jump testing method used, interventions, comparisons, and outcomes of the studies were not considered as inclusion or exclusion criteria. Regarding the study design, original articles were eligible for further analysis in which CMJ or SJ performance tests were used to assess lower limb muscular strength as part of PF evaluations in adolescents and not as training interventions. Due to the risk of involving other populations that were not adolescents, longitudinal studies were excluded.

No restriction criteria were applied for the country of origin, but only works written in English were considered. Reviews, meta-analyses, abstracts, citations from scientific conferences, statements, opinion pieces, commentaries, editorials, letters, book reviews, books, and non-peer reviewed journal articles were excluded.

Search Strategy

The databases consulted for relevant original articles were PubMed (NLM), Web of Science (TS), and Scopus. The search strategy included the use of the terms in the search field "title" and "topic" of each database. The terms used were divided into three groups. Group A used the following keywords: "countermovement jump*," "squat jump*," and "vertical jump*." Group B used the following keywords: "maximal dynamic strength," "field-based physical fitness test*," "fitness-test battery*," "field test*," "physical fitness," "muscle strength," "strength," "resistance training," "physical education," "reliability," and "validity." Finally, group C used the keywords: "youth," "preadolescence*," "adolescent*," "public health," and "health promotion." For each database, term by term of Group A was matched with each term of Group B and Group C using a Boolean operator (AND).

Data Sources, Studies Sections, and Data Extraction

Due to the growing interest in monitoring PF in young people in the last 10 years (Ruiz et al., 2011; Bianco et al., 2015; Ortega et al., 2015), databases were searched for studies published between the 1 January 2009 up to the 8 July 2019. All original articles selected from databases were transferred to the EndNote X8 software to check the presence of duplicates. In a second screening phase, two investigators, working independently, selected the articles against the inclusion and exclusion criteria described in the section "Participants, Interventions, Comparators," with a three steps process: (i) selection based on the titles; (ii) selection based on the abstract; and (iii) selection based on the full text. If there was disagreement between the two investigators, a third investigator took the final decision. A flow diagram that summarizes the selection process is reported in **Figure 1**.

The following information was extracted: first author, year of publication, sample size, participants' age (range, mean, and standard deviation), gender, aim of the study, active or sedentary, physical or sport activities practiced, jump method used, device employed, and main results. The information extracted from any section of the manuscript was consequently recorded in table format and descriptively summarized.

Following this stage, a descriptive analysis of the CMJ and SJ test technique was performed, and all common aspects between the VJs were considered.

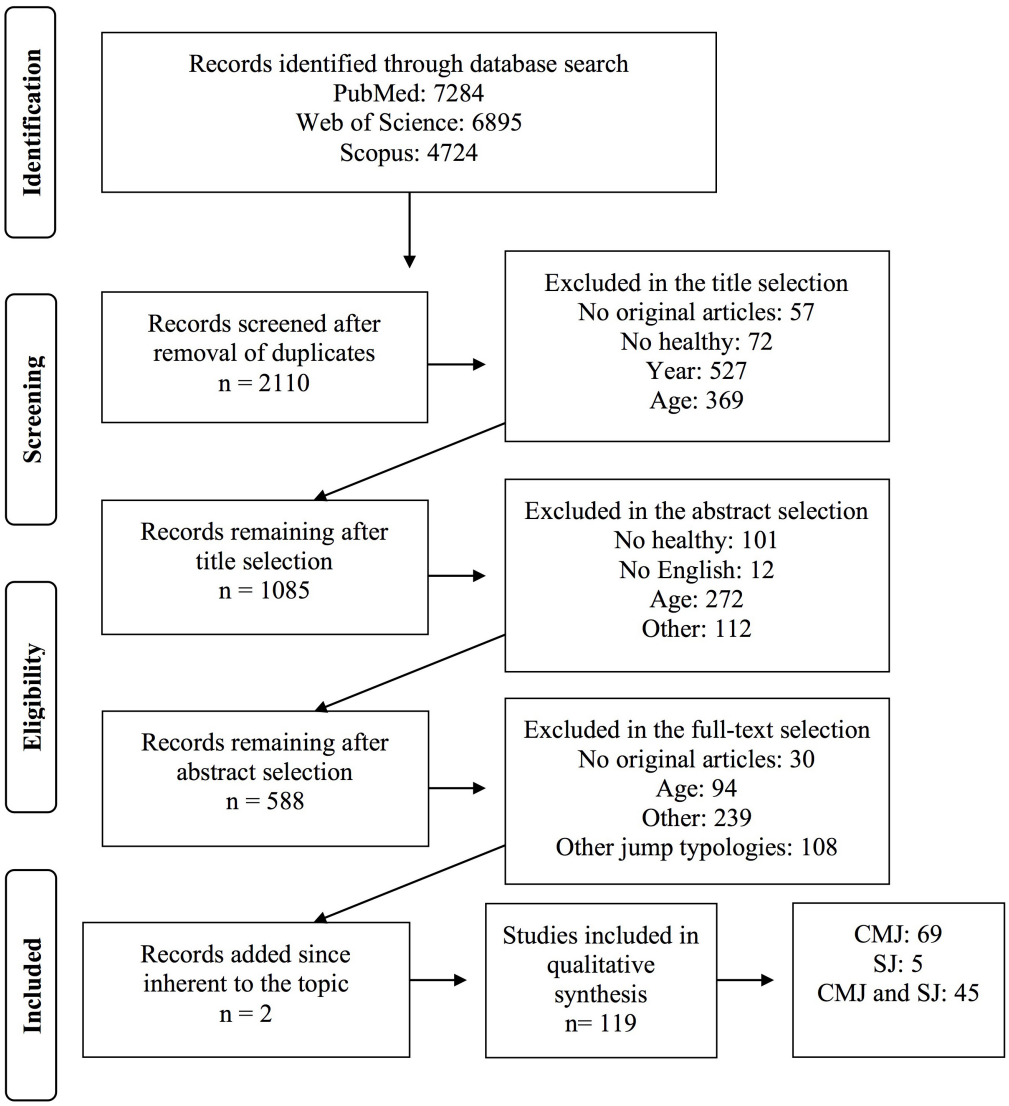

FIGURE 1 | Flow diagram representing the steps applied in the selection process of manuscripts.

RESULTS

Study Selection and Characteristics

After duplicate removal, title, abstract, and full-text comparison against inclusion and exclusion criteria, the number of publications was reduced from 18,903 to 1,860 with a final total of 119 articles being included. More details are provided in the diagram flow presented in **Figure 1**.

The 119 original studies were divided into subgroups as follows:

Sixty-nine studies solely using the CMJ test to evaluate muscular strength in adolescents (**Table 1**);

Five studies solely using the SJ test to evaluate muscular strength in adolescents (**Table 2**);

Forty-five studies where both the CMJ and the SJ test were employed to evaluate muscular strength in adolescents (**Table 3**).

A total of 9,940 individuals were considered. Of this 34% (3,373) were females, 57% (5,630) males, and for the remaining 9% (937) gender was not specified. The mean age was 15.2 years.

Synthesized Findings About Both Jumps

Study Characteristics for CMJ

There was no consistency in the description of the CMJ test method in the literature (**Tables 1**, **3**). The protocol developed by Bosco et al. (1983) was the one most commonly adopted (Coelho et al., 2010; Boccolini et al., 2013; Takai et al., 2013; Alberti et al., 2014; Padulo et al., 2016a; Gallo-Salazar et al., 2017; Perroni et al., 2017; Sanchez-Urena et al., 2017; Borges et al., 2018). According to the instructions of this protocol, participants have to stay in an upright position before the execution of the VJ, which starts with a CM until the legs are bent down to 90°. A more precise description, with information of the knee

TABLE 1 | General information containing the CMJ test only.

Study	Sample (female) [male]	Age range	Age mean (*SD*)	Physical activity level	Protocol	Device
Alvarez-San Emeterio and Gonzalez-Badillo, 2010	(15) [16]	13–16	14.6 (1.1)	Ski	OP	CM
Alvarez-San Emeterio et al., 2011	(19) [20]	13–16	14.7 (1.2)	Ski/none	OP	CM
Amaro et al., 2017	[21]	–	12.7 (0.8)	Swim	Garrido et al., 2010	CM
Boccolini et al., 2013	[23]	14–15	14.8 (0.1)	Basketball	Bosco et al., 1983	PS
Brännström et al., 2017	(19)	13–16	15.3 (0.7)	Soccer	OP	PS
Brunelli et al., 2014	[11]	–	13.3 (0.6)	Basketball	Breed and Young, 2003	CM
Bubanj et al., 2018	[60]	17–18		Mixed	No info	ABS
Buchan et al., 2010	(25) [64]	–	16.7 (0.6)	School	No info	PS
Buchheit et al., 2010	18	–	15.8 (0.9)	School	OP	PS
Castagna et al., 2013	20	–	15.5 (0.8)	Rugby	Domire and Challis, 2007	ABS, PS, FP
Chaouachi et al., 2017	[26]	13–14	13.9 (0.3)	Soccer	Chaouachi et al., 2014	CM
Claudino et al., 2016	[18]	14–17	15.2 (0.9)	Futsal	Ugrinowitsch et al., 2007	CM
Cortis et al., 2011	[10]	15–16	15.7 (0.2)	Basketball	OP	PS
Duncan et al., 2013	(51) [40]	12–16	14.3 (1.3)	None	OP	FP
Faigenbaum et al., 2010	[19]	–	16.5 (1.1)	Athlete	OP	Vanes
Franco-Márquez et al., 2015	[44]	14–18	14.7 (0.5)	Soccer	OP	PS
Gallo-Salazar et al., 2017	12	–	14.4 (0.9)	Tennis	Bosco et al., 1983	CF
Garcia-Pinillos et al., 2015	[43]	14–18	15.6 (1.5)	Soccer	OP	PS
Gavanda et al., 2019	[47]	–	17 (0.8)	Football	OP	PS
Gonzalez-Garcia et al., 2019	(24)	14–16	16.8 (1.6)	Soccer	OP	VA
Gonzalo-Skok et al., 2017	[30]	14–16	14.6 (0.5)	Basketball	OP	PS
Granacher et al., 2011	(15) [13]	–	16.7 (0.6)	School	OP	FP
Granacher and Gollhofer, 2011	28	–	16.8 (0.6)	None	OP	FP
Gorski et al., 2018	[31]	–	16.0 (0.2)	Handball	OP	FP
Hale et al., 2019	(15)	–	15.1 (2.7)	Volleyball	OP	Vanes
Hall et al., 2016	(20)	–	12.5 (1.7)	Gymnast	OP	JM
Harries et al., 2018	[16]	15–18	16.4 (1)	Rugby	Cormack et al., 2008	LPT
Hydren et al., 2013	(7) [4]	–	13.7 (0.5)	Ski	OP	Timing pad
Holden et al., 2015	(84) [97]	–	13 (1.4)	Mixed	OP	VA
Kinugasa and Kilding, 2009	28	–	14.3 (0.7)	Soccer	OP	Vanes
Klusemann et al., 2012	(21) [17]	–	14 (1)	Basketball	OP	FP
Idrizovic et al., 2018	(47)	–	16.6 (0.6)	Volleyball	OP	FP
Imai et al., 2014	[27]	–	16.3 (0.5)	Soccer	OP	Mat switch
Lago-Penas et al., 2014	[156]	13–15	15 (2)	Soccer	OP	CM
Lehnert et al., 2013	16	–	16.7 (0.7)	Soccer	OP	FP
Lyle et al., 2015	(14) [15]	–	16.1 (0.8)	Soccer	OP	Vanes
Marques et al., 2013	[52]	–	13.4 (1.4)	Soccer	Wisloff et al., 2004	CM
Marques et al., 2016	[167]	13–18	15.7 (1.7)	Soccer	Marques et al., 2008	TC
Martin et al., 2019	(37) [77]	–	16.6 (1.1)	Mixed	OP	Vanes
Matthys et al., 2013	472	13–16	13.5 (0.3)	Handball	OP	PS
McCormick et al., 2016	(14)	–	16 (0.8)	Basketball	OP	Vanes
Moraes et al., 2013	[38]	14–18	15.5 (1)	None	Castro-Pinero et al., 2009	Vanes
Muehlbauer et al., 2012	(15) [13]	16–17	16.7 (0.7)	None	OP	FP
Munivrana et al., 2015	(152) [154]	15–18	16.9 (1.7)	Tennis	OP	PS
Negra et al., 2016	24	–	12.8 (0.2)	Soccer	OP	PS
Negra et al., 2017	[37]	–	12.1 (0.5)	Soccer	OP	PS
Oliver et al., 2015	[11]	–	16.9 (0.8)	Rugby	Lloyd et al., 2009, 2011	CM
Padulo et al., 2015b	[17]	–	16	Soccer	Bosco et al., 1982	PS
Paradisis et al., 2014	47	–	14.6 (1.7)	Active	OP	Vanes
Paul et al., 2019	[19]	–	16.2 (0.8)	Soccer	OP	PS
Perroni et al., 2017	[112]	12–19	14 (2)	Soccer	Bosco et al., 1983	PS
Quagliarella et al., 2011	[123]	13–18	15.7 (1.4)	Soccer	OP	FP

(Continued)

TABLE 1 | Continued

Study	Sample (female) [male]	Age range	Age mean (*SD*)	Physical activity level	Protocol	Device
Saez de Villarreal et al., 2015	26	14–15	15.1 (0.2)	Soccer	OP	CF
Sanchez-Urena et al., 2017	[10]	14–15	14 (0.4)	Basketball	Bosco et al., 1983	PS
Sawczuk et al., 2017	(20) [39]	–	17.3 (0.7)	Sportive	OP	PS
Sekulic et al., 2014	[84]	12–13	15.2 (1.3)	None	OP	PS
Smart and Gill, 2013	82	13–18	15.2 (1.3)	Rugby	OP	Vanes
Struzik et al., 2017	(151) [154]	12–16	14.4 (0.8)	Mixed	OP	FP
Takai et al., 2013	[94]	12–16	13.7 (0.6)	None	Bosco et al., 1983	FP
Thomas et al., 2017b	[16]	–	17.3 (0.6)	Basketball	OP	FP
Till and Jones, 2015	[121]	12–16	14.4 (1.7)	Rugby	OP	JM
Tishukaj et al., 2017	(159) [195]	–	14.5 (0.4)	None	Castro-Pinero et al., 2009	FP
Torres-Luque et al., 2015	146	14–17	14.6 (1.1)	Judo	Aragon-Vargas, 2000	CF
Turner et al., 2017	(33) [46]	–	15.9 (0.7)	Fencer	OP	PS
Uthoff et al., 2018	[43]	13–15	14.6 (0.3)	Mixed	OP	Vanes
Weakley et al., 2017	[35]	–	16.9 (0.4)	Rugby	OP	FP
Wong et al., 2009	[70]	–	13.4 (0.7)	Soccer	OP	JM
Wong et al., 2010	[62]	13–14	13.7 (0.5)	Soccer	OP	JM
Yanci et al., 2016	(36) [28]	13–15	14.08 (1)	Mixed	Maulder and Cronin, 2005	PS

ABS, accelerometer-based system; CF, contact platform; CM, contact mat; FP, force plate; JM, jump mat; LPT, linear-position transducer; OP, own protocol; PA, physical activity; PF, physical fitness; PP, physical performance; PS, photoelectric system; VA, video analysis; VJ, vertical jump.

TABLE 2 | General information containing the SJ test only.

Study	Sample (female) [male]	Age range	Age mean (*SD*)	Physical activity level	Protocol	Device
Dayne et al., 2011	[11]	–	15.6 (0.5)	Mixed	OP	FP
Fischetti et al., 2019	[24]	12–14	13.2 (0.8)	Mixed	OP	ABS
Fischetti et al., 2018	[22]	13–14	13.6 (0.5)	Mixed	OP	ABS
Maciejewski et al., 2018	[14]	–	15.3 (0.6)	Rowers	OP	VA
Radnor et al., 2017	[8]	12–16	12.6 (0.2)	None	Lloyd et al., 2009	CM

ABS, accelerometer-based system; CM, contact mat; FP, force plate; OP, own protocol; VA, video analysis.

angle during the standing position, the landing (180°), and the CM (reach approximately 90°) phases, is given by Fernandez-Santos et al. (2015), cited one time (Nikolaidis and Knechtle, 2016). Yanci et al. (2016) and Ramirez-Campillo et al. (2018) cited the protocol of Maulder and Cronin (2005), giving general information on the take-off and the landing phases, which both had to be executed with extended knees and ankle joints. Regarding the CM phase, the protocols of Cormack et al. (2008) allowed participants to self-select the CM depth. Information regarding the speed of the CM phases are given by Ortega et al. (2008a) and Chaouachi et al. (2014). In the protocol proposed by Maulder and Cronin (2005) participants were asked to "sink as quickly as possible" reaching a knee angle of approximately 120°, which was similar to Ortega et al. (2008a) who instructed participants to perform a fast CM. The protocol of McGuigan et al. (2006) cited by Secomb et al. (2015) standardized the position of the hands by requiring the participants to perform the jump while holding a light weight (1.0 kg) over the shoulders. The protocol of Castro-Pinero et al. (2009) was employed twice (Moraes et al., 2013; Tishukaj et al., 2017). Because of the use of the arms, it is different from the protocols previously described, as participants had to touch and mark a wall with their fingertips at a highest possible point. Finally, the protocol by Aragon-Vargas (2000) was the only report requesting that the CMJ be executed barefoot.

Study Characteristics for SJ

Likewise, no standardized jump method was detected for the SJ (**Tables 2**, **3**). The protocol of Bosco et al. (1983) was used five times and required participants to perform the SJ from a half squat position with knees bent at 90°, torso straight, and both hands on their waist (Coelho et al., 2010; Alberti et al., 2014; Padulo et al., 2015c, 2016a; Borges et al., 2018). Additionally, three studies (Santos and Janeira, 2011, 2012; Hespanhol et al., 2013) used the 1983 protocol of Bosco (1994), but cited his work of 1994. The protocol of Lloyd et al. (2009) required the participants to take-off and land on the same spot. Furthermore, before the SJ test, some protocols instructed the participants to wait 4 (Maulder and Cronin, 2005), 3 (McGuigan et al., 2006), or 2 s (Lloyd et al., 2009) before executing the jump in order to control the assumed position. More information regarding the landing can be retrieved in the protocol of Fernandez-Santos et al. (2015). According to these researchers, the knees had to be kept extended at an angle of 180° and the ground contact during the

TABLE 3 | General information containing both, CMJ and the SJ tests.

Study	Sample (female) [male]	Age range	Age mean (*SD*)	Physical activity level	Protocol	Device
Alberti et al., 2014	[81]	–	16.9 (5.4)	Basketball	Bosco et al., 1983	PS
Aloui et al., 2013	[12]	–	13.3 (0.4)	Soccer	OP	CM
Battaglia et al., 2014	(51)	14–15	15 (0.9)	Mixed	Moir et al., 2004	PS
BenOunis et al., 2013	[42]	–	14.8 (0.4)	Soccer	OP	PS
Borges et al., 2018	(25) [64]	12–17	14.5 (0.5)	Soccer	Bosco et al., 1983	CF
Bouteraa et al., 2018	(26)	–	16.4 (0.5)	Basketball	OP	PS
Çakir-Atabek, 2014	(11) [13]	–	15.8 (0.8)	Mixed	OP	FP
Carvalho et al., 2012	[16]	–	14.5 (2.8)	Tennis	OP	JM
Chelly et al., 2009	[22]	–	17 (0.4)	Soccer	OP	CM
Coelho et al., 2010	80	12–14	12.9 (0.3)	Mixed	Bosco et al., 1983	CM
Comfort et al., 2014	[34]	–	17.2 (0.6)	Soccer	OP	JM
Cunha et al., 2017	[46]	12–18	14.2	Soccer	OP	Jump plate
Daneshfar et al., 2018	20	–	16.4 (0.7)	Handball	Bosco and Rusko, 1983	ABS
Dowse et al., 2017	(12)	–	14.2 (1.9)	Dancer	OP	FP
Garcia-Pinillos et al., 2014	[30]	–	15.9 (1.4)	Soccer	OP	Jump sensor
Girard and Millet, 2009	[12]	–	13.6 (1.4)	Soccer	OP	PS
Greco et al., 2019	(56)	13–18	16.8 (0.6)	Volleyball	OP	VA
Grgantov et al., 2013	(56)	13–15	14.6 (0.5)	Volleyball	OP	PS
Hammami M. A. et al., 2013	[50]	–	14.4 (0.3)	Soccer/none	OP	PS
Hammami M. A. et al., 2017	[40]	–	14.4 (0.3)	Soccer	OP	PS
Hammami R. et al., 2018	[56]	12–14	13.9 (1.4)	Handball	OP	FP
Hammami M. et al., 2017	[44]	12–14	16 (0.5)	Soccer	OP	FP
Hammami M. et al., 2018	[31]	15–17	16 (0.5)	Soccer	OP	FP
Hespanhol et al., 2013	[110]	13–18	15 (0.8)	Mixed	Bosco, 1994	CM
Hoshikawa et al., 2013	[28]	13–14	12.7	Soccer	OP	VA
Lesinski et al., 2016	(19)	–	14.7 (0.6)	Soccer	OP	ABS, PS, FP
Loturco et al., 2016	[10]	–	17 (0.7)	Swim	OP	CF
Maio Alves et al., 2010	[23]	–	17.4 (0.6)	Soccer	OP	CM
Makhlouf et al., 2016	[57]	–	13.7 (0.5)	Soccer	OP	FP
Maly et al., 2015	[22]	–	13	Active	CMJ: OP; SJ: no info	FP
Moliner-Urdiales et al., 2010	(180) [183]	12–17	14.8 (1.2)	None	OP	CF
Nikolaidis and Knechtle, 2016	[12]	–	12.2 (0.5)	Soccer	Fernandez-Santos et al., 2015	PS
Ortega et al., 2011	(1845) [1583]	12–18	14.9 (1.2)	None	Ruiz et al., 2006; Ortega et al., 2008a	
Padulo et al., 2015c	[18]	–	16	Soccer	Bosco et al., 1983	PS
Padulo et al., 2015a	[18]	–	16.4 (0.5)	Basketball	Bosco and Rusko, 1983	ABS
Padulo et al., 2016a	[18]	–	16	Soccer	Bosco and Rusko, 1983; Bosco et al., 1983	PS
Padulo et al., 2016b	(22) [14]	–	16 (1)	Basketball	Bosco and Rusko, 1983	ABS
Pino-Ortega et al., 2018	[15]	–	14.7 (0.2)	Soccer	OP	CF, ABS
Pojskic et al., 2018	[20]	–	17 (0.9)	Soccer	OP	CM
Ramirez-Campillo et al., 2018	[18]	17–18	17.4 (0.8)	Soccer	Maulder and Cronin, 2005	CM
Santos and Janeira, 2011	[24]	16–17	14.7 (0.4)	Basketball	Bosco, 1994	CM
Santos and Janeira, 2012	[25]	14–15	14.5 (0.6)	Basketball	Bosco, 1994	CM
Secomb et al., 2015	(7) [23]	–	14.8 (1.7)	Surf	Hasson et al., 2004; McGuigan et al., 2006; Sheppard et al., 2008	FP
Thomas et al., 2017a	(26)	–	16.1 (1.2)	Netball	OP	JM
Yousfi et al., 2018	[14]	–	16.9 (0.7)	Combact	Chamari et al., 2004	PS

ABS, accelerometer-based system; CF, contact platform; CM, contact mat; FP, force plate; JM, jump mat; OP, own protocol; PA, physical activity; PF, physical fitness; PP, physical performance; PS, photoelectric system; VA, video analysis; VJ, vertical jump.

landing had to be initiated with the toes. Straight legs in both take-off and landing was also used in the protocol of Maulder and Cronin (2005). Furthermore, according to Lloyd et al. (2009), the landing phase had to be performed with both legs fully extended while looking forward and, to maintain balance, to gaze at a specific point. Arms crossed against the chest was an instruction given by Secomb et al. (2015) citing the protocol of Hasson et al. (2004), while McGuigan et al. (2006) asked participants to hold a light weight (1.0 kg) over their shoulders.

Arm and Feet Information

Most of the CMJ and SJ tests were performed either with both hands positioned on the hips (number of articles = 41) or the waist (number of articles = 2). The arms placed in an akimbo position was also used in some works (number of articles = 4). Eight articles did not describe the hands/arms position and only stated that swinging of the arms was not permitted. However, an arm swinging movement was permitted in some CMJ-related research (number of articles = 16). A summary of information regarding the position of the upper limb for the CMJ and SJ is provided in **Table 4**.

Number of Jumps Performance Trials and Result Analysis

The number of trials proposed (**Table 4**) and the results taken for statistical analysis were either two (number of

TABLE 4 | Information regarding the position of the arms and the number of jumps used for analysis.

Information took in examination	Authors	Number of studies
Arm position		
Hands positioned on the hips	Alvarez-San Emeterio and Gonzalez-Badillo, 2010; Buchheit et al., 2010; Moliner-Urdiales et al., 2010; Alvarez-San Emeterio et al., 2011; Cortis et al., 2011; Quagliarella et al., 2011; Carvalho et al., 2012; Klusemann et al., 2012; Aloui et al., 2013; Hoshikawa et al., 2013; Hydren et al., 2013; Çakir-Atabek, 2014; Comfort et al., 2014; Lago-Penas et al., 2014; Paradisis et al., 2014; Sekulic et al., 2014; Franco-Márquez et al., 2015; Holden et al., 2015; Saez de Villarreal et al., 2015; Till and Jones, 2015; Lesinski et al., 2016; Loturco et al., 2016; Brännström et al., 2017; Cunha et al., 2017; Gonzalo-Skok et al., 2017; Thomas et al., 2017a,b; Turner et al., 2017; Weakley et al., 2017; Bouteraa et al., 2018; Fischetti et al., 2018, 2019; Hammami R. et al., 2018; Maciejewski et al., 2018; Pino-Ortega et al., 2018; Pojskic et al., 2018; Gavanda et al., 2019; Gonzalez-Garcia et al., 2019; Greco et al., 2019; Paul et al., 2019	41
Hands positioned on the waist	Maio Alves et al., 2010; Carvalho et al., 2012	2
Akimbo position	Grgantov et al., 2013; Lesinski et al., 2016; Negra et al., 2016, 2017	4
Swinging of the arms was not permitted	Maio Alves et al., 2010; Dayne et al., 2011; Lehnert et al., 2013; Garcia-Pinillos et al., 2014, 2015; Maly et al., 2015; Makhlouf et al., 2016; Dowse et al., 2017	8
Swing movement was permitted in the CMJ	Kinugasa and Kilding, 2009; Duncan et al., 2013; Grgantov et al., 2013; Hoshikawa et al., 2013; Lehnert et al., 2013; Matthys et al., 2013; Smart and Gill, 2013; Imai et al., 2014; Lyle et al., 2015; Maly et al., 2015; Munivrana et al., 2015; McCormick et al., 2016; Struzik et al., 2017; Gorski et al., 2018; Idrizovic et al., 2018; Uthoff et al., 2018	16
Number of jumps		
Two	Cortis et al., 2011; Lehnert et al., 2013; Smart and Gill, 2013; Imai et al., 2014; Lago-Penas et al., 2014; Makhlouf et al., 2016; Weakley et al., 2017	7
Three	Chelly et al., 2009; Kinugasa and Kilding, 2009; Wong et al., 2009, 2010; Buchheit et al., 2010; Maio Alves et al., 2010; Granacher and Gollhofer, 2011; Granacher et al., 2011; Carvalho et al., 2012; Muehlbauer et al., 2012; Aloui et al., 2013; BenOunis et al., 2013; Duncan et al., 2013; Hammami M. A. et al., 2013; Hoshikawa et al., 2013; Hydren et al., 2013; Matthys et al., 2013; Çakir-Atabek, 2014; Comfort et al., 2014; Garcia-Pinillos et al., 2014, 2015; Paradisis et al., 2014; Holden et al., 2015; Lyle et al., 2015; Till and Jones, 2015; Lesinski et al., 2016; McCormick et al., 2016; Brännström et al., 2017; Cunha et al., 2017; Dowse et al., 2017; Gonzalo-Skok et al., 2017; Hammami M. A. et al., 2017; Hammami M. et al., 2017, 2018; Thomas et al., 2017a,b; Bouteraa et al., 2018; Fischetti et al., 2018, 2019; Maciejewski et al., 2018; Pojskic et al., 2018; Gavanda et al., 2019; Gonzalez-Garcia et al., 2019; Greco et al., 2019; Hale et al., 2019; Martin et al., 2019; Paul et al., 2019	47
Above three	Alvarez-San Emeterio and Gonzalez-Badillo, 2010; Alvarez-San Emeterio et al., 2011; Quagliarella et al., 2011; Klusemann et al., 2012; Franco-Márquez et al., 2015; Saez de Villarreal et al., 2015; Loturco et al., 2016; Pino-Ortega et al., 2018	8
Jump/s to be considered for analysis		
Average of the jumps	Garcia-Pinillos et al., 2015	1
Out of five trials, lowest and highest were excluded and middle values averaged	Alvarez-San Emeterio and Gonzalez-Badillo, 2010; Alvarez-San Emeterio et al., 2011; Franco-Márquez et al., 2015; Saez de Villarreal et al., 2015	4
Highest jump	Chelly et al., 2009; Kinugasa and Kilding, 2009; Wong et al., 2009, 2010; Maio Alves et al., 2010; Cortis et al., 2011; Granacher and Gollhofer, 2011; Granacher et al., 2011; Klusemann et al., 2012; Muehlbauer et al., 2012; Aloui et al., 2013; BenOunis et al., 2013; Hammami M. A. et al., 2013; Hoshikawa et al., 2013; Hydren et al., 2013; Lehnert et al., 2013; Matthys et al., 2013; Smart and Gill, 2013; Çakir-Atabek, 2014; Garcia-Pinillos et al., 2014; Imai et al., 2014; Lago-Penas et al., 2014; Paradisis et al., 2014; Lyle et al., 2015; Till and Jones, 2015; Makhlouf et al., 2016; McCormick et al., 2016; Cunha et al., 2017; Dowse et al., 2017; Gonzalo-Skok et al., 2017; Hammami M. A. et al., 2017; Hammami M. et al., 2017, 2018; Struzik et al., 2017; Thomas et al., 2017a; Bouteraa et al., 2018; Fischetti et al., 2018, 2019; Maciejewski et al., 2018; Pojskic et al., 2018; Gavanda et al., 2019; Gonzalez-Garcia et al., 2019; Greco et al., 2019; Hale et al., 2019; Martin et al., 2019; Paul et al., 2019	46

articles = 7), three (number of articles = 47), or more than three (number of articles = 8). Some studies (number of articles = 1) used the mathematical average of the individual jump trials. Alternatively, out of five trials, the lowest and highest values were excluded averaging only the middle measurements (number of articles = 4). Most studies (number of articles = 46) only considered the highest jump.

Devices Used

Several measurement devices were employed for the jump assessment, both to measure and estimate the jump performance. Between the devices that measure the VJ performance based on the center of mass, there are the force plates (number of articles = 25). Between the devices that estimate the performance through the flight time there are: the motion caption system (number of articles = 5), photoelectric cell systems (number of articles = 36), contact mats (number of articles = 19), vanes (number of articles = 10), jump mats (number of articles = 7), contact platforms (number of articles = 7), accelerometer-based systems (number of articles = 6), and linear position transducers (number of articles = 1). More details are given in **Tables 1–3**.

Jump Phases Identification

The present review based the jump description and analysis according to the jump phases definition proposed by Bobbert et al. (1996): the starting position; the start of push-off; the toe-off; and the apex of the jump. Additionally, we identified the landing phase.

For the CMJ, the majority of studies (number of articles = 30) agreed in defining the starting position as a standing posture. Some authors provided more details about the position of the lower limbs, suggesting that participants maintain straight legs (Lago-Penas et al., 2014) or position their feet shoulder-width apart (Holden et al., 2015).

The SJ starting position was described by 17 authors as a squat position with knees flexed at 90° and by four authors as a semi-squatting position with knees bent at 90° using a ruler as measurement (Maciejewski et al., 2018). Some researchers required participants to remain in the squat position for either 3 (Comfort et al., 2014; Dowse et al., 2017; Pino-Ortega et al., 2018) or 2 s (Maciejewski et al., 2018) before executing the second phase, on command, i.e., the jump phase.

The CMJ push-off is described as a downward movement without an indication of the depth (number of articles = 26). Some authors indicated that the knee angle had to reach 90° (number of articles = 18) before starting the jump. A limited number of authors provided information regarding the speed of the downward movement, i.e., that it had to perform with a rapid descend (Alvarez-San Emeterio and Gonzalez-Badillo, 2010; Alvarez-San Emeterio et al., 2011; Negra et al., 2016, 2017; Dowse et al., 2017). Regarding the push-off phase for the SJ, most studies performed the jump without a CM (number of articles = 14).

The toe-off phase was described as a maximal effort, i.e., as high as possible (29 works related to the CMJ and 11 works related to the SJ).

For the apex of the jump phase, a requirement of both the CMJ and the SJ was that the participants maintain extended legs (number of articles = 11).

Likewise, to provide reliable results during the execution of the jump landing, standardization is required (Borras et al., 2011). Descriptions of the landing phase were similar for the CMJ and the SJ, with most works reporting a fully extended knee landing (number of articles = 8). Similarly, six works required participants to land without legs flexed. Landing with the toes on the same spot as the take-off (number of articles = 4) and in an upright position (number of articles = 1) were other variants of the instructions given to the participants.

To emphasize the use of the leg extensors, participants were asked to maintain the torso in an upright position (Moliner-Urdiales et al., 2010; Cortis et al., 2011).

A summary of information regarding each CMJ and SJ phase is provided in **Table 5**.

DISCUSSION

The main finding of this review is that results and recommendations for both the CMJ and the SJ published in the literature are derived using a vast variety of testing methods and devices to evaluate lower body muscular strength in adolescents. It is questionable, therefore, whether results and, where applicable, normative jump height values, recommended for adolescents and used to assess PF are comparable. Therefore, it is necessary to create SOPs for CMJ and SJ tests that can be used in the context of health promotion and health investigations.

With this in mind, we recommend participants start the CMJ from an erect standing position with a straight torso, knees fully extended, with hands-on-hips and feet shoulder-width apart. We also recommend maintaining this position for at least 2 s before the descending phase. The CMJ push-off phase should be characterized by a downward movement until the knee angle reaches 90° and this should be visually inspected by the examiner and where possible, the use of accelerometer that emits audio feedback when the angle is reached (Cular et al., 2018). Instructions for the toe-off phase should explicitly state that it has to be performed with a maximal effort. Furthermore, during the apex of the jump, participants have to keep their legs fully extended. Finally, the landing phase has to occur with both feet together and with knees fully extended.

The SJ starting position is recommended with a knee flexion angle of 90°, torso straight, hands-on-hips, and feet shoulder-width apart. This position should be maintained for 2 s before jumping. The push-off phase has to be executed avoiding any kind of counter-movement. As with the CMJ test, instructions for the toe-off phase should explicitly state that it has to be executed with maximal effort. During the apex of the jump phase, participants should keep their legs fully extended. The landing phase has to occur with both feet together in an upright position with knees fully extended.

TABLE 5 | Information regarding the jump phases.

Phase of the jump	Authors	Number of studies
Starting position CMJ		
Standing position	Chelly et al., 2009; Girard and Millet, 2009; Wong et al., 2009, 2010; Alvarez-San Emeterio and Gonzalez-Badillo, 2010; Maio Alves et al., 2010; Moliner-Urdiales et al., 2010; Alvarez-San Emeterio et al., 2011; Cortis et al., 2011; Granacher and Gollhofer, 2011; Granacher et al., 2011; Muehlbauer et al., 2012; BenOunis et al., 2013; Garcia-Pinillos et al., 2014; Lago-Penas et al., 2014; Paradisis et al., 2014; Sekulic et al., 2014; Holden et al., 2015; Loturco et al., 2016; Negra et al., 2016, 2017; Hammami M. et al., 2017; Weakley et al., 2017; Bouteraa et al., 2018; Gorski et al., 2018; Hammami M. et al., 2018; Hammami R. et al., 2018; Idrizovic et al., 2018; Pino-Ortega et al., 2018; Pojskic et al., 2018	30
Starting position SJ		
Squat position with a knee flexion of 90°	Chelly et al., 2009; Maio Alves et al., 2010; Dayne et al., 2011; Carvalho et al., 2012; BenOunis et al., 2013; Grgantov et al., 2013; Çakir-Atabek, 2014; Lesinski et al., 2016; Loturco et al., 2016; Negra et al., 2016; Hammami M. et al., 2017, 2018; Bouteraa et al., 2018; Fischetti et al., 2018, 2019; Hammami R. et al., 2018; Pojskic et al., 2018	17
Semi-squat position (knees bent at 90°)	Girard and Millet, 2009; Moliner-Urdiales et al., 2010; Maciejewski et al., 2018; Greco et al., 2019	4
Push-off CMJ		
Downward movement without indication on the depth	Girard and Millet, 2009; Alvarez-San Emeterio and Gonzalez-Badillo, 2010; Buchheit et al., 2010; Moliner-Urdiales et al., 2010; Wong et al., 2010; Cortis et al., 2011; Granacher and Gollhofer, 2011; Granacher et al., 2011; Carvalho et al., 2012; Muehlbauer et al., 2012; Hoshikawa et al., 2013; Smart and Gill, 2013; Çakir-Atabek, 2014; Holden et al., 2015; Saez de Villarreal et al., 2015; Till and Jones, 2015; Lesinski et al., 2016; Loturco et al., 2016; McCormick et al., 2016; Cunha et al., 2017; Gonzalo-Skok et al., 2017; Sawczuk et al., 2017; Thomas et al., 2017a,b; Weakley et al., 2017; Pojskic et al., 2018	26
Knee flexion angle to reach 90°	Chelly et al., 2009; BenOunis et al., 2013; Grgantov et al., 2013; Hammami M. A. et al., 2013; Comfort et al., 2014; Lago-Penas et al., 2014; Paradisis et al., 2014; Sekulic et al., 2014; Garcia-Pinillos et al., 2015; Brännström et al., 2017; Hammami M. A. et al., 2017; Hammami M. et al., 2017, 2018; Bouteraa et al., 2018; Hammami R. et al., 2018; Idrizovic et al., 2018; Pino-Ortega et al., 2018; Greco et al., 2019	18
Push-off SJ		
Squat jump position	Moliner-Urdiales et al., 2010; Hammami M. A. et al., 2013, 2017; Hoshikawa et al., 2013; Comfort et al., 2014; Lesinski et al., 2016; Thomas et al., 2017a; Bouteraa et al., 2018; Maciejewski et al., 2018; Pino-Ortega et al., 2018; Pojskic et al., 2018	11
Toe-off CMJ		
Maximal effort, i.e., as high as possible	Alvarez-San Emeterio and Gonzalez-Badillo, 2010; Buchheit et al., 2010; Faigenbaum et al., 2010; Maio Alves et al., 2010; Alvarez-San Emeterio et al., 2011; Cortis et al., 2011; Granacher and Gollhofer, 2011; Granacher et al., 2011; Carvalho et al., 2012; Klusemann et al., 2012; Muehlbauer et al., 2012; Aloui et al., 2013; Duncan et al., 2013; Hoshikawa et al., 2013; Smart and Gill, 2013; Comfort et al., 2014; Garcia-Pinillos et al., 2014; Imai et al., 2014; Sekulic et al., 2014; Holden et al., 2015; Saez de Villarreal et al., 2015; Dowse et al., 2017; Thomas et al., 2017a,b; Weakley et al., 2017; Hammami R. et al., 2018; Pino-Ortega et al., 2018; Greco et al., 2019; Martin et al., 2019	29
Toe-off SJ		
A maximal effort, i.e., as high as possible	Maio Alves et al., 2010; Dayne et al., 2011; Carvalho et al., 2012; Grgantov et al., 2013; Negra et al., 2016; Dowse et al., 2017; Thomas et al., 2017a; Fischetti et al., 2018, 2019; Gorski et al., 2018; Greco et al., 2019	11
Not to perform a CM	Chelly et al., 2009; Girard and Millet, 2009; BenOunis et al., 2013; Grgantov et al., 2013; Çakir-Atabek, 2014; Loturco et al., 2016; Hammami M. et al., 2017, 2018; Fischetti et al., 2018, 2019; Maciejewski et al., 2018; Pino-Ortega et al., 2018; Pojskic et al., 2018; Greco et al., 2019	14
Fast extension of the legs	Negra et al., 2016, 2017; Pino-Ortega et al., 2018; Pojskic et al., 2018	4
Apex of the jump		
Maintenance of extended legs CMJ	Chelly et al., 2009; Klusemann et al., 2012; Hammami M. et al., 2017, 2018; Sawczuk et al., 2017; Struzik et al., 2017; Turner et al., 2017; Gavanda et al., 2019; Gonzalez-Garcia et al., 2019	9
SJ	Chelly et al., 2009; Greco et al., 2019	2
Landing		
Fully extended knee landing	Buchheit et al., 2010; Cortis et al., 2011; Grgantov et al., 2013; Hammami M. et al., 2017, 2018; Turner et al., 2017; Maciejewski et al., 2018; Pino-Ortega et al., 2018	8
Without any leg flexion	Imai et al., 2014; Gonzalo-Skok et al., 2017; Fischetti et al., 2018, 2019; Gavanda et al., 2019; Greco et al., 2019	6
With toes on the same spot as the take-off	Fischetti et al., 2018, 2019; Pojskic et al., 2018; Greco et al., 2019	4
Upright position	Saez de Villarreal et al., 2015	1

Finally, even though different kinds of shoe material can result in artificially deflated jump power and height measurement (LaPorta et al., 2013), to avoid injuries for both CMJ and SJ tests, these should not be performed barefoot (in case of test/retest participants are required to wear the same shoes).

Regarding the measurement device, a photoelectric system is the most commonly employed technology, is less costly, and is

very user-friendly. We, therefore, recommend it a part of the SOPs. Furthermore, the equation $H = g^*t^2/8$ [H: VJ height (m); t: flight time (s); g is 9.81 m/s^2] presents high coefficients of determination in the prediction of the VJ height (Attia et al., 2017), and is consequently suggested. Five jumps should be performed during the testing session for both CMJ and SJ, with a 1-min passive rest between jumps to ensure muscular recovery. Due to possible learning effects and consequently higher jump performances, only the best jump should be used for further analysis. Furthermore, we recommend starting the CMJ, and SJ testing session with a standardized warm-up as this can influence jump performance (i.e., a short warm-up can improve the jump height, while a high-intensity plyometric protocol deteriorates the performance) (Romero-Franco and Jimenez-Reyes, 2017). Stretching also seems to potentially cause injury rather than prevent it (Shrier, 1999). Our advice is to perform the same standardized warm-up protocol before any VJ. An example of a suitable warm-up protocol has been suggested by Pinfold et al. (2018). This comprises of two sets of the following exercise: (a) standing on one leg and nod head gently for 30 s; (b) single leg airplane squat with hip thrust (20 repetitions); (c) single leg airplane squat with trunk rotation (20 repetitions); (d) single leg airplane squat with a black theraband resistance applied to the knee that includes trunk rotation with a dumbbell held in hand (10 repetitions); (e) monster walk with a black theraband resistance positioned around the forefoot, forward, and backward (3 m each way); (f) monster walk with a black theraband positioned around the forefoot, side-to-side, i.e., left and right (3 m each way). A summary of the first part of the section "Discussion" can be seen in **Table 6**.

Concerning the proposed SOPs testing method, the upright position of the torso during the starting position phase for both jumps emphasizes the use of the leg extensors (Moliner-Urdiales et al., 2010; Tounsi et al., 2015). Importantly, this upright position prevents the inclination of the torso segment, a common mistake during the jump performance. In the case of a reduction of forwarding torso inclination by 50%, this can result in a 13% increase of the maximal power (Vanrenterghem et al., 2008). In contrast, hip extensors, upper body, and thigh muscles reduce their contribution on the jump performance when the torso is in a vertical position during the push-off phase, and the plantar flexors contribute mainly to the positive work while knee and hip joint muscles cannot contribute to this positive work (Kopper et al., 2012). In a simulation model, Blache and Monteil (2014) demonstrated that a non-consideration of the erector spinae muscle contribution resulted in a ∼15% reduced SJ height and, if a torso inclination was restricted, the anticipated movement and higher knee joint torque development was possible which resulted in a higher maximal power (Vanrenterghem et al., 2008). A standardized position of the knee angle in the SOPs for the CMJ and the SJ is required during the push-off phase, due to the impact it can have on either increasing or decreasing jump height (Krahenbuhl and Pangrazi, 1983; Gheller et al., 2015) caused by the hip and ankle working differently (Hara et al., 2006, 2008). The execution of the VJ with a lower knee angle compromises the jump performance as a deeper squat starting

TABLE 6 | Standard operating procedures proposed for the countermovement jump (CMJ) and the squat jump (SJ).

Phase	CMJ	SJ	
Starting position	Erect position with trunk straight. Knee angle of 180°. Feet shoulder width apart. Maintain the position for at least 2 s	Squat position with trunk straight. Knee angle flex at 90°. Feet shoulder width apart. Maintain the position for at least 2 s	
Push-off	Downward movement until the knees angle are flexed (approximately) 90°	No CM	
Toe-off	Maximal effort and explosive VJ	Jump vertically as high as possible	
Apex of the jump	Maintain legs extended	Maintain legs extended	
Landing	Feet together. Knees extended at an angle of about 180°	Feet together. Knees extended at an angle of about 180°	
Warm-up suggested	Two sets of the following exercise: (a) standing on one leg and nod head gently for 30 s; (b) single leg airplane squat with hip thrust (20 repetitions); (c) single leg airplane squat with trunk rotation (20 repetitions); (d) single leg airplane squat with a black theraband resistance applied to the knee that includes trunk rotation with a dumbbell held in the hand (10 repetitions); (e) monster walk with a black theraband resistance positioned around the forefoot, forward, and backward (3 m each way); (f) monster walk with a black theraband positioned around the forefoot, side-to-side, i.e., left and right (3 m each way) (Pinfold et al., 2018)		
Hands position	On hips		
Barefoot	No		
Number of jumps	Best of 5		
Rest time	1 min between		
Measurement device	Photoelectric system		
Jump suggested	CMJ		

position results in a higher jump, maximum force, and power output (Gheller et al., 2015). For this precise reason, the SJ has to be carefully monitored. In addition, participants tend to perform a small-amplitude CM (Bobbert et al., 1996; Hasson et al., 2004) and jumps with a CM should be discarded. As the SJ is a purely explosive VJ, some researchers (Hasson et al., 2004; Fitzgerald et al., 2018) argued that the SJ could not be influenced, and consequently performing the SJ with a standardized knee angle or a self-selected jump, apparently present no meaningful difference (Fitzgerald et al., 2018). Based on the discussion above, we strongly advocate the need for the standardized starting position of a 90^{o} knee flexion, feet shoulder-width apart, hands-on-hips, and with a straight torso.

Furthermore, this standardized arm position avoids the contribution of the upper limbs as well as coordinative issues as a confounding variable which, as a result of the shoulder, elbow, hip, and ankle muscles working together, can impact on jump performances between 8 and 11% (Harman et al., 1990; Lees et al., 2004; Hara et al., 2006, 2008). Also, the instruction to maintain both legs fully extended starting from the toe-off to the landing phase (i.e., for the entire duration of the apex of the jump phase) is of crucial importance as this can affect the accuracy of the flight time (Borras et al., 2011). Likewise, the landing phase also has to be standardized to obtain equal results during the execution of the jumps (Borras et al., 2011). According to Bui et al. (2015), there are different factors such as the landing with the feet nearly flat or with the legs bent that can alter the flight time, altering the calculated jump height.

Therefore, it is important to land with straight legs, on the forefoot (Bui et al., 2015) and at the same time to amortize the movement because a stiffer technique increases the risk of injuries (Aerts et al., 2013).

Regarding the devices used, a video analysis technique that measures the displacement of the center of body mass from the standing position to the highest vertical point has been proposed as a gold standard (Aragon-Vargas, 2000). However, this specialized equipment is costly, difficult to calibrate, and transport but also requires a complex procedure to obtain data (Aragon-Vargas, 2000). Less expensive, easy to use devices are contact mats which detect the flight time. These have been reported to produce highly reliable and valid results (Markovic et al., 2004). Force plates derive jump height from the flight time, and they can measure the velocity at take-off (Mcgown et al., 1990; Kibele, 1998; Lara et al., 2006). Furthermore, force plates accurately assess ground reaction forces, and can thus provide a preferred solution in pediatric populations (Fricke et al., 2006). Compared to force plates, photoelectric cell systems present a similar level of validity and they provide excellent test–retest reliability for the estimation of the jump height (Glatthorn et al., 2011). Vertec devices (Sports Imports, Hilliard, OH, United States) are also valid (Leard et al., 2007) and reliable (Young et al., 1997) even though not recommended to use for different reasons. Firstly, the measurement device consists of a metal stand and a height scale composed of color-coded vanes that are displaced by the participant when jumping (Klarova, 2000) which requires the use of the arms. Secondly, and in contrast to force plates, the Vertec device (Sports Imports, Hilliard, OH, United States) does not demonstrate an accurate representation of jump height (Buckthorpe et al., 2012).

Claudino et al. (2017) analyzed the average of five CMJs, but, as opposed to the researchers that use jumps to investigate fatigue or super-compensation effects, we recommend to perform the same number of jumps and to use only the best performance.

The proposal of creating SOPs is supported in the literature by researchers who attempted to develop normative gender or country-related VJ test data (Taylor et al., 2010; Holden et al., 2015; Ramirez-Velez et al., 2017). However, considering the different factors that influence maximal jump height performance (e.g., different test methods or assessment criteria), it is unclear whether results are generally comparable with other populations. Furthermore, Claudino et al. (2017) proposed the CMJ to monitor the neuromuscular status using the average of five jump heights but different jumping methods thus compromising the possibility of comparing the VJ. The above further highlights the need for the development of SOPs, which offers researchers a more rigorous and robust test approach. Indeed, it has to be considered that the developed CMJ test protocol by Bosco et al. (1983) was used in only 5 out of the reviewed 102 original works (**Table 4**). A similar situation applies to the SJ test whereby the two developed protocols by Bosco et al. (1983) and Bosco (1994) were fully replicated in only 3 of the considered 46 works (**Table 5**).

Strengths and Limitations

The strength of this review is the stipulation of SOPs for both the CMJ and the SJ test to facilitate the evaluation of the lower limb muscular strength, in a public health context, for adolescents. If these are followed, future communications, sharing of data, result comparisons, and the development of normative data could be made easier and, importantly, these procedures should be more effective in assessing adolescents' PF. With such a vast variety of testing methods and measurement devices used, results, in fact, are not comparable which present a significant limitation of this review as it was not possible to perform a meta-analysis. The argument above is the rationale for the choice of a quantitative analysis approach of this review. Future works are recommended to review normative data using the stipulated SOPs. A second limitation of the present work is that, due to the mixed samples within the studies analyzed, gender was not considered. Future works should therefore extend their investigation to males, females, and other age groups.

CONCLUSION

The present review considered the variety of CMJ and SJ testing method procedures published in the literature, making it impossible to identify standard procedures. Consequently, SOPs for both CMJ and SJ tests have been provided in **Table 6** and these are strongly recommended to researchers and health practitioners alike. It is, however, always preferred to study the context first before proposing one protocol over another, especially in the context of sporting performance.

AUTHOR CONTRIBUTIONS

LP, APal, and AB developed the research concept and study design. LP, BK, and GM performed the literature review, and data analysis and interpretation. LP and APal performed the data collection. LP, BK, GM, APao, and GD wrote the manuscript. All authors contributed to the revision and approved the submitted version of the manuscript.

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Evaluating Individual Level Responses to Exercise for Health Outcomes in Overweight or Obese Adults

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Background: Understanding group responses to a given exercise exposure is becoming better developed; however, understanding of individual responses to specific exercise exposures is significantly underdeveloped and must advance before personalized exercise medicine can become a functional reality. Herein, utilizing data from the STRRIDE studies, we address some of the key issues surrounding our efforts to develop better understanding of individual exercise responsiveness.

Methods: We assessed individual cardiometabolic and cardiorespiratory fitness responses in subjects successfully completing STRRIDE I (n = 227) and STRRIDE II (n = 155). Subjects were previously sedentary, overweight or obese men and women with mild-to-moderate dyslipidemia. Subjects were randomized to either an inactive control group or to an exercise training program. Training groups varied to test the differential effects of exercise amount, intensity, and mode on cardiometabolic health outcomes. Measures included fasting plasma glucose, insulin, and lipids; blood pressure, minimal waist circumference, visceral adipose tissue, and peak VO_2. Absolute change scores were calculated for each subject as post-intervention minus pre-intervention values in order to evaluate the heterogeneity of health factor responsiveness to exercise training.

Results: For subjects completing one of the aerobic training programs, change in peak VO_2 ranged from a loss of 37% to a gain of 77%. When ranked by magnitude of change, we observed discordant responses among changes in peak VO_2 with changes in visceral adipose tissue, HDL-C, triglycerides, and fasting plasma insulin. There was also not a clear, direct relationship observed between magnitudes of individual response in the aforementioned variables with aerobic training adherence levels. This same pattern of highly variable and discordant responses was displayed even when considering subjects with adherence levels greater than 70%.

Conclusion: Our findings illustrate the unclear relationship between magnitude of individual response for a given outcome with training adherence and specific exercise

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exposure. These discordant and heterogeneous responses highlight the difficult nature of developing understanding for how individuals will respond to any given exposure. Further investigation into the biological, physiological, and genetics factors affecting individual responsiveness is vital to making personalized exercise medicine a reality.

Keywords: exercise prescription, lifestyle medicine, individual variation, cardiovascular health, precision medicine, training response

INTRODUCTION

Understanding the benefits of exercise at a group or population level has several purposes. At a population level – for the purposes of public health – understanding group responses and the variability around these responses provides information about cost-benefit and what health messages and initiatives should be provided to a population so as to be able to obtain the greatest benefit for the population as a whole. This is the goal of the recently released 2018 Physical Activity Guidelines for Americans (U.S Department of Health and Human Services, 2018) and the science that supports these guidelines (Physical Activity Guidelines Advisory and Committee, 2018).

Understanding the benefits of exercise at the individual level has other purposes. Assessing the heterogeneity of exercise training effects on health benefits at an individual level and being able to predict what benefits will accrue to an individual in response to a given exercise regimen may allow one to prescribe a specific program for a specific health benefit to accomplish one of the main goals of personalized exercise medicine – the right exercise regimen, for the right condition, for the right person, at the right time (Kraus, 2017). However, to use physical activity or exercise prescriptions to reduce risk for disease or treat a developing health problem, one needs to be able to predict with some precision what magnitude of response in health parameters one might expect to see at an individual level from any one of a number of different exercise regimens. We are now just beginning to develop these understandings to be able to approach this question with some precision; nonetheless, there is still some way to go (Buford et al., 2013; Ozemek and Arena, 2019).

Both intensity and amount effects of aerobic exercise contribute to improvements in cardiorespiratory fitness and in the various components of the metabolic syndrome (Duscha et al., 2005; Johnson et al., 2007; Bateman et al., 2011). At the group level, greater amounts of aerobic exercise drive improvements in HDL-cholesterol (Kraus et al., 2002), waist circumference (Slentz et al., 2004) and visceral adiposity (Slentz et al., 2005); and intensity of aerobic exercise drives reductions in fasting insulin and triglycerides in a paradoxical fashion, whereby moderate intensity has a more beneficial effect than vigorous intensity when the same amount (energy expenditure) of exercise is performed (Kraus et al., 2002; Houmard et al., 2004). As the relationship of various characteristics of exercise to health improvements is multifaceted at the group level, the heterogeneity of response at the individual level introduces even greater complexity. Within an individual, the magnitude of favorable or adverse change in any given health parameter may bear an unpredictable relationship to favorable or adverse responses to the same exercise regimen for other equally important health parameters. The purpose of this paper is to discuss these concepts using a secondary analysis of data from the STRRIDE studies (Kraus et al., 2001; Slentz et al., 2011), including our understanding of the challenges and limitations of a personalized approach to lifestyle medicine as they currently exist.

MATERIALS AND METHODS

We assessed the heterogeneity of cardiometabolic and cardiorespiratory fitness responses in data from STRRIDE I (Kraus et al., 2001) and II (Slentz et al., 2011). STRRIDE I (1999–2003) and STRRIDE II (2004–2008) enrolled previously sedentary, overweight or obese men and women with mild-to-moderate dyslipidemia (classified by low-density lipoprotein-cholesterol (LDL-C): 130–190 mg/dL or high-density lipoprotein cholesterol (HDL-C): $\leq$40 mg/dL for men and $\leq$45 mg/dL for women). In STRRIDE I, subjects were randomized into one of four groups: (1) inactive control; (2) low amount/moderate intensity aerobic exercise: 14 kcal of energy expenditure/kg of body weight/week (KKW) at 40–55% peak oxygen consumption (VO_2); (3) low amount/vigorous intensity aerobic exercise: 14 KKW at 65–80% peak VO_2; (4) high amount/vigorous intensity aerobic exercise: 23 KKW at 65–80% peak VO_2. Of the 334 subjects randomized, 227 subjects successfully completed STRRIDE I (32.0% drop out rate). In STRRIDE II, subjects were randomized to one of four groups: (1) low amount/vigorous intensity aerobic exercise: 14 KKW at 65–80% peak VO_2; (2) high amount/vigorous intensity aerobic exercise: 23 KKW at 65–80% peak VO_2; (3) resistance training only: 3 days/week, 8 exercises, 3 sets/exercise, 8–12 repetitions/set; (4) linear combination of the low amount/vigorous intensity aerobic and resistance training programs. Of the 210 subjects randomized in STRRIDE II, 155 subjects successfully completed the study (26.2% drop out rate). Both STRRIDE I and II study protocols were approved by the institutional review boards at Duke University and East Carolina University. Subjects provided both verbal and signed written informed consent.

Laboratory Measurements

Subjects underwent multiple laboratory measures at baseline and post-intervention. Body mass and height were assessed while subjects were wearing light clothing and no shoes. Blood pressure was measured at rest in a seated position. Waist circumference was measured at the minimal waist, which

is the least circumference measurement obtained above the umbilicus and below the xiphoid (Willis et al., 2007). Computed tomography scans were performed by a radiological technologist and the images were analyzed using Slice-o-matic imaging software from Tomo Vision to determine the area of visceral adipose tissue (Slentz et al., 2005).

Fasting blood samples were obtained from the beginning of an intravenous glucose tolerance test at baseline and 16–24 h after the final exercise bout. Fasting plasma glucose was determined via a YSI analyzer (Yellow Springs, OH, United States), and HDL-C as well as triglycerides were determined via nuclear magnetic resonance spectroscopy (LipoScience, Raleigh, NC, United States). Fasting plasma insulin was determined via immunoassay (Access Immunoassay System, Beckman Coulter, Fullerton, CA, United States).

Subjects completed graded maximal cardiopulmonary exercise tests on a treadmill with 12-lead electrocardiography and expired gas analysis (TrueMax Parvomedics; Provo, UT, United States). Within STRRIDE I and STRRIDE II, the same study staff members performed all tests. The graded treadmill test protocol consisted of 2-min stages, starting at 3 mph and 0% grade, then increased speed and/or grade by approximately one metabolic equivalent per stage until the subject reached volitional exhaustion. The two greatest, consecutive 15-s readings were averaged to determine peak VO_2.

Exercise Training

For the aerobic training groups, subjects underwent an initial ramp period of 2–3 months in order to allow subjects to gradually adapt to their exercise prescription. The ramp period was followed by six additional months of training at the appropriate exercise prescription. The intensity of prescribed exercise was based on the individual baseline cardiopulmonary exercise test results. Aerobic exercise modes included treadmills, elliptical trainers, cycle ergometers, or any combination of these.

For the resistance training groups, the ramp period started with one set during weeks 1–2, two sets during weeks 3–4, and built up to the three set prescription on week 5. The prescription included three sessions per week (non-consecutive days), of three sets of 8–12 repetitions on eight Cybex weight-lifting machines. The resistance exercises were designed to target all major muscle groups. To ensure a progressive resistance training stimulus throughout the intervention, the amount of weight lifted was increased by 2.75 kg each time the subject performed 12 repetitions properly on all three sets during two consecutive workout sessions.

For the aerobic training groups, exercise intensity and duration for all exercise sessions were verified by direct supervision and/or with the use of downloadable heart rate monitors (Polar Electro, Woodbury, NY, United States). Aerobic training adherence was calculated for each subject as the number of minutes completed within the prescribed heart rate range, divided by the number of total weekly minutes prescribed. Resistance training sessions were verified by direct supervision and/or the FitLinxx Strength Training Partner (FitLinxx, Norwalk, CT, United States). The "training partner" automatically sent data from each session to the FitLinxx server computer. The computers recorded total weight lifted via laser weight plate detection, and total number of repetitions (which were only counted when subjects lifted through the full range of motion), and finally speed of weight lifting motion was monitored and alerts were given when subjects lifted too quickly.

Statistical Analyses

To further illustrate the heterogeneity of cardiometabolic response to exercise intervention, we assessed both group and individual level changes in the metabolic syndrome *z*-score in the STRRIDE I cohort. As described in a previous STRRIDE study (Johnson et al., 2007), the metabolic syndrome *z*-score was used as a continuous score of the five metabolic syndrome variables. A modified *z*-score was calculated for each metabolic syndrome variable using individual subject data, the Adult Treatment Panel (ATP) III criteria (Grundy et al., 2005), and baseline standard deviations for the entire STRRIDE I cohort. To account for variations in ATP III criteria between men and women, we used sex-specific metabolic syndrome *z*-score equations. For women, metabolic syndrome *z*-score = [(50 − HDL-C)/14.1] + [(triglycerides − 150)/81.0] + [(fasting plasma glucose − 100)/11.3] + [(waist circumference − 88)/9.0] + [(mean arterial pressure − 100)/9.1]. For men, metabolic syndrome *z*-score = [(40 − HDL-C)/9.0] + [(triglycerides − 150)/81.0] + [(fasting plasma glucose − 100)/11.3] + [(waist circumference − 102)/7.7] + [(mean arterial pressure − 100)/9.1].

For all outcome variables, absolute change scores were calculated for each subject as the difference between the post- minus pre-intervention values. We also calculated percent change for peak VO_2 as the absolute change score divided by the pre-intervention value. At the group level, paired *t* tests were performed to determine significant mean changes within groups; a *p*-value of 0.05 was used to indicate statistical significance (STATVIEW, SAS, Cary, NC, United States). To illustrate the individual variability of responses to exercise training, waterfall plots were created for peak VO_2 and metabolic syndrome *z*-score, in which each bar represents an individual subject's change score.

RESULTS

Overall, subjects completing STRRIDE I (n = 227; 46.7% female; 80.6% Caucasian) were 52.4 ± 6.4 years old and had a body mass index of 29.7 ± 3.0 kg/m^2 at baseline. STRRIDE I graduates also had an average baseline peak VO_2 of 27.8 ± 5.9 mL/kg/min, mean systolic blood pressure of 128.9 ± 14.8 mmHg, mean diastolic blood pressure of 83.3 ± 8.0 mmHg, minimal waist circumference of 95.2 ± 9.7 cm, HDL-C of 45.9 ± 13.8 mg/dL, triglycerides of 152.5 ± 82.5 mg/dL, and fasting plasma glucose of 93.0 ± 10.2 mg/dL.

At baseline, STRRIDE II graduates (n = 155; 55.5% female; 84.5% Caucasian) were 48.9 ± 10.2 years old and had a body mass index of 30.5 ± 3.3 kg/m^2. In addition, STRRIDE II graduates had an average peak VO_2 of 27.4 ± 6.0 mL/kg/min, mean systolic blood pressure of 119.3 ± 13.6 mmHg, mean diastolic blood pressure of 78.1 ± 8.9 mmHg, minimal waist

circumference of 96.4 ± 9.5 cm, HDL-C of 45.3 ± 12.2 mg/dL, triglycerides of 139.7 ± 71.0 mg/dL, and fasting plasma glucose of 96.4 ± 11.8 mg/dL at baseline.

When assessing mean change at the group level in STRRIDE I (Johnson et al., 2007), both the low amount/moderate intensity and high amount/vigorous intensity groups significantly decreased their metabolic syndrome *z*-scores following 8 months of exercise training, while the inactive control and low amount/vigorous intensity groups did not significantly change compared to baseline (**Figure 1**). However, when plotting individual change scores ordered by magnitude of change, all four groups display a broad range of responses, from negative to positive (**Figure 2**). As shown in **Figure 2D**, even the group with the greatest average improvement in metabolic syndrome *z*-score (−1.5 ± 1.6 points for the high amount/vigorous intensity group) included individuals who either had no significant improvement or even an increase in their metabolic syndrome *z*-score.

We also evaluated the heterogeneity of responsiveness of peak VO_2 in both STRRIDE I and STRRIDE II. By order of magnitude, we ranked all graduates who participated in an aerobic training group by percent change peak VO_2. Across both cohorts, change in peak VO_2 ranged from a loss of 37.0% to a gain of 76.8%. We plotted these individual change scores for those with the greatest positive change in peak VO_2 (ranging from 25.2 to 76.8%; $n = 29$) with the corresponding training adherence in **Figures 3A,B**. We also plotted the individual change scores for the 29 subjects with the greatest negative to no response in peak VO_2 (ranging from −37.0 to −0.2%) and their corresponding training adherence (**Figures 3C,D**). To further explore the substantial variability in peak VO_2 response and confirm that the subjects performed ideal maximal exercise tests, we evaluated the peak respiratory exchange ratios (RER) achieved during the exercise tests to serve as an objective measure of maximal effort. For the 58 subjects listed in **Figure 3**, we found that pre-intervention peak RER values averaged 1.16 and the post-intervention peak RER values averaged 1.13. In addition, only five individual tests out of 116 tests had RER's below 1.0.

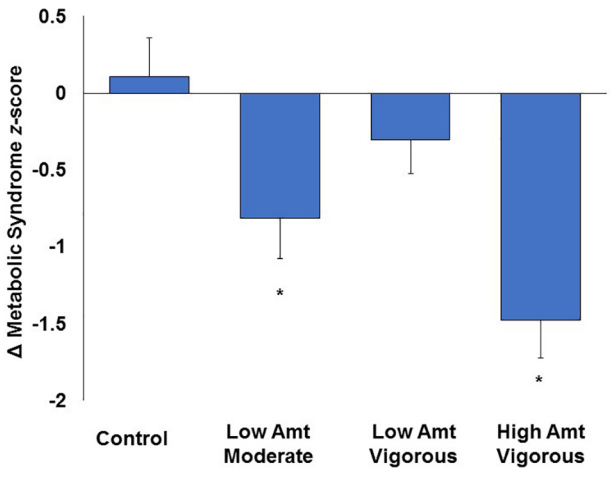

FIGURE 1 | Effects of amount and intensity of exercise on changes in metabolic syndrome *z*-score at the group level in STRRIDE I. Amt, amount; * represents $p < 0.05$ for within-group change with exercise training. Reprinted from Johnson et al. (2007) with permission from Elsevier, Copyright 2007.

In addition, we assessed these 58 subjects with the most negative and positive peak VO_2 responses by their corresponding changes in the following cardiometabolic risk factors: visceral adipose tissue, HDL-C, triglycerides, and fasting plasma insulin. In both tables, change scores that were greater than the technical variation in the test in the favorable direction are shaded green. Change scores greater than the technical variation in an unfavorable direction are shaded in red. As illustrated in **Tables 1**, **2**, there was discordance in response across all variables within individual subjects. For any given group of outcome measures, subjects having a negative response in one outcome variable did not necessarily have a negative response in the other risk factors. Taking together, the illustrations in **Figure 3** and **Tables 1**, **2**, there does not appear to be a clear, direct relationship between magnitude of individual response for a given outcome variable with aerobic training adherence nor specific training stimulus group. Even when considering only the subjects who had aerobic training adherence levels greater than 70%, the same pattern is observed: large heterogeneity and discordance of responses across a variety of important cardiometabolic health risk factors.

DISCUSSION

From our work in STRRIDE, we have several key observations bearing on the issues surrounding our efforts to develop better understanding of individual exercise responsiveness. First, hidden beneath the well-defined health benefits of exercise at the group level reported in exercise training studies – where groups may be defined as different modes, intensities, or amounts of exercise exposure – there is significant heterogeneity of response at an individual level.

Second, this heterogeneity of response is defined by the error bars routinely reported in our study figures (e.g., **Figure 1**). The heterogeneity of the response clearly can be appreciated in a series of waterfall plots of response by group (see **Figure 2** for metabolic syndrome *z*-score). The fact that this heterogeneity is intrinsic to the individual biology or physiology of an individual, and not a reflection of exposure, can be seen when the individual responses are plotted over the individual intervention adherence metrics (**Figure 3** for change in cardiorespiratory fitness, peak VO_2). Interestingly, even when we assessed the subjects with aerobic training adherence levels greater than 70%, we observed large variability and discordant responses across peak VO_2, visceral adipose tissue, HDL-C, triglycerides, and fasting insulin (**Tables 1**, **2**). At least part of the individual biology responsible for the individual heterogeneity of response is genetic (Sarzynski et al., 2016, 2017; Ross et al., 2019). In the case of change in cardiorespiratory fitness in response to aerobic exercise, approximately 50% is genetic (Timmons et al., 2010; Bouchard, 2012). Work is just now beginning to define the genetic predictors of response to other elements of the metabolic syndrome (Sarzynski et al., 2016). That said, because they do not yet replicate well across study populations,

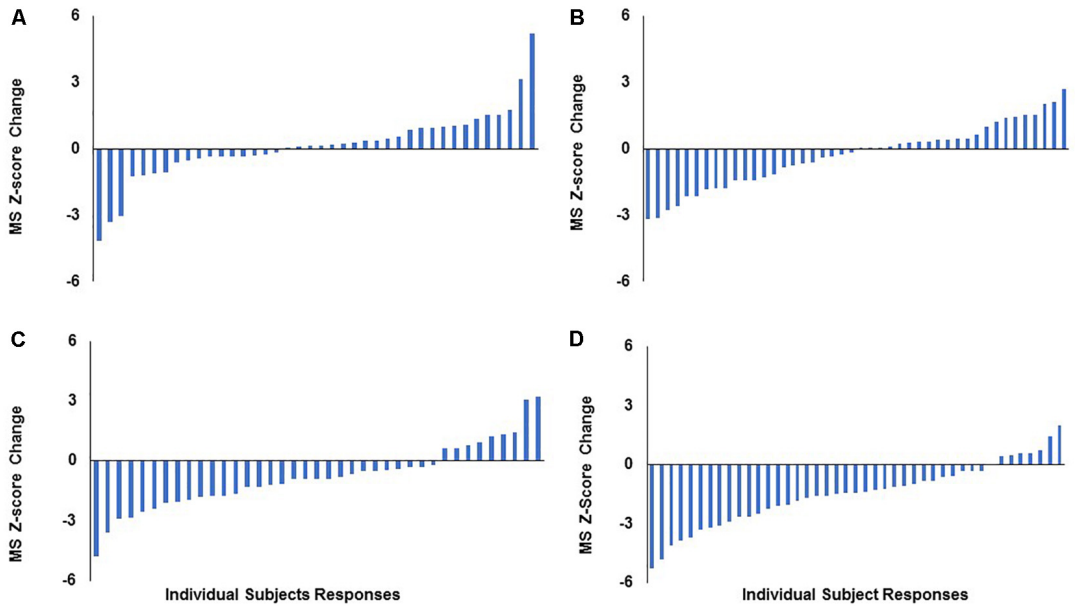

FIGURE 2 | Distribution of individual responses for change in metabolic syndrome *z*-score following 8 months of aerobic exercise training in STRRIDE I. Each bar represents an individual subject's change score. **(A)** Control group; **(B)** low-amount/vigorous-intensity group; **(C)** low-amount/moderate-intensity group; **(D)** high-amount/vigorous-intensity group. MS, metabolic syndrome.

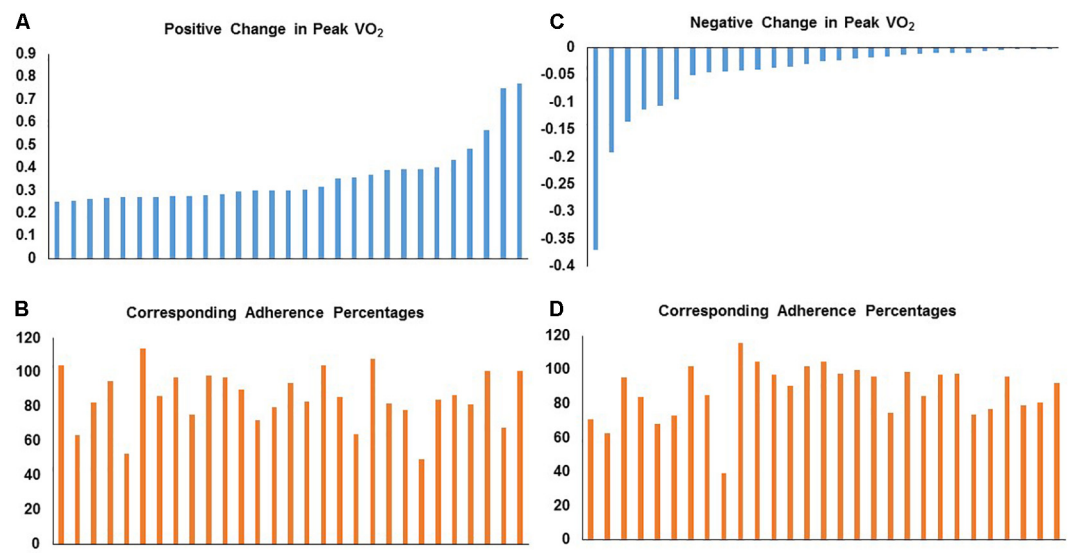

FIGURE 3 | Distribution of individual responses for fractional change in peak VO_2 with corresponding aerobic training adherence (percent) in STRRIDE I and STRRIDE II. Each bar represents an individual subject's change score or adherence level. Panel **(A)** represents individuals with the greatest positive change in peak VO_2 with their corresponding training adherence in panel **(B)**. Panel **(C)** represents individuals with the greatest negative to no response in peak VO_2 with the corresponding training adherence in panel **(D)**.

polygenic predictors of response for even change in peak VO_2 to exercise are not well developed enough to be useful clinically (Timmons et al., 2010). In fact, this is true for even the strongest polygenic risk predictors for obesity and cardiovascular disease, where the predictive capacity for the best polygenic risk for obesity predicts a mere 29.2% of cases (Emdin et al., 2017; Khera et al., 2019).

Third, this heterogeneity is expected in continuous variables and is characteristic of a Gaussian distribution of responses across a population. The tails of this distribution are defined as being outside two standard deviations of the mean response (positive or negative), where 5% of the population will be at the tails of the response. When this extreme distribution is wide, and the lower tail crosses the technical variation of the

TABLE 1 | Non-cordance of responses – negative peak VO_2 change.

Percent change in peak VO_2 (%)	Training group	Aerobic training adherence (%)	Delta VAT (cm^2)	Delta HDL-C (mg/dL)	Delta TG (mg/dL)	Delta FI (uU/mL)
−37.0	Vig/Low	71		2.9	35.9	6.0
−19.2	Mod/Low	63		8.7	−15.4	−0.7
−13.6	Vig/Low /Resistance	96	−89	10.2	−39.8	−4.1
−11.4	Vig/Low /Resistance	84		6.4	−10.6	−3.7
−10.7	Mod/Low	68	−19			0.2
−9.5	Mod/Low	73		−3.0	48.0	1.5
−5.0	Mod/Low	102	5	−1.6	3.5	−1.5
−4.6	Vig/Low	85	13	−0.3	−1.0	−5.0
−4.4	Vig/High	40	−14	0.2	15.3	−0.1
−4.2	Vig/Low	116	−23	13.2	−65.3	−1.3
−4.0	Mod/Low	105	−48	−2.0	4.0	−3.4
−3.7	Vig/Low	97	−53	17.1	106.7	−4.9
−3.5	Vig/Low	91	−46	−0.4	−31.2	−5.2
−3.1	Mod/Low	102	−69	3.1	−20.0	−6.7
−2.6	Mod/Low	105	−69	−5.8	−66.0	−3.7
−2.4	Mod/Low	98	3	−11.5	−24.0	−3.4
−2.0	Mod/Low	100	−21	−1.0	−224.0	−4.8
−1.8	Vig/Low	96	−4	2.9	−118.1	−1.3
−1.7	Mod/Low	75		−1.0	15.0	−4.1
−1.3	Mod/Low	99	23	−1.5	−38.0	−1.9
−1.2	Vig/Low /Resistance	85		−3.1	−64.0	−1.2
−1.1	Mod/Low	97	−11	−1.1	38.0	1.2
−1.0	Mod/Low	98	5	9.0	−50.0	−0.2
−0.9	Vig/Low /Resistance	74	40	12.6	37.2	−1.3
−0.6	Vig/High	77	−7	−3.9	−10.0	0.3
−0.5	Mod/Low	96	10	0.8	54.0	0.4
−0.3	Mod/Low	79		1.5	−15.2	−2.7
−0.3	Mod/Low	81		−1.0	101.0	
−0.2	Mod/Low	92	−1	−11.0	91.0	−1.2
0.6	Mod/Low	84	−1	−2.5	−30.7	−3.7
0.6	Mod/Low	85	−72	−7.0	−52.0	7.7
0.6	Mod/Low	101		3.7	−31.0	−2.0
0.7	Mod/Low	104		4.0	1.0	−0.3
0.8	Mod/Low	71	−5	−2.0	−30.2	−11.9

Each row represents an individual subject's data; Vig/Low, vigorous intensity and low amount of aerobic exercise training; Mod/Low, moderate intensity and low amount; Vig/Low/Resistance, vigorous intensity and low amount aerobic plus resistance exercise training; Vig/High, vigorous intensity and high amount; Delta VAT, change in visceral adipose tissue; Delta HDL–C, change in high-density lipoprotein-cholesterol; Delta TG, change in triglycerides; Delta FI, change in fasting insulin.

health outcome variable, it can be termed an adverse or negative response (Bouchard et al., 2012). When the intervention exposure is modest or weak, even a greater number may be characterized as non-responders; however, this is more a characteristic of the exposure, not the individual. As an example, a recent study evaluated individual patterns of fitness response in a randomized, cross-over study in 21 healthy young adults comparing two different exercise exposures: 3 weeks of endurance cycling versus 3 weeks of sprint interval cycling (Bonafiglia et al., 2016). Both training protocols elicited similar main group effects, while the individual responses varied widely and the patterns of individual response differed by protocol. Although the study assessed the initial response to short-term training, the results support the hypothesis that individuals who do not favorably respond to a given exercise exposure may undergo more beneficial fitness responses to a different exercise exposure. However, we do not know whether these results would extend to longer duration training interventions and populations with additional risk factors, like the present study includes.

Fourth, the response of a given health parameter to a given exercise regimen for any given individual does not predict the response to a related health variable to the same intervention. As illustrated in **Tables 1**, **2**, there is little correlation between cardiorespiratory fitness responses and some elements of the metabolic syndrome. For example, a high responder in cardiorespiratory fitness does not necessarily mean any given individual will be a high responder in metabolic syndrome or the constituent elements thereof (HDL-C, triglycerides, fasting insulin or visceral adiposity; **Table 2**). Similarly, a high responder in one element of the metabolic syndrome (e.g., HDL-C) is not

TABLE 2 | Non-cordance of responses – positive peak VO_2 change.

Percent change in peak VO_2 (%)	Training group	Aerobic training adherence (%)	Delta VAT (cm^2)	Delta HDL-C (mg/dL)	Delta TG (mg/dL)	Delta FI (uU/mL)
76.8	Mod/Low	101	9	−19.0	37.0	−0.6
74.7	Vig/Low /Resistance	68		−2.0	−14.1	
56.7	Vig/High	101	−42			−10.4
48.2	Vig/Low /Resistance	81	18	8.2	45.9	−6.7
43.6	Vig/Low	87	−6	1.9	19.1	−7.0
40.0	Vig/High	84		5.0	−8.0	−3.7
39.5	Vig/High	49		2.6	−42.4	−2.9
39.3	Vig/Low	78		10.5	13.0	1.3
39.0	Vig/High	82	−49	2.0	−10.0	1.7
37.0	Mod/Low	108	40			
35.5	Vig/High	64	21	−2.1	−21.7	0.3
35.1	Vig/Low /Resistance	86	44	4.3	−86.8	−5.1
31.4	Vig/Low	104	−99	7.8	−154.4	−9.2
30.2	Mod/Low	83		2.1	−20.6	1.0
30.1	Vig/High	94	−1	1.4	12.6	−3.6
29.8	Vig/High	80	5	1.0	−12.0	0.8
29.8	Vig/High	72		−5.9	7.0	0.6
29.7	Vig/High	90	30	6.5	57.9	5.7
28.3	Vig/High	97	−18	12.0	−23.0	−0.4
27.8	Mod/Low	98	−30	5.6	−81.0	3.6
27.5	Vig/Low /Resistance	76	−48	7.7	−8.5	−6.5
27.3	Vig/High	97	−58	5.0	−118.0	−0.3
27.2	Vig/Low /Resistance	57	−39	−2.9	−24.6	2.2
27.2	Mod/Low	114	−1	10.5	−27.4	−1.1
27.0	Vig/High	53		−2.2	12.7	2.7
26.6	Vig/High	95	−61	6.6	−13.1	2.3
26.1	Vig/High	83		5.0	−36.0	−3.4
25.5	Mod/Low	64		7.7	−104.0	−2.5
25.2	Mod/Low	104	−34	−4.6	−143.7	−0.3
24.7	Mod/Low	103	−29	−6.2	−68.3	0.4
24.3	Vig/High	65	−39	9.6	8.0	0.0
23.9	Mod/Low	111	−25	2.1	−92.9	0.1
23.5	Mod/Low	71	4			2.4
23.3	Mod/Low	87	−6			−0.9
23.2	Vig/Low	96	−38			

Each row represents an individual subject's data; Vig/Low, vigorous intensity and low amount of aerobic exercise training; Mod/Low, moderate intensity and low amount; Vig/Low/Resistance, vigorous intensity and low amount aerobic plus resistance exercise training; Vig/High, vigorous intensity and high amount; Delta VAT, change in visceral adipose tissue; Delta HDL-C, change in high-density lipoprotein-cholesterol; Delta TG, change in triglycerides; Delta FI, change in fasting insulin.

necessarily a high responder, or responder at all, in the other elements of the metabolic syndrome.

Fifth, there is heterogeneity in the health outcome responses to the components of exercise exposure (intensity, amount, frequency, and mode). At the group level, both intensity and amount influence improvements in cardiorespiratory fitness in response to aerobic exercise training, while intensity influences responses in fasting insulin, triglycerides, and metabolic syndrome score in an opposite direction to that of how it influences cardiorespiratory fitness: greater intensity when controlling for amount leads to greater improvements in cardiorespiratory fitness; whereas, the converse is true (less intense exercise training controlling for amount) for fasting insulin, triglycerides, and metabolic syndrome score at a group level (**Figure 1**).

Sixth, in addition to the biological and physical contributors to the heterogeneity of the response of health outcomes to exercise training, there are biological – in fact, genetic – predictors of exercise and physical activity, even in humans (de Geus et al., 2014). The identification of such genetic variants may promote better targeted and individualized messaging for individuals less likely from a constitutional standpoint to engage in favorable physical activity behaviors. For instance, using a candidate gene approach, we identified a genetic variant in the *acid ceramidase* gene predicting non-completion – or drop-out – from our STRRIDE studies (Lewis et al., 2018). As has been demonstrated for other lifestyle behaviors such as smoking and nutrition (e.g., coffee consumption) (Cornelis et al., 2011; Gustavsson et al., 2012; Shenker et al., 2013; Matoba et al., 2019), this result provides evidence for the presence of biological predictors

of exercise adherence. We are now underway in attempts to identify other genetic variants predicting exercise behavior in human STRRIDE subjects using a genome-wide approach.

Limitations

We recognize some limitations of our study. In STRRIDE I and II, subjects were allowed to perform aerobic training on a combination of aerobic equipment, while exercise testing occurred solely on a treadmill. We recognize that cross-training effects may have occurred, which can lead to bias in the exercise testing results; however, we believe the aerobic training stimuli from treadmill and elliptical trainer use, which were the primary modes of aerobic training, should yield similar peak VO_2 responses. We also recognize that the STRRIDE studies were not designed to specifically investigate individual-level exercise training responsiveness. Thus, the current paper is a secondary analysis highlighting the observed heterogeneity of responses.

CONCLUSION

There are inherent individual biological, physiological, and genetics factors involved in determining the responses in a myriad of health parameters to exercise training of a given mode, intensity and amount. Indeed, not only is there heterogeneity at the individual level to exercise-induced health responses and behaviors, there is also heterogeneity at a group level for specific health parameters to exercise programs that differ in mode, intensity and amount. All of this heterogeneity makes predicting, at an individual level, favorable responses to exercise training inherently difficult, but not impossible. Developing more information in this arena may eventually make personalized exercise medicine a reality, permitting us not only to suggest exercise programs of a given mode, intensity or amount for a particular health condition, but also to target intensive behavioral reinforcement strategies for exercise to those most likely to be poorly adherent, or who avoid exercise altogether.

DATA AVAILABILITY STATEMENT

The datasets analyzed in this manuscript are not publicly available. Requests to access the datasets should be directed to william.kraus@duke.edu.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

LR conceived the project, developed the data, and assisted in writing the manuscript. CS collected the data, synthesized the data, and assisted in editing the manuscript. WK conceived the project, obtained funding for the project, collected the data, and wrote the manuscript. All authors contributed to data analysis, interpretation of results, figure preparation, drafting and editing the manuscript, and approved the final version of the manuscript.

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Identification of Potential Performance-Related Predictors in Young Competitive Athletes

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Introduction: Systematic training is an essential demand for the individual success of an athlete. However, similar training modalities cause individual responses, and finally, decide on athletes' success or failure. To predict performance development, potential influencing parameters should be known. Therefore, the purpose of this study was to identify performance-related parameters in young competitive athletes.

Methods: Individual performance developments of 146 young athletes (m: $n = 96$, f: $n = 50$, age V1: 14.7 ± 1.7 years) of four different sports (soccer: $n = 45$, cycling: $n = 48$, swimming: $n = 18$, cross-country skiing: $n = 35$) were evaluated by analysis of 356 visits in total (exercise intervention periods, 289 ± 112 d). At V1 and V2 several performance parameters were determined. Based on the relative performance progress (Δ), potential influencing predictors were analyzed: training load, health sense, stress level, clinical complaints, hemoglobin, vitamin D, hs-CRP and EBV serostatus. Data were collected within a controlled, prospective study on young athletes, which was conducted between 2010 and 2014.

Results: Athletes improved their performance by 4.7 ± 10.7%. In total, 66.3% of all athletes represented a positive performance progress. This group demonstrated, despite similar training loads ($p = 0.207$), enhanced health senses ($p = 0.001$) and lower stress levels ($p = 0.002$). In contrast, compared to athletes with an impaired performance progress, no differences in hemoglobin values (m: $p = 0.926$, f: $p = 0.578$), vitamin D levels (0.787) and EBV serostatus ($p = 0.842$) were found. Performance progress was dependent on extents of health senses ($p = 0.040$) and stress levels ($p = 0.045$). Furthermore, the combination of declined health senses and rised stress levels was associated with an impaired performance development ($p = 0.018$) and higher prevalences of clinical complaints ($p < 0.001$) above all, in contrast to hs-CRP ($p = 0.168$).

Discussion: Athletes with an improved performance progress reported less pronounced subjective sensations and complaints. In contrast, objective known performance-related indicators, offered no differences. Therefore, subjective self-reported data, reflecting health and stress status, should be additionally considered to regulate training, modify intensities, and finally, predict and ensure an optimal performance advance.

Keywords: athlete, competitive sport, intervention, training load, health, stress, performance, immune system

INTRODUCTION

The goal of every competitive athlete is success in his sport. Athletes' aims are individually different (Bompa, 1995). Senior success requires an optimal performance development over numerous years of systematic training starting at a young age. An uncomplicated passage through this period is indispensable. Therefore, predictors of performance progress and potential risk factors, including their complex interactions, should be known and considered.

Diverse parameters define and characterize an elite athlete: e.g., sport-specific skills, physical performance, anthropometric and physiological characteristics, maturation, genetic predisposition, length of training, experience, health, and psychosocial factors (Armstrong and McManus, 2011; McManus and Armstrong, 2011). In the medical context, health plays an important role. Especially in adolescence, there are known parameters, which can negatively affect the risk of physical and psychological illness and injury: high training loads (Fleisig et al., 2011; Hjelm et al., 2012), an early specialization (Bompa, 1995; Jayanthi et al., 2013), previous illnesses, environmental factors, and negative stressors such as school problems, parental conflicts, pressure to perform, and competition failure (Cohn, 1990; Scanlan et al., 1991).

An improvement of performance can be assessed at different levels: long term until reaching top-level status, short term during training periods for season or competition preparation. Beside sport-specific skills, the development of an adequate endurance capacity is necessary to ensure resilience and to affect health, training and ability for a fast regeneration progress (Borresen and Lambert, 2009; Dhabhar, 2014). For example, road cyclists establish their base for the season in the winter months. This requires a systematic training and the knowledge of strengths, weaknesses and limitations. Inadequate strains, such as to intensive training loads, can lead to a diminished immune competence affecting recurrent infections. Ignoring medical issues can cause frequent interruptions, lack or stagnation of performance, up to retirement from competitive sports (Maffulli et al., 2010).

As part of annual systematic medical examinations, mostly organized only in adulthood, physiological conditions can be evaluated and disorders excluded. In addition, performance tests are used to determine parameters for regulating training (e.g., heart rate, lactate thresholds). Nevertheless, despite apparent similar conditions and regular participation in training, performance developments differ between athletes. In the absence of performance or appearing complaints, diagnostic evaluations are initiated, not infrequently too late or with unremarkable results. During the season frequent medical follow-up examinations are not feasible. Therefore, simple diagnostic tools are necessary to characterize athletes' status and well-being for ensuring an optimal performance development.

Based on these facts, a high performance capacity and an optimal health status are necessary requirements for a continuous progress and success. The aim is to determine valid predictors of performance development, which are easy to identify and apply in practice. High hemoglobin and vitamin D values are associated with an enhanced performance. Furthermore, hs-CRP may indicate an inflammatory process, and diminished performance and fatigue with concurrent unspecific flu-like symptoms are often combined with an Epstein Barr Virus (EBV) infection in competitive athletes (Gleeson et al., 2002; Balfour et al., 2015). Therefore, these potential predictors, in addition subjective health-related parameters, were recorded in a cohort of young competitive athletes with the purpose to determine whether these parameters can predict performance progress.

MATERIALS AND METHODS

Participants

Individual performance developments of 146 young athletes (male [m]: n = 96 [65.8%], female [f]: n = 50 [34.2%]) of four different sports (cross-country skiing [CCS]: n = 35, cycling [CYC]: n = 48, soccer [SOC]: n = 45, swimming [SWI]: n = 18) were evaluated. With the exception of soccer, in each sport both genders were considered. Every exercise intervention period counted as one assessment, hence, the analyzes included possibly several examinations of one athlete. Data with missing values and/or inadequate details in recording were excluded. In total, 356 visits (= exercise intervention periods) were analyzed (mean age at visit 1 [V1]: 14.7 $\pm$ 1.7 years, gender distribution m/f: 57.6/42.4%, CCS: n = 77 [21.6%], CYC: n = 169 [47.5%], SOC: n = 81 [22.8%], SWI: n = 29 [8.1%]).

Athletes belonged to a controlled, prospective, longitudinal study, which was conducted between 2010 and 2014 (Blume et al., 2018). For individual characterization, participants (n = 274) were examined up to three times each year (during regeneration, training, and competitive season) regarding selected parameters to determine the effects of certain stress factors (e.g., training load), plus their dynamics, on chosen outcome measures (e.g., clinical, immunological, physiological, psychological, and

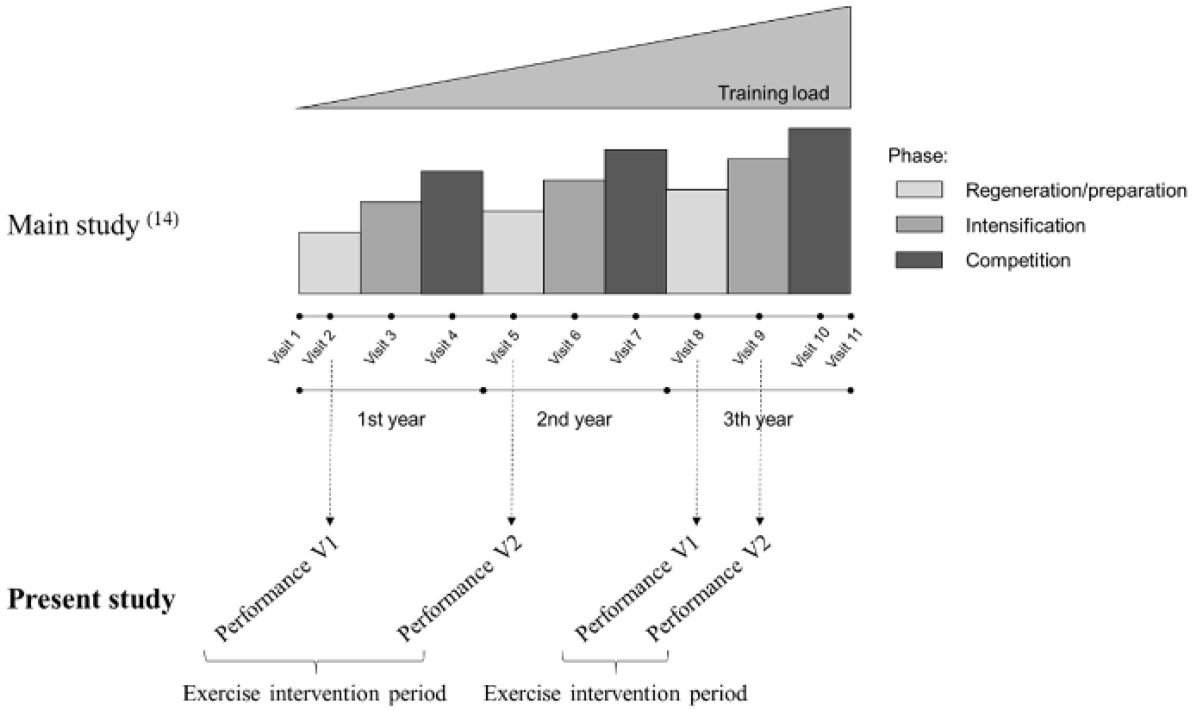

FIGURE 1 | Study timeline (main and present study).

performance end points). The main and present study timeline is shown in **Figure 1**.

Sports

For the analyzes, sports were considered, which benefit from an improved endurance performance. In addition to classic endurance sports (cross-country skiing, cycling, swimming), soccer players were referred to the analyzes due to the knowledge that an aerobic endurance training improves individuals' soccer performance (Helgerud et al., 2001). As a further study requirement, this sport is subject to a training macrocycle permitting the analyze of defined periods. Here, soccer players pass through weeks, which consist of specific endurance training. The focus of the study was the development of athletes' endurance capacity. This has only a restricted value on sport-specific performance.

Eligibility Criteria

Prior to commencement of the investigations, each athlete underwent a comprehensive clinical examination and were examined to assess inclusion (at V1 age $\leq$ 18 years, competing successfully at international or national level competitions for at least 2 years, belonging to training groups to ensure systematic training, future perspective of the athlete, written informed consent from parents and athletes) and exclusion criteria (e.g., chronic pathology and/or disability that affected their athlete's career, long-lasting injury or illness at V1). For present analyzes, exclusion criteria have been extended: different performance diagnostic tests at V1 and V2 (e.g., due to illness/injury), not reaching the lactate thresholds (e.g., lack of motivation, achieving termination criteria such as ecg abnormalities), exceeding the examination periods. All subjects were fully informed about the rationale for the study and of all procedures to be undertaken. Before study participation, athletes and their parents signed a written informed consent form. The study was approved by the Medical Research Ethics Committee (TU Müenchen) and was in agreement with the principles outlined in the Declaration of Helsinki.

Exercise Intervention Period

Once a year each athlete underwent a comprehensive sports medical examination. Depending on the kind of sport, this investigation (V1) was scheduled at the beginning of the season (CCS: June, CYC: November, SOC: August, SWI: December), and was repeated, for monitoring, after one year (V2). Between V1 and V2, at defined examination times (interval: 4 months), selected parameters, regarding training, health, stress, performance, were collected (**Figure 2**). This observation period (V1-V2, long exercise intervention time) had the duration of 365 $\pm$ 22 days [d]. In addition, for assessing e.g., performance progress during the preparation (pre-season) period, certain sports (CCS, CYC) got more than one performance test per year (CCS: October, CYC: March). These short intervention periods (136 $\pm$ 40 d) accounted for 33.4% (n = 119) of the total analysis. In summary, athletes were prospectively followed for 289 $\pm$ 112 days.

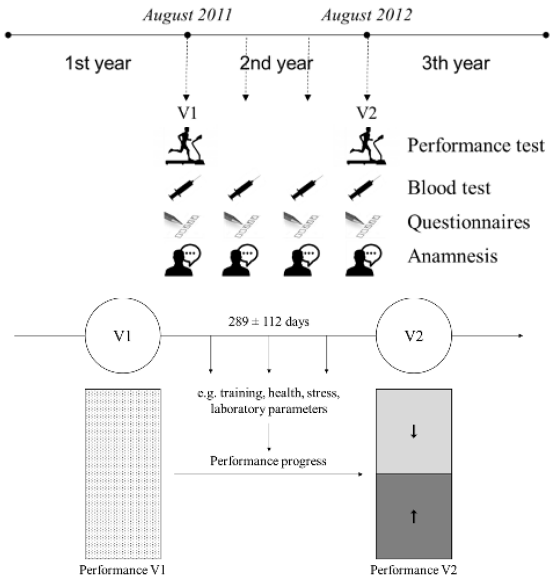

FIGURE 2 | Study design: exercise intervention period. V1, Visit 1 (e.g., *SOC: August 2011*); V2, Visit 2 (e.g., *SOC: August 2012*).

Determined Parameters

At V1, V2 and defined examination times between (**Figure 2**), selected parameters were collected. For this purpose, certain tools were used: anamnesis (medical history), questionnaires (training, health-related parameters, stress), blood draw, performance test. To evaluate the study end point (performance progress), individual performance parameters, in particular aerobic threshold, were measured, compared between V1 and V2, and the relative progress (Δ) calculated. To identify potential outcome-related predictors, selected parameters were analyzed: training hours (Th/w [h], Training hours per week), health sense (Hs [%]), stress level (Sl [%]), prevalences of recurrent infections (Ri [%]), feeling "unhealthy" (Fu [%]), fatigue (Fa [%]), and sleep disorders (Sd [%]), hemoglobin (Hb [g/dl]), high sensitivity C-reactive protein (hs-CRP [mg/dl]), vitamin D (VD [ng/ml], and Epstein-Barr-Virus serostatus (EBV [%]).

The used questionnaires were created for the study. Each athlete received a detailed verbally description.

Performance Progress (End Point)

Athletes performed before (V1) and after (V2) the exercise intervention period a standardized incremental test on a cycle ergometer (E) or a treadmill (T). Depending on gender and sport, initial load, incremental load, stage duration and incline differed. Exemplary, soccer players started the treadmill test (1% incline) with 6 kilometers per hours (km/h). Every three minutes, load was increased by 2 km/h until athletes' exertion. Beside, swimmers performed the test on a cycle ergometer with an initial load of 50 Watt (W) and an incremental load of 30 W. Test protocols were chosen depending on sport-specific demands and were predetermined by national sports federations (SOC: T, 6 km/h [=initial load], 2 km/h [=incremental load], 3 min [=stage duration], 1% [=incline]; CCS male: T, 8 km/h, 1 km/h, 3 min, 5%; CCS female: T, 6 km/h, 1 km/h, 3 min, 5%; SWI: E, 50 W, 30 W, 3 min; CYC male: E, 80 W, 20 W, 3 min; CYC female: E, 60 W, 20 W, 3 min).

Participants were instructed to avoid intensive physical training 24 h prior the test. At defined times, before, during and after the test, selected parameters were assessed: heart rate, blood pressure, blood lactate (capillary blood samples from earlobe), and rating of perceived exertion (RPE). After analyzing the lactate concentrations, lactate thresholds were calculated. To determine the individual aerobic performance (P_{aerob}), two fixed lactate thresholds were used (E: 3 mmol/l, T: 4 mmol/l), and declared as performance output, relative in watt per kilogram (E: W/kg), or rather, absolute in kilometers per hour (T: km/h) (Heck et al., 1985). Finally, individual thresholds between V1 and V2 were compared. The calculated differences were defined as performance progresses (Δ [%]) representing the study end point. For analyzes, performance development was categorized into two groups: impairment and improvement. An impairment corresponded with a difference (Δ) of 0% or lower (performance V2 = / < V1), an improvement of more than 0% (performance V2 > V1).

Potential Outcome-Related Parameters

Training hours

As part of every visit, individuals' training hours per week (average number) were recorded using standardized questionnaires, training logs and interviews. In addition to current data, the average number of training hours per week (Th/w) of the last four ones were reported. For analyzes, the mean number of Th/w, evaluated between V1 and V2, was used. Furthermore, beside metric evaluation, training hours were categorized into three groups same size for each gender (tertiles [T]).

Health sense/stress level

To evaluate subjective sensitivities, directly prior every visit, athletes were required to complete a questionnaire regarding, amongst others, subjective health sense (Hs) and stress level (Sl). The answers should reflect the condition of the last 4 weeks. Here, visual analog scales (VAS) were used, with a range between 0 and 100 percentages. High scores indicated an elevated stress level or rather an improved health sense (0%: "no stress"/"ill", 100%: "highest stress level"/"healthy"). Athletes marked subjective feelings with a cross. For analyzes, mean values of health senses and stress levels, determining during V1 and V2, were applied. In addition, both parameters were categorized into five groups reflecting ordinal gradation (1: high health sense/low stress level, 5: low health sense/high stress level). Based on that, a sum score was calculated (**Figure 3**).

Other health-related parameters

Using a questionnaire, additional health-related parameters were requested: recurrent infections/susceptibility to infections, feeling "unhealthy", fatigue, and sleep disorders. For that, athletes were asked if they felt more often sick (compared to the past/to others), felt unhealthy, suffered from fatigue (prolonged tiredness), and whether sleep disorders existed. The questions

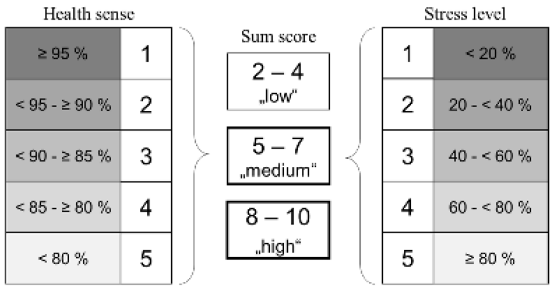

FIGURE 3 | Categorizations of health sense (Hs) and stress level (Sl) to calculate sum score.

could be answered with "*yes*", "*no*", "*I don't know*." When responding with "*yes*" at least once between V1 and V2, athletes were categorized as clinically noticeable. Finally, based on this, prevalences were determined.

Laboratory parameters

Beside subjective information, biochemical data were determined at each examination. Thus, blood samples were taken standardized. All blood collections were obtained from the antecubital vein. Regarding performance, selected parameters were selected: hemoglobin concentration ([Hb], g/dl), high sensitivity C-reactive protein (hs-CRP, mg/dl), vitamin D (VD, ng/ml) and Epstein-Barr-Virus serostatus (EBV). All values were considered and mean values calculated. In addition, prevalence of EBV positive athletes was detected [detailed methods description in previous publication (Blume et al., 2018)].

Rationales of the selected biochemical parameters:

Hemoglobin: There exists a strong relationship between hemoglobin concentration ([Hb]), hemoglobin mass (Hb_{mass}), maximal oxygen uptake (VO_{2max}) and, hence, endurance performance (Gore et al., 1997; Heinicke et al., 2001; Jacobs et al., 2011). So, studies demonstrated an increase of VO_{2max} by 3.6 ml/min per 1 g hemoglobin (Prommer et al., 2018). Furthermore, low hemoglobin concentrations indicate anemia, which can negatively affect athletes' performance (Schumacher et al., 2002; Novack et al., 2007). Because of the simple and low-cost assessment, [Hb] was used to examine its dependence on athletes' performance. It was assumed that high [Hb] values correlate with a better outcome.

High-sensitivity C-reactive protein: CRP is a general marker for inflammation and infection, moreover, increased high-sensitivity CRP (hs-CRP) values reflect a low-grade systemic inflammation (Mazurek et al., 2011). Exercise-induced muscle damage and airway inflammation, which can negatively affect athletes' training and performance, can induce an elevation of hs-CRP (Kasapis and Thompson, 2005; Ko et al., 2016). Therefore, it was assumed that high hs-CRP levels are associated with a poor performance development because of potentially underlying disorders.

Vitamin D: High vitamin D supplies are associated with an enhanced performance, furthermore, low values are discussed as a potentially limiting factor (Cannell et al., 2009; Dahlquist et al., 2015). Regarding this, vitamin D deficiency can negatively affect athletes' health (Cannell et al., 2009; Ogan and Pritchett, 2013). Therefore, we examined the association between vitamin D values and athletes' performance development.

Epstein Barr Virus: EBV is often associated with a diminished performance and fatigue in competitive athletes (Gleeson et al., 2002; Balfour et al., 2015). There has been an ongoing controversy, whether elite athletes suffer a higher rate of EBV infections, which can negatively affect athletes' health and performance (Farrokhyar et al., 2015). It was assumed that athletes with a positive EBV serostatus showed lower performance developments.

Statistical Analyzes

The data were compiled using Microsoft Excel® and evaluated using the SPSS® software package (version 25.0; SPSS Lead Technologies Inc., Chicago, IL, United States). Frequency distributions of all continuous variables were examined to detect outlying values, and the Kolmogorov-Smirnov test was used to check the normal distribution of variables. All results, assuming normal distribution, were presented as mean ± standard deviation (SD). Differences between groups were analyzed using an independent samples *t*-test. To determine the differences in the group analysis, ANOVA was used. The chi-square test was performed to verify possible differences between nominal scaled variables. Significance was accepted at the $p < 0.05$ level. Depending on the analysis, data were stratified by gender and sport, were presented by tertiles, and variables were categorized in ordinal gradation. All tables and graphics were generated with Microsoft Excel® and SPSS®.

RESULTS

Basic Characteristics

Participants

Individual performance developments of 146 young athletes (m: $n = 96$ [65.8%], f: $n = 50$ [34.2%]) of four different sports (SOC: $n = 45$, CYC: $n = 48$, SWI: $n = 18$, CCS: $n = 35$) were evaluated. In total, 356 exercise intervention periods were considered (**Table 1**). At V1 adolescent competitive athletes showed a mean age of 14.7 ± 1.7 years (m: 14.2 ± 1.7 years, f: 15.4 ± 1.4 years, $p < 0.001$), at V2 of 15.5 ± 1.8 years. Athletes were prospectively followed for 289 ± 112 days.

Performance Development

In summary, athletes achieved a mean performance development of 4.7 ± 10.7%. Here, no gender differences were found (m: 5.3 ± 11.0%, f: 3.9 ± 10.3%, $p = 0.238$). Performance advances differed between the four sports ($p = 0.001$, **Table 1**). The highest relative performance progress reached soccer players (7.0 ± 10.0%), followed by male (6.0 ± 11.4%) and female cyclists

TABLE 1 | Basic characteristics of the total athletes' collective and dependent on sport and gender.

		n (%)	Age V1 [yrs]	Th/w [h]	Duration [d]	P_{aerob} V1	P_{aerob} V2	Δ [%]
Total		356 (100)	14.7 ± 1.7	12.4 ± 4.6	289 ± 112			
	m	205 (57.6)	14.2 ± 1.7	11.2 ± 4.3	313 ± 98			
	f	151 (42.4)	15.4 ± 1.4	13.9 ± 4.6	256 ± 123			
	p^1		<0.001**	<0.001**	<0.001**			
SOC	m	81 (22.8)	13.8 ± 1.4	9.6 ± 2.8	362 ± 19	13.2 ± 1.4 km/h	14.1 ± 1.1 km/h	7.0 ± 10.0
CYC	m	71 (19.9)	13.6 ± 2.0	12.2 ± 3.8	245 ± 118	3.7 ± 0.5 W	3.9 ± 0.5 W	6.0 ± 11.4
	f	98 (27.5)	15.2 ± 1.4	14.2 ± 4.5	233 ± 123	3.1 ± 0.5 W	3.3 ± 0.5 W	5.6 ± 11.6
	p^1		<0.001**	=0.005*	$=0.529^{ns}$	<0.001**	<0.001**	$=0.834^{ns}$
SWI	m	16 (4.5)	14.7 ± 1.6	14.2 ± 8.1	373 ± 24	2.8 ± 0.5 W	2.8 ± 0.6 W	1.1 ± 18.3
	f	13 (3.7)	15.1 ± 1.0	17.0 ± 4.5	377 ± 16	2.7 ± 0.5 W	2.7 ± 0.5 W	0.9 ± 8.1
	p^1		$= 0.443^{ns}$	$=0.276^{ns}$	$=0.652^{ns}$	$=0.485^{ns}$	$=0.565^{ns}$	$=0.968^{ns}$
CCS	m	37 (10.4)	15.7 ± 1.2	10.6 ± 3.7	309 ± 99	12.5 ± 1.0 km/h	12.7 ± 1.0 km/h	2.2 ± 6.8
	f	40 (11.2)	16.1 ± 1.4	11.7 ± 3.7	273 ± 117	11.1 ± 0.6 km/h	11.2 ± 0.8 km/h	0.9 ± 5.8
	p^1		$=0.153^{ns}$	$=0.295^{ns}$	$=0.152^{ns}$	<0.001**	<0.001**	$=0.388^{ns}$
p^2			<0.001**	<0.001**	<0.001**			=0.001*

V1, visit 1; V2, visit 2; SOC, soccer; CYC, cycling; SWI, swimming; CCS, cross-country skiing; m, male; f, female; yrs, years; Th/w, training hours per week; h, hours; d, days; P_{aerob}, aerobic performance on lactate threshold of 3 mmol/l (E) or rather 4 mmol/l (T); Δ = delta P_{aerob} V1 vs. P_{aerob} V2; km/h, kilometers per hour; W, watt. Data are shown as mean ± SD and percentage values. p^1, significance between genders; p^2, significance between sports; $^{**}p < 0.001$, $^{*}p < 0.01$, $^{\#}p < 0.05$, $^{ns}p \geq 0.05$ (non-significant).

(5.6 ± 11.6%). **Table 1** represents all performance developments subdividing in dependence of sport and gender. 66.3% ($n = 236$) of all athletes showed an improvement of performance (Δ > 0%), 33.1% ($n = 118$) of at least 7.5%.

Potential Outcome-Related Predictors

Female athletes, compared to males, showed higher training loads per week (13.9 ± 4.6 h vs. 11.2 ± 4.5 h, $p < 0.001$), an impaired health sense (83.5 ± 11.1% vs. 87.5 ± 10.0%, $p = 0.001$), and elevated stress levels (50.1 ± 24.0% vs. 42.0 ± 23.1%, $p = 0.003$). During the intervention period, 15.0% complained recurrent infections, one in five athletes (20.7%) felt "unhealthy", and 12.3% reported fatigue. Only 2.7% of all participants referred sleep disorders. Thereby, no gender differences were found. Male athletes possessed a mean hemoglobin value of 14.6 ± 0.9 g/dl, females of 13.5 ± 0.9 g/dl ($p < 0.001$). Almost two-thirds (64.0%) of all athletes was detected as EBV-positive, with a similar distribution between both genders (m: 65.9%, f: 61.6%, $p = 0.407$). Also, no gender differences in vitamin D levels (m: 33.2 ± 9.7 ng/ml, f: 37.0 ± 12.5 ng/ml, $p = 0.168$) and hs-CRP values (m: 0.096 ± 0.217 mg/dl, f: 0.077 ± 0.149 mg/dl, $p = 0.452$) were found. All potential outcome-related predictors dependent on sports are demonstrated in **Table 2**.

Performance Improvement vs. Impairment

Training Hours, Sports

Athletes with a positive performance advance (Δ > 0%) trained similar loads weekly compared to unsuccessful participants during the intervention period (Δ > 0%: 12.1 ± 4.7 h, Δ ≤ 0%: 12.9 ± 4.5 h, $p = 0.207$, **Table 3**). Furthermore, soccer players offered the lowest number of training hours (9.6 ± 2.8 h, $p < 0.001$, **Table 2**), but showed the highest performance development (7.0 ± 10.0%, $p = 0.001$, **Table 1**). 76.5% of all soccer players demonstrated a positive performance advance (CYC: 68.6%, SWI: 55.5%, CCS: 54.5%, $p = 0.014$), 44.4% a development of minimum 7.5% (CYC: 40.8%, SWI: 31.0%, CCS: 14.3%, $p = 0.001$). The parameter training hours showed sex-specific differences (Th: m: 11.2 ± 4.3 h, f: 13.9 ± 4.6 h, $p < 0.001$), therefore, this variable was categorized into three groups same size individually for each gender (m: Th1: ≤ 9 h, Th2: 9.1–12.7 h, Th3: ≥ 12.8 h; f: Th1: ≤ 12.5 h, Th2: 12.6–15.4 h, Th3: ≥ 15.5 h). Regarding this, performance development was unaffected by training load (m: Th1: 4.2 ± 9.7%, Th2: 5.8 ± 10.9%, Th3: 4.7 ± 12.3%, $p = 0.748$; f: Th1: 4.8 ± 10.6%, Th2: 6.2 ± 10.9%, Th3: 1.2 ± 10.4%, $p = 0.092$, **Table 4**).

Subjective Health-Related Variables

Athletes with a positive performance development reported higher health sense levels during the exercise intervention period compared to the group with an impairment (Δ > 0%: 87.2 ± 10.4%, Δ ≤ 0%: 83.3 ± 10.7%, $p = 0.001$, **Table 3**). This result was confirmed for cyclists (Δ > 0%: 86.1 ± 10.3%, Δ ≤ 0%: 82.1 ± 12.5%, $p = 0.040$, **Figure 4**). Similar tendencies were evident in the other groups, but without significance (SOC: $p = 0.185$, SWI: $p = 0.247$, CCS: $p = 0.321$, **Figure 4**). Health sense differences were detectable in both genders, particularly significant among female athletes (m: Δ > 0%: 88.4 ± 10.4%, Δ ≤ 0%: 85.4 ± 8.8%, $p = 0.056$; f: Δ > 0%: 85.3 ± 10.1%, Δ ≤ 0%: 80.7 ± 12.2%, $p = 0.023$). Furthermore, athletes with an improvement of at least 7.5% offered the highest subjective health senses (Δ ≥ 7.5%: 88.0 ± 10.5%, Δ 0 to <7.5%: 86.2 ± 10.2%, Δ < 0%: 83.3 ± 10.7%, $p = 0.004$, **Figure 5**). Notably, also significant differences among male athletes were demonstrated (Δ ≥ 7.5%: 89.8 ± 10.1%, Δ 0 to < 7.5%: 86.8 ± 10.5%, Δ < 0%: 85.3 ± 8.8%, $p = 0.034$). For further analyzes, health sense levels were divided into five groups. Here, performance

TABLE 2 | Potential outcome-related predictors (training, subjective health-related, and biochemical parameters, stress) dependent on sport.

		SOC	CYC	SWI	CCS	*P*
Training hours (Th/w)		9.6 ± 2.8	13.3 ± 4.3	15.4 ± 6.8	11.1 ± 3.7	<0.001**
Stress level (%)		42.0 ± 23.2	46.0 ± 24.8	49.1 ± 22.8	45.9 ± 22.3	=0.491^{ns}
Subjective health variables						
Health sense (%)		89.3 ± 9.4	84.8 ± 11.2	86.0 ± 9.0	84.2 ± 10.7	=0.010$^{\#}$
Recurrent infections (%)		15.1	12.7	10.3	22.4	=0.263^{ns}
Feeling "unhealthy" (%)		15.4	21.2	24.1	24.6	=0.545^{ns}
Fatigue (%)		15.6	9.9	21.4	9.1	=0.241^{ns}
Sleep disorders (%)		3.9	2.1	/	3.6	=0.668^{ns}
Biochemical markers						
Hemoglobin (g/dl)	m	14.3 ± 0.8	14.7 ± 1.0	14.6 ± 0.9	15.1 ± 0.7	<0.001**
	f	/	13.4 ± 0.7	13.3 ± 1.1	13.9 ± 1.0	=0.016$^{\#}$
hs-CRP (mg/dl)		0.094 ± 0.177	0.075 ± 0.140	0.065 ± 0.062	0.112 ± 0.292	=0.601^{ns}
Vitamin D (ng/ml)		40.3 ± 8.9	31.4 ± 8.5	31.4 ± 16.5	38.0 ± 8.6	=0.015$^{\#}$
EBV-positive *n* (%)		71.6	46.7	75.9	89.6	<0.001**

*SOC, soccer; CYC, cycling; SWI, swimming; CCS, cross-country skiing; m, male; f, female; Th/w, training hours per week. Data are shown as mean ±SD and percentage values. p, significance between sports; **$p < 0.001$, $^{\#}p < 0.05$, $^{ns}p \geq 0.05$ (non-significant).*

developments increased with elevating health sense categorizes ($p = 0.040$, **Table 4**).

Compared to the group with an improved performance development, almost twice as many athletes reported a "unhealthy" feeling ($\Delta > 0\%$: 15.7%, $\Delta \leq 0\%$: 30.3%, $p = 0.002$, **Table 3**, **Figure 6**). This difference was replicable among female athletes ($\Delta > 0\%$: 12.5%, $\Delta \leq 0\%$: 35.3%, $p = 0.002$), in the male collective without significance ($\Delta > 0\%$: 17.7%, $\Delta \leq 0\%$: 25.9%, $p = 0.198$).

Considering the prevalences of recurrent infections and fatigue, there were noticeably differences between the performance progress groups. So, athletes with an improvement reported fewer recurrent infections (13.7% vs. 17.4%) and less fatigue (9.7% vs. 17.0%), but without significance (Ri: $p = 0.379$, Fa: $p = 0.068$, **Table 3**). However, in the group of cyclist a significant difference of reported recurrent infections was found ($\Delta > 0\%$: 8.4%, $\Delta \leq 0\%$: 22.0%, $p = 0.017$). In contrast, there was no relationship between the outcome performance advance and the parameter sleep evident (**Table 3**).

TABLE 3 | Potential outcome-related predictors (training, subjective health-related parameters, biochemical markers, stress) dependent on performance development.

Aerobic performance		Δ ≤ 0% Impairment	Δ > 0% Improvement	*p*
n (%)		120 (33.7)	236 (66.3)	
Training hours (Th/w)		12.9 ± 4.5	12.1 ± 4.7	=0.207^{ns}
Health sense (%)		83.3 ± 10.7	87.2 ± 10.4	=0.001*
Recurrent infections *n* (%)		20 (17.4)	29 (13.7)	=0.379^{ns}
Feeling "unhealthy" *n* (%)		33 (30.3)	33 (15.7)	=0.002*
Fatigue *n* (%)		18 (17.0)	19 (9.7)	=0.068^{ns}
Sleep disorders *n* (%)		2 (1.9)	6 (3.1)	=0.535^{ns}
Stress level (%)		50.8 ± 22.4	42.3 ± 24.0	=0.002*
Hemoglobin (g/dl)	m	14.6 ± 0.8	14.6 ± 1.0	=0.926^{ns}
	f	13.5 ± 0.9	13.6 ± 0.9	=0.578^{ns}
hs-CRP (mg/dl)		0.124 ± 0.284	0.072 ± 0.132	=0.043$^{\#}$
Vitamin D (ng/ml)		33.9 ± 12.0	34.6 ± 9.9	=0.787^{ns}
EBV-positive *n* (%)		76 (63.3)	152 (64.4)	=0.842^{ns}

*Collective: total cohort. Δ = delta P_{aerob} V1 vs. P_{aerob} V2; Th/w, training hours per week. Data are shown as mean ± SD and percentage values. **$p < 0.001$, *$p < 0.01$, $^{\#}p < 0.05$, $^{ns}p \geq 0.05$ (non-significant).*

Stress Level

Athletes with an impaired performance development felt more stressed during the exercise intervention period compared to them with a positive advance ($\Delta > 0\%$: 42.3 ± 24.0%, $\Delta \leq 0\%$: 50.8 ± 22.4%, $p = 0.002$, **Table 3**). This result was confirmed for soccer players ($\Delta > 0\%$: 39.0 ± 23.0%, $\Delta \leq 0\%$: 51.1 ± 22.1%, $p = 0.048$, **Figure 4**). Similar tendencies were evident in the other groups, but without significance (CYC: $p = 0.063$, SWI: $p = 0.956$, CCS: $p = 0.119$, **Figure 4**). Furthermore, with increase in performance, a less extent of stress levels was observed ($\Delta \geq 7.5\%$: 40.0 ± 23.1%, Δ 0 to <7.5%: 45.1 ± 24.5%, $\Delta < 0\%$: 51.0 ± 22.6%, $p = 0.002$, **Figure 5**). After stress level categorization into five groups, an increasing performance development with declined stress levels were observed ($p = 0.045$, **Table 4**, **Figure 7**). Here, an improved performance development prevalence ($\Delta > 0\%$) was found comparing the highest and lowest stress level category with each other ($p = 0.014$, **Figure 7**).

Biochemical Markers

There were no differences of hemoglobin values between athletes with performance improvements and impairments (m: $\Delta > 0\%$: 14.6 ± 1.0 g/dl, $\Delta \leq 0\%$: 14.6 ± 0.8 g/dl, $p = 0.926$; f: $\Delta > 0\%$: 13.6 ± 0.9 g/dl, $\Delta \leq 0\%$: 13.5 ± 0.9 g/dl, $p = 0.578$, **Table 3**), in all sports (SOC: $p = 0.086$; CYC: m: $p = 0.060$, f: $p = 0.363$; SWI: m: $p = 0.600$, f: $p = 0.933$; CCS: m: $p = 0.211$, f: $p = 0.571$). Furthermore, in both genders, also a high performance progress was not associated with higher hemoglobin values (m: $\Delta \geq 7.5\%$: 14.6 ± 0.9 g/dl, Δ 0 to < 7.5%: 14.7 ± 1.0 g/dl, $\Delta < 0\%$: 14.6 ± 0.8 g/dl, $p = 0.910$; f: $\Delta \geq 7.5\%$: 13.5 ± 0.9 g/dl, Δ 0 to < 7.5%: 13.7 ± 0.9 g/dl, $\Delta < 0\%$: 13.5 ± 0.9 g/dl, $p = 0.827$).

TABLE 4 | Performance development dependent on potential outcome-related predictors (stress, health sense, training hours, subjective health-related parameters, biochemical markers).

Health sense (%)	≥ 95	≥90 to <95	≥ 85 to <90	≥80 to <85	<80	
Performance Δ (%)	7.6 ± 10.3	4.8 ± 12.7	5.5 ± 10.2	2.9 ± 8.8	2.4 ± 11.2	$=0.040^{\#}$
Stress level (%)	< 20	20 to <40	40 to <60	60 to <80	≥80	
Performance Δ (%)	7.1 ± 9.4	7.0 ± 11.0	3.3 ± 8.8	3.7 ± 11.5	1.8 ± 14.8	$=0.045^{\#}$
Hemoglobin (g/dl)	m: ≥15.0		m: 14.3 to 14.9		m: ≤14.2	
	f: ≥13.9		f: 13.3 to 13.8		f: ≤13.2	
Performance Δ (%)	5.1 ± 10.8		4.9 ± 12.8		4.2 ± 8.6	$=0.772^{ns}$
hs-CRP (mg/dl)	≤0.026		0.027 to 0.058		≥0.059	
Performance Δ (%)	6.1 ± 8.8		5.3 ± 10.0		4.5 ± 11.8	$=0.588^{ns}$
Vitamin D (ng/ml)	> 20				≤20	
Performance Δ (%)	4.9 ± 11.6				5.4 ± 23.7	$=0.578^{ns}$
EBV serostatus	Negative				Positive	
Performance Δ (%)	5.4 ± 12.1				4.3 ± 9.8	$=0.374^{ns}$
Training hours (Th/w)	m: ≤9.0		m: 9.1 to 12.7		m: ≥12.8	
	f: ≤12.5		f: 12.6 to 15.4		f: ≥15.5	
Performance Δ (%)	m: 4.2 ± 9.7		m: 5.8 ± 10.9		m: 4.7 ± 12.3	$=0.748^{ns}$
	f: 4.8 ± 10.6		f: 6.2 ± 10.9		f: 1.2 ± 10.4	$=0.092^{ns}$

m, male; f, female; Th/w, training hours per week; Δ = delta P_{aerob} V1 vs. P_{aerob} V2. Data are shown as mean ± SD and percentage values. $^{\#}p < 0.05$, $^{ns}p \geq 0.05$ (non-significant).

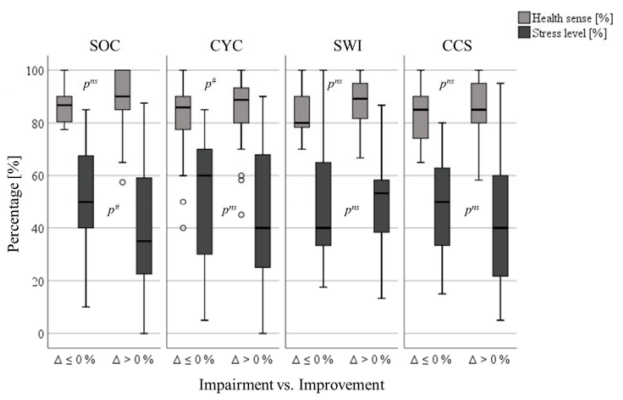

FIGURE 4 | Sport-specific health senses and stress levels dependent on performance development. Δ = delta P_{aerob} V1 vs. P_{aerob} V2; SOC, soccer; CYC, cycling; SWI, swimming; CCS, cross-country skiing. $^{\#}p < 0.05$, $^{ns}p \geq 0.05$ (non-significant).

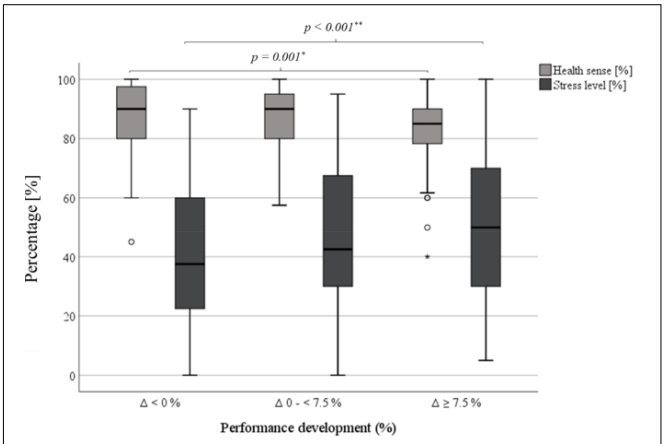

FIGURE 5 | Health senses and stress levels dependent on performance development (total cohort). Δ = delta P_{aerob} V1 vs. P_{aerob} V2. $^{**}p < 0.001$, $^{*}p < 0.01$.

For further analyzes, hemoglobin values were divided into three groups (ordinal gradation). Here, performance advances were similar between the groups ($p = 0.772$, **Table 4**).

Also, vitamin D values (Δ > 0%: 34.6 ± 9.9 ng/ml, Δ ≤ 0%: 33.9 ± 12.0 ng/ml, $p = 0.787$, **Table 3**) and EBV-serostatus (Δ > 0%: 64.4%, Δ ≤ 0%: 63.3%, $p = 0.842$, **Table 3**) showed no noticeably differences between the performance development groups in the total collective. As an exception, cross-country skiers with a performance improvement offered higher vitamin D values (Δ > 0%: 46.1 ± 3.5 ng/ml, Δ ≤ 0%: 33.2 ± 6.7 ng/ml, $p = 0.022$). This result was not found in the other groups (SOC: $p = 0.510$, CYC: $p = 0.445$, SWI: $p = 0.910$). Vitamin D values below 20 ng/ml are defined as deficiency. Based on this cut-off value, 8.2% of all athletes offered too low levels. However, this group showed no impaired performance progress (VD ≤ 20 ng/ml: 5.4 ± 23.7%, VD > 20 ng/ml: 4.9 ± 11.6%, $p = 0.578$, **Table 4**). EBV-positive athletes improved their performance by 4.3 ± 9.8% (m: 4.9 ± 10.4%, f: 3.6 ± 8.9%), seronegative youths by 5.4 ± 12.1% (m: 6.1 ± 12.0%, f: 4.5 ± 12.3%), in any case without significance (total: $p = 0.374$, m: $p = 0.426$, f: $p = 0.608$, **Table 4**).

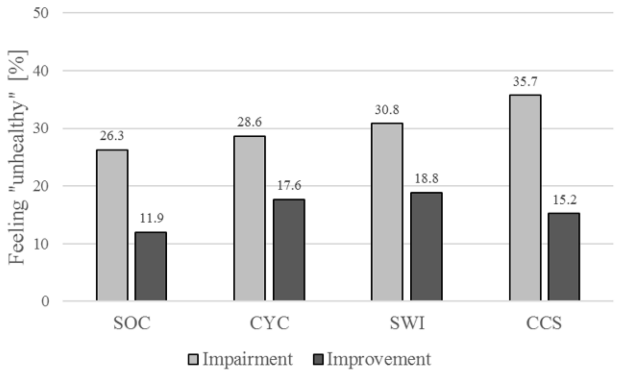

FIGURE 6 | The subjective health-related parameter feeling "unhealthy" dependent on performance development and sport. SOC, soccer; CYC, cycling; SWI, swimming; CCS, cross-country skiing.

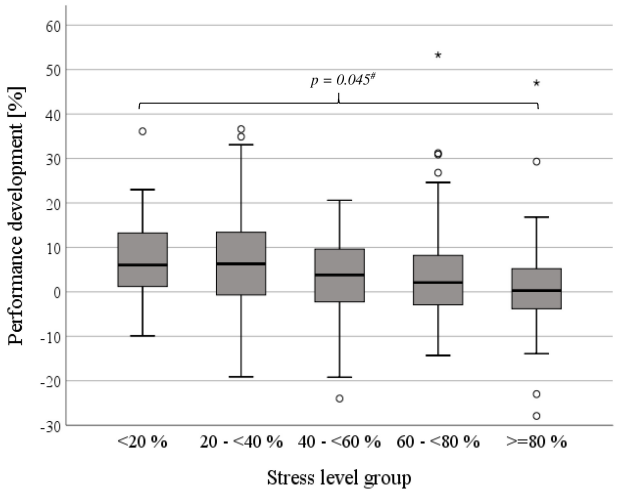

FIGURE 7 | Performance development dependent on stress level group. #$p < 0.05$.

In athletes with an improvement of performance lower hs-CRP values were measured ($\Delta > 0\%$: 0.072 ± 0.132 mg/dl, $\Delta \leq 0\%$: 0.124 ± 0.284 mg/dl, $p = 0.043$, **Table 3**). This result was found in the group of soccer players ($\Delta > 0\%$: 0.064 ± 0.097 mg/dl, $\Delta \leq 0\%$: 0.204 ± 0.314 mg/dl, $p = 0.003$), but not in the other sports (CYC: $p = 0.488$, SWI: $p = 0.261$, CCS: $p = 0.328$). However, there was no dependence regarding the extent of the positive performance progress ($\Delta \geq 7.5\%$: 0.082 ± 0.164 mg/dl, Δ 0 to $< 7.5\%$: 0.061 ± 0.085 mg/dl, $\Delta < 0\%$: 0.125 ± 0.286 mg/dl, $p = 0.090$). For further analyzes, the values of hs-CRP, in ascending order, were categorized into three groups. There were no effects on performance progress, neither after comparison of all three groups (hs-CRP1: 6.1 ± 8.8%, hs-CRP2: 5.3 ± 10.0%, hs-CRP3: 4.5 ± 11.8%, $p = 0.588$, **Table 4**), nor between group hs-CRP1 and hs-CRP3 ($p = 0.310$).

Interaction Between Training Hours, Health Senses, Stress Levels, Other Health-Related Parameters and Biochemical Markers

Decreased subjective health senses were associated with elevated stress levels (Hs < 80%: 54.4 ± 19.9%, Hs ≥ 95%: 33.4 ± 25.2%, $p < 0.001$). Also, an impaired health sense, in particular values below 80%, was combined with higher prevalences of recurrent infections (Hs < 80%: 36%, Hs ≥ 95%: 2.7%, $p < 0.001$), fatigue (Hs < 80%: 28.8%, Hs ≥ 95%: 2.8%, $p < 0.001$), and the feeling "unhealthy" (Hs < 80%: 61.9%, Hs ≥ 95%: 0%, $p < 0.001$). In contrast, there were no significant differences of hs-CRP values (Hs < 80%: 0.143 ± 0.343 mg/dl, Hs ≥ 95%: 0.087 ± 0.185 mg/dl, $p = 0.168$) and vitamin D levels (Hs < 80%: 31.9 ± 12.4 ng/ml, Hs ≥ 95%: 31.4 ± 9.2 ng/ml, $p = 0.067$) between the groups. Beside health senses, stress levels showed no associations to the other health-related parameters (Ri: $p = 0.295$; Fu: $p = 0.139$; Fa: $p = 0.145$). However, just stress levels of less than 20% were linked with moderate prevalences (Ri: Sl < 20%: 9.3%, Sl ≥ 80%: 19.2%; Fu: Sl < 20%: 9.3%, Sl ≥ 80%: 22.2%; Fa: Sl < 20%: 7.7%, Sl ≥ 80%: 11.1%). The parameter training hours showed sex-specific differences (Th: m: 11.2 ± 4.3 h, f: 13.9 ± 4.6 h, $p < 0.001$), therefore, this parameter was categorized into three groups same size individually for each gender (m: Th1: ≤ 9 h, Th2: 9.1–12.7 h, Th3: ≥ 12.8 h; f: Th1: ≤ 12.5 h, Th2: 12.6 – 15.4 h, Th3: ≥ 15.5 h). Regarding these classifications, neither stress levels (m: Tl1: 39.8 ± 24.9%, Tl2: 47.7 ± 22.0%, Tl3: 42.2 ± 19.6%, $p = 0.187$; f: Tl1: 49.0 ± 20.9%, Tl2: 50.9 ± 24.6%, Tl3: 52.0 ± 26.6%, $p = 0.850$) nor health senses (m: Tl1: 88.5 ± 9.9%, Tl2: 84.7 ± 10.2%, Tl3: 88.4 ± 8.6%, $p = 0.074$; f: Tl1: 82.8 ± 9.3%, Tl2: 86.3 ± 11.1%, Tl3: 83.6 ± 10.1%, $p = 0.308$) were dependent on extent of training load. Also, there were no prevalence differences of recurrent infections (m: Th1: 11.3%, Th2: 18.4%, Th3: 7.3%, $p = 0.216$; f: Th1: 11.4%, Th2: 18.9%, Th3: 29.5%, $p = 0.101$), feeling "unhealthy" (m: Th1: 20.7%, Th2: 27.7%, Th3: 18.2%, $p = 0.495$; f: Th1: 17.1%, Th2: 22.9%, Th3: 23.3%, $p = 0.746$), and fatigue (m: Th1: 14.5%, Th2: 8.9%, Th3: 18.4%, $p = 0.416$; f: Th1: 8.1%, Th2: 17.6%, Th3: 11.6%, $p = 0.467$).

Sum Score Health Sense/Stress Level

Because of their significant impact on performance development and their interaction each other, a sum score of health sense and stress level was formed. This score was based on the group numbers (1–5) with a range between 2 and 10 (**Figure 3**). Considering the sum score values, three groups were categorized: "low" (sum 2–4, high health sense/low stress level), "medium" (sum 5–7), and "high" risk (sum 8–10, low health sense/high stress level). The "low" risk group possessed a mean health sense of 95.1 ± 4.5% and a mean stress level of 22.4 ± 14.0%, the "high" risk group of 74.3 ± 7.3%, respectively 66.7 ± 12.3% (**Table 5**). In summary, 44.5% of all athletes demonstrated scores between 5 and 7 ("medium" risk), almost one quarter (23.7%) belonged to the "high" risk group. The distribution pattern differed significantly between

TABLE 5 | Potential outcome-related predictors (training, stress, subjective health-related parameters) and performance development dependent on sum of health sense and stress level group (sum 2–10).

Health sense + Stress level (2–10)	2, 3, 4 "low"	5, 6, 7 "medium"	8, 9, 10 "high"	p^a	p^b
%	31.9	44.5	23.7		
Training load (Th/w)	12.2 ± 5.0	12.8 ± 4.3	12.0 ± 4.7	$=0.433^{ns}$	$=0.858^{ns}$
Health sense (%)	95.1 ± 4.5	85.5 ± 8.8	74.3 ± 7.3	<0.001**	<0.001**
Stress level (%)	22.4 ± 14.0	50.1 ± 20.1	66.7 ± 12.3	<0.001**	<0.001**
Recurrent infections (%)	5.2	16.8	26.0	=0.001*	<0.001**
Feeling "unhealthy" (%)	5.0	17.0	48.0	<0.001**	<0.001**
Fatigue (%)	2.2	13.1	23.6	<0.001**	<0.001**
Sleep disorders (%)	2.2	2.2	4.2	$=0.651^{ns}$	$=0.459^{ns}$
Performance development (%)	6.9 ± 10.9	4.7 ± 10.8	2.1 ± 10.9	$=0.015^{\#}$	=0.004*

*Th/w, training hours per week. Data are shown as mean ± SD and percentage values. [a]significance between all three groups, [b]significance between group "low" (sum 2–4) and "high" (sum 8–10) risk. **$p < 0.001$, *$p < 0.01$, $^{\#}p < 0.05$, $^{ns}p \geq 0.05$ (non-significant).*

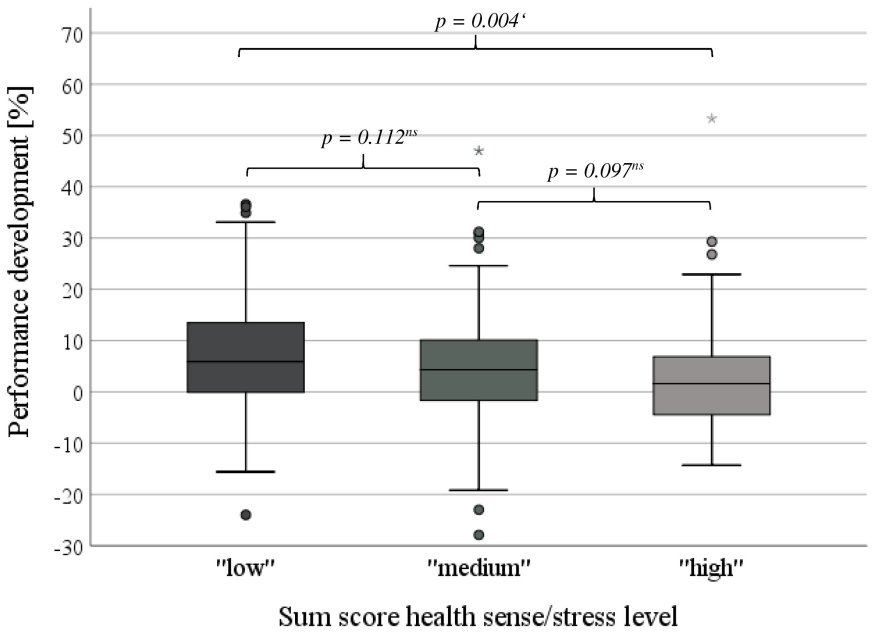

FIGURE 8 | Performance development dependent on sum of health sense and stress level group ("low" [sum 2–4] vs. "medium" [sum 5–7] vs. "high" [sum 8–10] risk). **$p < 0.001$, *$p < 0.01$, $^{\#}p < 0.05$, $^{ns}p \geq 0.05$ (non-significant).

male and female athletes (m/f: "low" 39.4/20.9%, "medium" 39.9/51.2%, "high" 20.7/27.9%, $p = 0.002$). There were no differences in training hours ("low" 12.2 ± 5.0 h, "medium" 12.8 ± 4.3 h, "high" 12.0 ± 4.7 h, $p = 0.839$, **Table 5**) between the three groups. In contrast, significant distinctions of the other health-related parameters were found (**Table 5**). Comparing the "high" with the "low" risk group, the prevalence of recurrent infections was fivefold ("low" 5.2%, "high" 26.0%, $p < 0.001$), furthermore, nearly tenfold of feeling "unhealthy" ("low" 5.0%, "high" 48.0%, $p < 0.001$) and twice as much of fatigue ("low" 2.2%, "high" 4.2%, $p < 0.001$). For all applicable, an ordinal increase in prevalence was evident (**Table 5**). Finally, performance development diminished in dependence of group affiliation ("low" 6.9 ± 10.9%, "medium" 4.7 ± 10.8%, "high" 2.1 ± 10.9%, $p = 0.015$, **Figure 8**).

DISCUSSION

Systematic training is an indispensable requirement for the development of a successful performance. However, similar training modalities, such as achieved training hours, lead to individual responses, and finally, decide on athletes' success or failure. To foresee performance development, potential predictors should be known. For that, the evaluation of subjective parameters, in addition to known performance-related variables, such as biochemical markers, was focus of the analysis.

To increase individual performance over time, sufficient training loads are necessary. Competitive sport is associated with frequent, prolonged and intensive training sessions, even at a young age. 13-year-old or younger athletes in several sports train 15–20 h per week (Armstrong and McManus, 2011). Due

to the earlier professionalization (e.g., *Youth Olympic Games*, *European Youth Olympics Festival*), the danger of a premature training cumulation is obvious (Myer et al., 2015). These physical strains can be linked with an increased risk for illness and injury (Hastmann-Walsh and Caine, 2015). Consistently, physical stress activates the immune system more or less, resulting in weakness or stabilization. However, the clinical relevance of such immunological changes, triggered by exercise, remains controversial, because no direct associations to increased infection rates could be clearly demonstrated (Fricker et al., 2005; Cox et al., 2008). Furthermore, it is unclear, from which load (e.g., duration, intensity, frequency) the immune system is negatively influenced, from when clinical complaints occur, and therefore, the performance development will be diminished (Konig et al., 2000). The average training load of the analyzed athletes was nearly 12 h per week, with a maximum of 30 h. The results presented an unaffected performance development by number of training hours. Here, the impairment and improvement groups showed similar amounts of mean training hours weekly. In addition, soccer players, who offered the lowest training hours, demonstrated the highest performance advances. Nevertheless, besides the quantitative evaluation of training loads, quality (e.g., intensity, type of training) should be considered as well. Too low individual intensities induce missing impulses, and thus, avoid required adaptations. In contrast, excessive intensities can negatively affect recovery and health causing frequent interruptions and lack or stagnation of performance. Therefore, training loads should be individually determined and adjusted regularly. In addition, the initial performance status should be taken into account, because performance and relative development show no linear relationship to each other. So, weaker athletes demonstrate possible greater performance improvements in less time. In summary, for further analyzes the inclusion of training quality, the evaluation of basic values (requirement of standardized performance diagnostic tests) and the assessment of detailed objective training logs (e.g., by digital data transfer) is recommended. The results are emphasized by a lack of dependence of neither subjective stress levels, health senses, nor of the occurrences of clinical complaints on extent of training load. In adolescence, overall strains of 60 h per week, including schooldays, may be present. Hence, perceived increased stress levels can have a negative impact on health (Main et al., 2010; Kellmann et al., 2018). The detected missing relation between training load and stress confirm previous published data (Blume et al., 2018). Here, athletes demonstrated similar stress levels despite higher training loads compared to a control group. This illustrates the individual and multi-factorial etiology of stress, the discrepancy among existing and perceiving stress, respectively, the individuals' handling with stress factors. In future analyzes, possible further triggering factors regarding stress (e.g., psychological parameters) should be identified and analyzed. Despite this, results can be based on known positive effects of exercise on stress sense (e.g., vagotonic increase, structured everyday life, social environment, recognition, mental stability) and on the young age of the collective (e.g., less pressure, "playful" component) (Blume et al., 2018). These assumptions require the examination of adult elite athletes using a similar study design. In summary, the parameter training hours possessed no negative influence regarding athletes' performance and health.

The aim of an exercise intervention, respectively of a pre-season period, is to improve individual performance. In particular, in endurance athletes, performance is directly associated with a high maximum oxygen consumption (VO_{2max}) (Bassett and Howley, 2000; Mairbäurl, 2013). Beside oxygen utilization, one key factor of a high VO_{2max} is an enhanced oxygen transport capacity accomplished by hemoglobin (Wagner, 1996). Previous studies proved the predictive value of a high hemoglobin mass in endurance sports regarding performance (Steiner et al., 2019; Zelenkova et al., 2019). In contrast, most athletes demonstrate normal hemoglobin concentrations because of plasma volume changes, so called hemodilution (Schumacher et al., 2002). Therefore, an evident relationship between concentration values and competitive success is missing (Kuipers et al., 2007). Nevertheless, in clinical practice, an assessment of the hematological profile, notably of hemoglobin concentrations, is used to estimate and predict athletes' performance, and in particular to detect disorders such as anemia. The results showed gender-specific differences of hemoglobin concentrations, but at no time an association with the endpoint performance development. Here, athletes with significant performance improvements ($\geq$7.5%) offered similar values compared to the others, moreover, the lowest concentrations had none negative impact on progress. Furthermore, athletes with diminished hemoglobin concentrations (<12 g/dl, prevalence 2.2%) demonstrated no enlarged performance loss. Thus, present results are consistent with previous studies (Kuipers et al., 2007). Regular monitoring of hematological parameters is still recommended, not for the prediction of performance, but to detect abnormalities such as anemia. At once, further parameters should be considered, especially the evaluation of iron metabolism (e.g., ferritin), because a non-anemic iron depletion may impair performance (DellaValle and Haas, 2011).

In the last decade, vitamin D has been given special attention. Low values are discussed as a potentially performance limiting factor, in contrast, high vitamin D supplies are associated with an enhanced performance (Cannell et al., 2009; Dahlquist et al., 2015). Furthermore, a vitamin D deficiency can negatively affect athletes' health (Cannell et al., 2009; Ogan and Pritchett, 2013). The importance regarding athletes performance remains uncertain, because placebo-controlled studies showed no improved physical performance of athletes with raised vitamin D serum concentrations (Dubnov-Raz et al., 2015; Fairbairn et al., 2018). Confirming this, the present analysis demonstrated no consistent relationship between vitamin D values and performance progress. Only in the group of cross-country skiers, an elevated vitamin D value was associated with an improved performance development. Beside this, none impaired outcome of vitamin D deficient athletes were found (Dubnov-Raz et al., 2015; Fairbairn et al., 2018). Compared to the literature, the prevalence of deficiency was low (Farrokhyar et al., 2015). Finally, prospective, controlled studies are required to evaluate long-term effects of a chronic vitamin D deficiency in young age.

In contrast to hemoglobin and vitamin D, Epstein Barr Virus (EBV) is often associated with a diminished performance and fatigue in competitive athletes (Gleeson et al., 2002; Balfour et al., 2015). There has been an ongoing controversy, whether elite athletes suffer a higher rate of EBV infections (Farrokhyar et al., 2015). Furthermore, previous studies interpreted slightly elevated EBV-specific IgG titers over the competition season as a reaction to increased EBV activity, possibly inducing an increased susceptibility to infections and an impaired performance (Pottgiesser et al., 2006; Farrokhyar et al., 2015). The results showed no dependence of performance progress on EBV serostatus. Moreover, EBV positive athletes demonstrated similar performance developments compared to seronegative participants. This is consistent with our previous publication, in which young elite athletes presented no different EBV-specific serological parameters compared to controls (Blume et al., 2018). Also, no direct relationships between training loads, clinical complaints, and EBV-specific immune responses (e.g., extent of IgG titers) were found (Blume et al., 2018).

Elite athletes are exposed to high strains, not only physically. The known stress factors include e.g., psychological pressure (e.g., annual selection, performing in competition), unstable financial support, training and competition environment factors (e.g., weather, inadequate training facilities), travel, nutrition, mismatch between internal and external expectations, protracted injuries, and repeated illnesses (Sabato et al., 2016). In young age, also puberty, a changed physical development, school stress, surrounding conflicts (with e.g., parents, friends, coach, and teachers) and an upcoming prioritization (less leisure time) should not be underestimated (Armstrong and Mc Manus, 2011). In addition, the trend of recent years shows an increased duration, intensity, and difficulty of training, a high-frequency participation in sports events, and an earlier specialization and professionalization (Armstrong and Mc Manus, 2011). All these potential risk factors can negatively affect the risk of illness and injury (Armstrong and Mc Manus, 2011; Sabato et al., 2016). While exercise has various positive effects on athletes health and well-being (cardiovascular fitness, muscular strength, bone health, weight control, self-confidence stabilization, reduced morbidity) (Wartburton and Bredin, 2016, 2017), prolonged and intensive training, in competitive sports unavoidable, can diminish the immune competence with following higher rates of infections. Furthermore, recurrent infections can cause frequent interruptions, lack or stagnation of performance, up to retirement from competitive sport (Maffulli et al., 2010). However, the phenotypes of athletes differ significantly among each other: only a small percentage of junior athletes achieve senior level, participate internationally, or even win medals at Olympics. Others are permanent sick or injured, moreover, despite apparently similar conditions, are not able to access their real performance in competition. In this regard, recent studies have shown fewer episodes of infections in successful top-ranking athletes, potentially based on selection mechanisms of "talented" ones (Hellard et al., 2015; Schwellnus et al., 2016). Unfortunately, a comprehensive definition of "talented" athletes is lacking, therefore, an identification in young age represents still a huge challenge (Johnston et al., 2018). The analyzes showed a significant relationship between health sense, stress level and performance development. Here, poor subjective health senses or elevated stress levels, and moreover their combination, result to an impaired performance progress comparing to athletes without reported disturbances. Regardless of hemoglobin values, subjective stress levels were a key factor modifying performance. Training loads possessed none influence on subjective stress levels, therefore, in case of increased stress levels, an exact diagnostics of potential provoking risk factors should be occurred. Subsequently, trigger factors can be minimized by specific interventions.

Competitive athletes, in particular cyclists and cross-country skiers, requires superior physiological skills, such as a trained aerobic and anaerobic performance, and accordingly a high maximum oxygen uptake (Bell et al., 2017). For the analyzes, aerobic performance capacity (relative performance at 3 mmol/l, or rather 4-mmol/l lactate) as outcome was chosen. The decision for this parameter was based on its standardized determination in contrast to competition results, although a high aerobic performance capacity does not reflect individual overall performance and sport-specific skills. However, also an aerobic endurance training improves soccer performance (Helgerud et al., 2001). Furthermore, an enhanced endurance performance reduces athletes' regeneration time resulting in training advantages (Kellmann et al., 2018). It still remains to be considered, that missing performance developments are based on differently selected training priorities. Nevertheless, the analyzed exercise intervention periods had the focus on improvements of endurance performance, because, in adolescence, athletes' capacity should be stepwise developed and optimized (Myer et al., 2015). Future evaluations should assess longitudinal data including individual senior success to identify performance predictors and drop-out reasons.

It must be noted, that the presented results are partially based on self-reported data, in particular the parameters health sense and stress level. The aim was to detect abnormalities, so an impaired health sense could indicate an existing infection. In the analyzes, low health senses were associated with increased prevalences of clinical complaints, but not with higher hs-CRP values. However, in contrast to hs-CRP, the parameter health sense showed a relationship to the end point performance progress. Subjective self-reported data will not replace objective parameters, but they are able to suggest individual impaired sensations. In this regard, training can be modulated to avoid further complications. Therefore, the monitoring of subjective self-reported data, in particular because of their close association to performance development, can represent an appropriate diagnostic tool to control training. Nevertheless, the clinical relevance of such health-related parameters remains to be further investigated (e.g., validated questionnaires [WURSS], blood samples) (Barrett et al., 2005).

Despite similar training modalities, performance development varies among each athlete. The degree of progress can be influenced by numerous parameters, such as training quality and quantity, genetic disposition, health, physiological conditions,

and psychosocial factors. Nearly two-thirds of all athletes demonstrated an improvement of performance, one-third of at least 7.5%. These groups reported improved health senses and lower stress levels. In contrast, objective known indicators, such as hemoglobin concentrations and vitamin D values, offered no differences between the groups. Therefore, subjective parameters, which reflect athletes' health, should be considered to regulate training, and finally, to predict performance potential. Hence, already at a young age ensuring health should be one superior aim, resulting in a higher training quality, and thus, in a better performance development.

The analyzes based on data from a large, prospective, controlled study of adolescent athletes (Blume et al., 2018). Therefore, "real-life" results could be determined, which in turn can be transferred and applied directly in practice. Beside known, or rather assumed biochemical markers regarding performance development prediction, the evaluation of subjective parameters was focus of the analysis. Although these were determined subjectively, the assessment was performed standardized and, in addition, longitudinal. The results emphasize the consideration of such parameters for health evaluation, training monitoring and performance prediction. However, biochemical markers should not be disregarded, in particular for the detection of abnormalities, such as deficiency, anemia or a systemic inflammation. Rather, the individuals' performance development is based on numerous influencing factors, which all should be estimated. In contrast to the benefits of a "real-life" study, it comprises limitations. In order to maintain compliance and to ensure practicability, long questionnaires and complex laboratory measurements were left unconsidered. In further analyzes these methods should be compared with the used tools for validation. The results were confirmed for various athletes of different sports. Nevertheless, a larger homogeneous collective is needed to verify the results (e.g., similar basic values, same performance test). Also, individuals' long-term and sport-specific performance should be identified. The focus of this analysis was the development of the athletes' endurance capacity, therefore, endurance-related sports and training periods were included. As already mentioned, training hours should not equalize with the total training load. For this purpose, further parameters should be determined (e.g., detailed training information such as frequency, intensity and duration). Finally, the evaluation of subjective parameters represents, in combination with other variables, such as known biochemical markers, a practicable tool for training monitoring und performance prediction.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Research Ethics Committee (TU München). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KB and BW conceived the present idea, developed the theory, performed the computations, verified the analytical methods, discussed the results, and contributed to the final manuscript.

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Effect of Interleukin-15 Receptor Alpha Ablation on the Metabolic Responses to Moderate Exercise Simulated by *in vivo* Isometric Muscle Contractions

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Lack of interleukin 15 receptor alpha (IL15RA) increases spontaneous activity, exercise capacity and protects from diet-induced obesity by enhancing muscle energy metabolism, suggesting a role as exercise mimetic for IL15RA antagonists. Using controlled *in vivo* muscle stimulation mimicking moderate exercise in normal and Il15ra$^{-/-}$ mice, we mapped and contrasted the metabolic pathways activated upon stimulation or deletion of IL15RA. Stimulation caused the differential regulation of 123 out of the 321 detected metabolites (FDR $\leq$ 0.05 and fold change $\geq \pm 1.5$). The main energy pathways activated were fatty acid oxidation, nucleotide metabolism, and anaplerotic reactions. Notably, resting Il15ra$^{-/-}$ muscles were primed in a semi-exercised state, characterized by higher pool sizes of fatty acids oxidized to support muscle activity. These studies identify the role of IL15RA in the system-wide metabolic response to exercise and should enable translational studies to harness the potential of IL15RA blockade as a novel exercise mimetic strategy.

Keywords: muscle, exercise, interleukin-15 receptor alpha, metabolomics, *in vivo* stimulation, exercise mimetics, metabolism, fatty acid oxidation

INTRODUCTION

Regular exercise is a safe and effective disease modifier for managing obesity and many preventable chronic diseases related to energy metabolism, as it promotes fat utilization, weight control, and ameliorates insulin sensitivity (Borghouts and Keizer, 2000). In practice, however, the people who would benefit the most from exercising often have exercise intolerance or limited mobility. In the United States, only 52% of adults perform some kind of aerobic activity (Source: Behavioral Risk Factor Surveillance System BRFSS, Centers for Disease Control and Prevention, Atlanta). From a translational perspective, making the beneficial effects of exercise universally available in the form

of pharmacological intervention (exercise mimetics) is an important goal. Research on exercise mimetics has led to the identification of genes and pathways that, when modulated, can increase exercise performance and simulate the exercised condition (North et al., 1999; Lagouge et al., 2006; Quinn et al., 2013; Fan and Evans, 2017; Amoasii et al., 2019). Activators of PGC1a, AMPK and PPARδ are well-known examples of exercise mimetics (Narkar et al., 2008; Matsakas and Narkar, 2010) that can promote oxidative metabolism. Precise control over the experimental conditions is necessary because energy metabolism is influenced by multiple factors such as circadian rhythms (Dyar et al., 2018), intensity (Horowitz and Klein, 2000; van Loon et al., 2001), and duration of exercise (Romijn et al., 1993). While studies in human subjects have the highest translational value, they often face issues with recruitment, compliance and controlling for lifestyle factors, disease features or co-morbidities (Anziska and Inan, 2014). Animal research, on the other hand, offers controlled and homogeneous experimental conditions and the possibility to adopt appropriate genetically engineered models, *in vivo* physiological techniques and high-throughput analyses.

Increasing evidence in humans and mice suggests that another strategy to mimic exercise is by modulating the cytokine interleukin 15 (IL15) or its primary binding partner, IL15 receptor alpha (IL15RA). IL15 and IL15RA have a well-recognized immunological role in mediating the activation of T and natural killer cells, and constitutive ablation of either of them leads to blunted immune response to pathogens (Lodolce et al., 1998; Schluns et al., 2005). However, consistent with their widespread tissue distribution, IL15/IL15RA have also been shown to participate in the regulation of energy processes of a variety of tissues (He et al., 2010), including brain (Wu et al., 2010; Nguyen et al., 2017), bone (Djaafar et al., 2010; Loro et al., 2017), adipose tissue (Sun and Liu, 2015; Lacraz et al., 2016), and muscle (Pistilli and Quinn, 2013; Loro et al., 2015, 2018; O'Connell et al., 2015). We and others have shown that ablation of IL15RA in mice increases spontaneous activity, exercise capacity and protects against diet-induced obesity (He et al., 2010; Pistilli et al., 2011; Loro et al., 2015). Similar to the muscles of endurance-trained athletes (van Loon and Goodpaster, 2006), fast muscles from mice lacking IL15RA (Il15ra$^{-/-}$) have higher intramuscular triglycerides (IMTG) content compared to controls. We have proposed that the ability to use IMTG stores efficiently enhances Il15ra$^{-/-}$ recovery capacity after exercise. Supporting our hypothesis, we found that lipases, fatty acid binding proteins and the AMPK/ACC pathway were significantly activated in Il15ra$^{-/-}$ fast muscles (Loro et al., 2015, 2018). Little is known, however, about the impact of these changes on the metabolome of resting and exercised muscles.

In the current study, we have developed an experimental model of moderate-intensity isometric exercise using *in vivo* stimulation of the sciatic nerve. Following stimulation, we have performed a detailed metabolomic analysis of the changes evoked by contraction in extensor digitorum longus (EDL) muscles. Finally, we have compared the obtained contraction-induced metabolomic signature with the changes occurring in the muscles of Il15ra$^{-/-}$ mice, highlighting the metabolic reactions associated with their exercise performance.

RESULTS

Establishment of the *in vivo* Moderate Muscle Stimulation Protocol

Muscle contractile activity is supported by a cascade of biochemical and metabolic events fueled by the conversion of energy substrates to ATP. To study the effects of controlled exercise on muscle energy metabolism, we developed an *in vivo* stimulation protocol that mimics moderate exercise in fed anesthetized mice (**Figure 1**). The protocol consists of controlled bursts of muscle activity alternating with periods of rest, repeating over 22 min. When applied to control and Il15ra$^{-/-}$ mice, the stimulation evoked the simultaneous contraction of all the muscles of the anterior and posterior compartments of the targeted limb. For each animal, one limb underwent stimulation while the contralateral was used as unstimulated control. The protocol did not induce complete muscle exhaustion, as indicated by the only partial decrement of EMG amplitude and root mean square (RMS) (**Figure 1B**). Consistent with moderate exercise, peripheral capillary oxygen saturation (SpO_2) remained stable, while heart rate (HR) increased moderately as a consequence of the repeated stimulation (**Figure 1B**).

Generation of C57BL/6-Il15ra$^{-/-}$ Mice With Fatigue-Resistant Muscles and Higher Spontaneous Activity

Previous studies performed on a B6;129×1-based Il15ra$^{-/-}$ model had raised the concern of potential differences in background compared to the control strain used (B6129SF2/J). To address this concern, we generated a new C57BL/6-based Il15ra$^{-/-}$ line by crossing IL15RA-flox mice to mice expressing Cre driven by the ubiquitous promoter EIIA (**Figure 2A**) and performed every experiment using control and Il15ra$^{-/-}$ littermates. Genomic PCR detected the successful recombination and full excision of exons 2/3 (**Figure 2B**), the region coding for the IL15 binding site in IL15RA. We confirmed the successful excision in all tissues, including muscle, brain, liver, spleen and adipose tissue (**Figure 2C**). To test whether the C57BL/6-Il15ra$^{-/-}$ line (from now on called Il15ra$^{-/-}$) retained the previously described muscle phenotype, we compared EDL muscles from Il15ra$^{-/-}$ and littermate controls in terms of isometric strength (**Supplementary Table S1**), resistance to fatigue (**Figure 2D**) and recovery capacity (**Figure 2E**). Measurements of muscle strength and weight were similar between genotypes (**Supplementary Table S1**). Consistently with the previous reports, Il15ra$^{-/-}$ EDL retained higher fatigue resistance (**Figure 2D**) and recovery capacity (**Figure 2E**), suggesting differences in energy metabolism. *In vivo*, Il15ra$^{-/-}$ retained the overall normal circadian rhythm of locomotor activities, although they were more active than controls when tested for cumulative wheel running activity (**Figure 2F**) and beam breaks (**Figure 2G**, – top panels), with higher oxygen

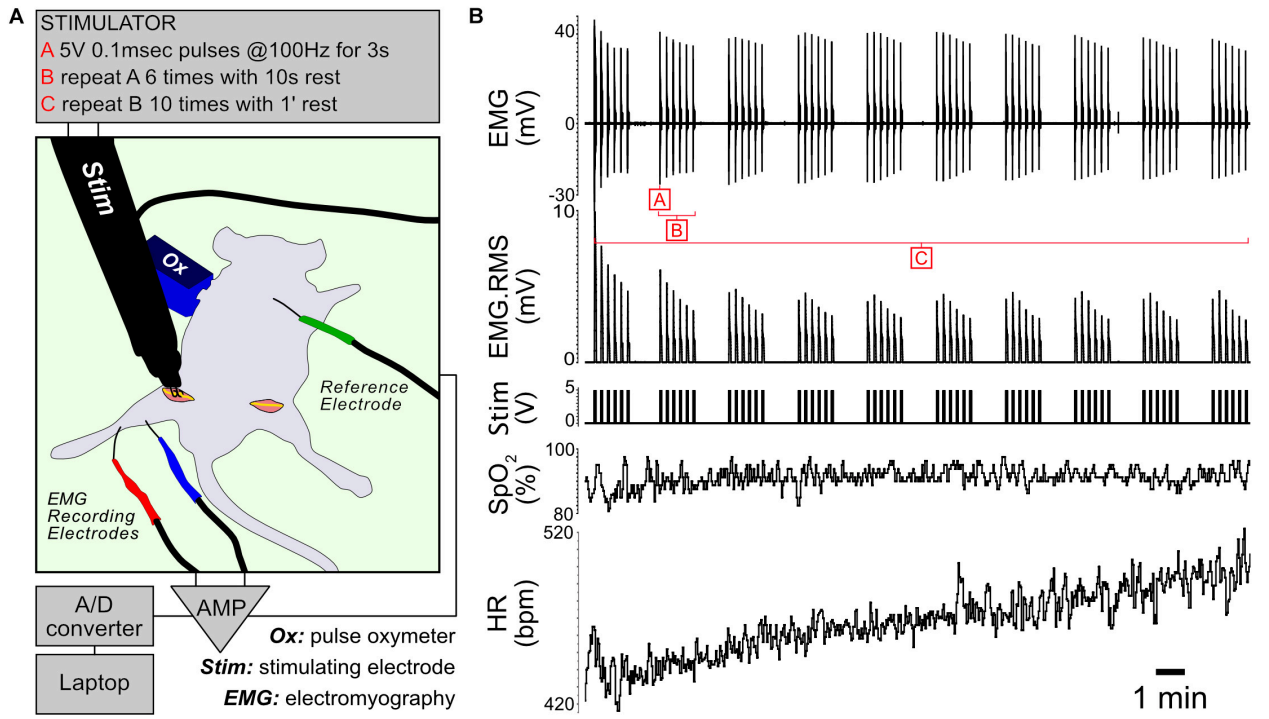

FIGURE 1 | *In vivo* stimulation protocol. **(A)** Diagram of the experimental setup. The unstimulated limb underwent a sham procedure before dissecting the muscles of interest. **(B)** Representative traces showing EMG, RMS (root mean square) of the EMG trace, stimulation, capillary oxygen saturation and heart rate measured during the experiment.

consumption and energy expenditure especially during the dark phase (**Figure 2G**, – bottom panels).

Metabolomic Analysis of EDL Muscles

Because changes in transcriptomics or proteomics are unlikely to drive the acute response to a single bout of muscle stimulation by themselves, our approach focused on the analysis of muscle metabolome. We performed targeted metabolomics on unstimulated and stimulated EDL muscles (**Figure 3A**). A total of 321 metabolites were detected and identified by GC-TOF MS analysis (**Supplementary Table S2**). To model the effects of stimulation and genotype, we applied a supervised partial least squares discriminant analysis (PLS-DA). The significance of class discrimination, assessed using a permutation test based on prediction accuracy, was calculated to be P = 0.007. The PLS-DA model distinguished between the contribution of two main components, accounting respectively, for 54 and 12% of the total variance. Component 1 best separated the effect of stimulation, while component 2 separated the effect of genotype (**Figure 3B**). A loading plot representation (**Figure 3C** and **Supplementary Table S3**) highlighted the contribution of carnitine species and TCA cycle intermediates to the variance associated with component 1 (stimulation), and the contribution of gut microbiota metabolites to the variance of component 2 (genotype). A ranked representation of the 25 highest variable importance projection (VIP) scores for each component confirmed the contribution of carnitines, fatty acids, and TCA intermediates to the variation associated with component 1 (**Figure 3D** and **Supplementary Table S4**). Carnitines and fatty acids were also among the 25 highest VIPs for component 2 (**Figure 3E** and **Supplementary Table S4**), suggesting an interaction between genotype and stimulation.

Effects of Moderate-Intensity Stimulation

By applying a two-way mixed ANOVA analysis (Genotype × Stimulation) with statistical significance at $\alpha \leq 0.05$ after FDR adjustment, 195 metabolites were significantly affected by stimulation (123 more than 1.5×), while only 7 were significantly different between genotypes (5 more than 1.5×) (**Supplementary Table S2**). Because of the magnitude of the changes associated with stimulation, we initially dropped the genotype factor and focused on the comparison between stimulated and unstimulated muscles. The majority of stimulation-induced changes were toward higher metabolite levels (107 up and 16 down – **Figure 4A**). The most represented metabolite superclasses were fatty acids (34.1%), amino acids (23.6%), carbohydrates (14.6%), and nucleotides (8.9%) (**Figure 4B**). Consistent with the PLS-DA model, carnitines were predominant within the group of upregulated metabolites (**Figure 4C**). Pathway analysis suggested that the most represented pathways impacted by stimulation were amino acids metabolism (Asp – P = 4.20E-12; Phe, Tyr – P = 1.79E-09; Glu – P = 1.03E-06; Arg, Pro – P = 8.57E-07), purine metabolism (P = 1.13E-09), citric acid cycle (P = 5.00E-07), urea cycle (P = 2.30E-06), glycolysis (P = 1.36E-04) and oxidation of

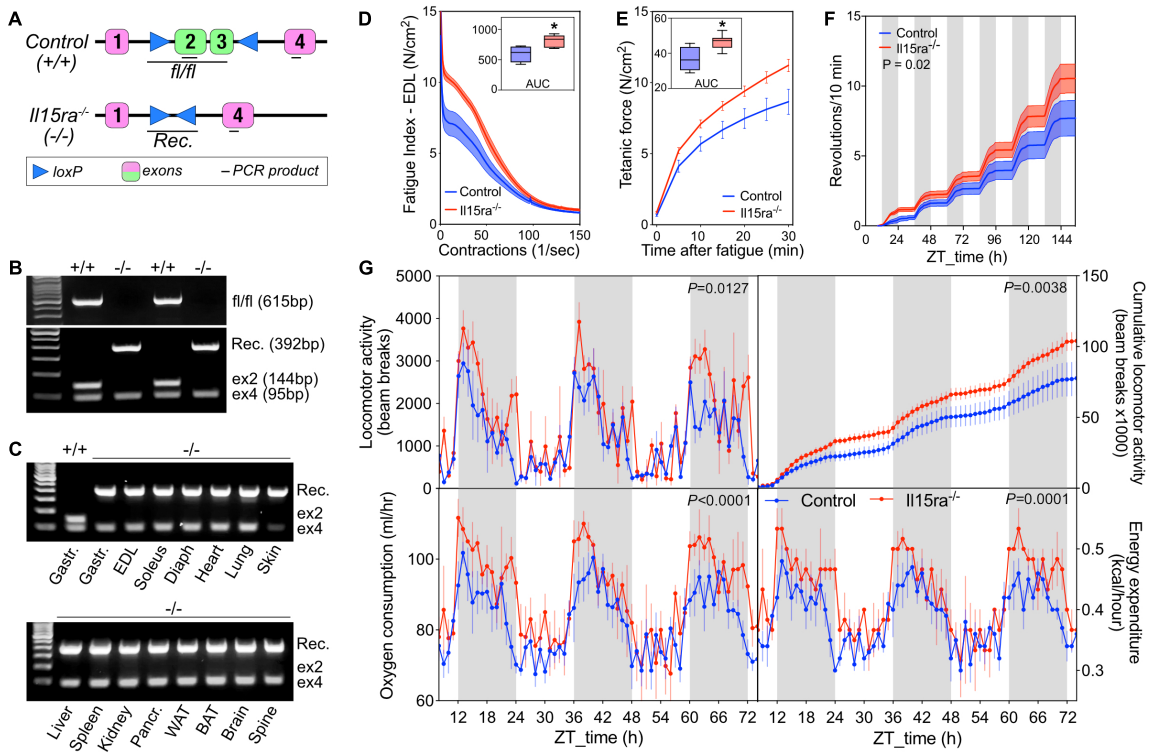

FIGURE 2 | Generation of C57BL/6-Il15ra$^{-/-}$ mice with enhanced exercise capacity. **(A)** Diagram of EIIA-cre-mediated genomic deletion of exons 2 and 3 of the *Il15ra* gene. **(B)** Genomic PCR for the detection of the intact Il15ra-flox alleles (615 bp), recombined genomic sequence after the cre-mediated flox excision (392 bp), exon 4 (95 bp), and exon 2 (144 bp, absent in fully recombined samples). **(C)** Genomic PCR to confirm the successful recombination in multiple tissues of Il15ra$^{-/-}$ mice, with consequent excision of the exon 2 sequence. Representative *ex vivo* EDL fatigue **(D)** and recovery **(E)** traces of control (n = 4) and Il15ra$^{-/-}$ (n = 6) mice. Insets show the average area under the curve (AUC). **(F)** Cumulative wheel running traces (n = 5 mice per group). Cumulative distributions were compared with the Kolmogorov–Smirnov test. **(G)** Representative indirect calorimetry assay of control (n = 5) and Il15ra$^{-/-}$ (n = 3) mice. Data are represented as mean ± SEM. *P < 0.05 and **P < 0.01.

branched-chain fatty acids (P = 1.10E-06) (**Figure 4D**). Due to the lack of Kegg identifiers for the fatty acids in our dataset, both measures of enrichment and impact on the entries associated with fatty acids metabolism were likely underpowered. A ranked visualization of the most significantly regulated metabolites highlighted the important role of TCA and purine metabolism in the response to stimulation in fast muscles (**Figure 4E**). The most differentially regulated metabolites in the dataset were inosine monophosphate (IMP, 54×, P = 2.34E-11) and deoxyadenosine monophosphate (dAMP, −5.7×, P = 3.10E-05). IMP and dAMP are intermediates in the reactions catalyzed by adenylate kinase (which converts 2 ADP – >ATP and AMP) and myoadenylate deaminase (AMP or dAMP – >NH_3 and IMP), which are activated during medium to high-intensity exercise in response to ATP utilization (−1.8×, P = 5.85E-05) and changes in pH (Sahlin et al., 1989; Tullson and Terjung, 1991, 1999). Metabolism of amino acids such as glutamine, glycine, and aspartate plays an essential anaplerotic role in muscle recovery by providing intermediates for the TCA, e.g., by converting aspartate (−4×, P = 2.69E-07) to fumarate (7×, P = 1.48E-09) (Gibala et al., 1997). The moderate exercise-mimicking stimulation also decreased the creatine-phosphate/creatine ratio by ∼50% and caused a 1.6× accumulation of intramuscular lactate in both genotypes. Together, these results show that the activation of oxidative metabolism following the application of our *in vivo* stimulation protocol is consistent with a bout of moderate-intensity exercise.

A Schematic Overview of the Effects of Stimulation on Muscle Metabolome

A pathway diagram of the main muscle energy metabolites affected by stimulation (**Figure 5**) suggested that, upon moderate exercise, glucose derived from increased uptake and glycogenolysis was shunted from glycolysis to the pentose phosphate pathway, generating reducing equivalents and ribose-5-phosphate that entered the purine nucleotide pathway as ZMP (also known as AICAR; 3.78×, P = 2.77E-04), a potent activator of AMPK and a known regulator of energy metabolism (Ezagouri et al., 2019). Pyruvate levels were not affected by stimulation, suggesting that the increased acetyl-CoA and TCA cycle intermediates were mainly derived from fatty acid degradation and anaplerotic reactions. Interestingly, glycolytic intermediates accumulated upstream of GAPDH. GAPDH enzymatic activity may be allosterically inhibited by multiple mechanisms, including nitrosylation in presence of high levels of NADH (Mohr et al., 1996) (25.95×, P = 1.05E-03), or by increased concentrations

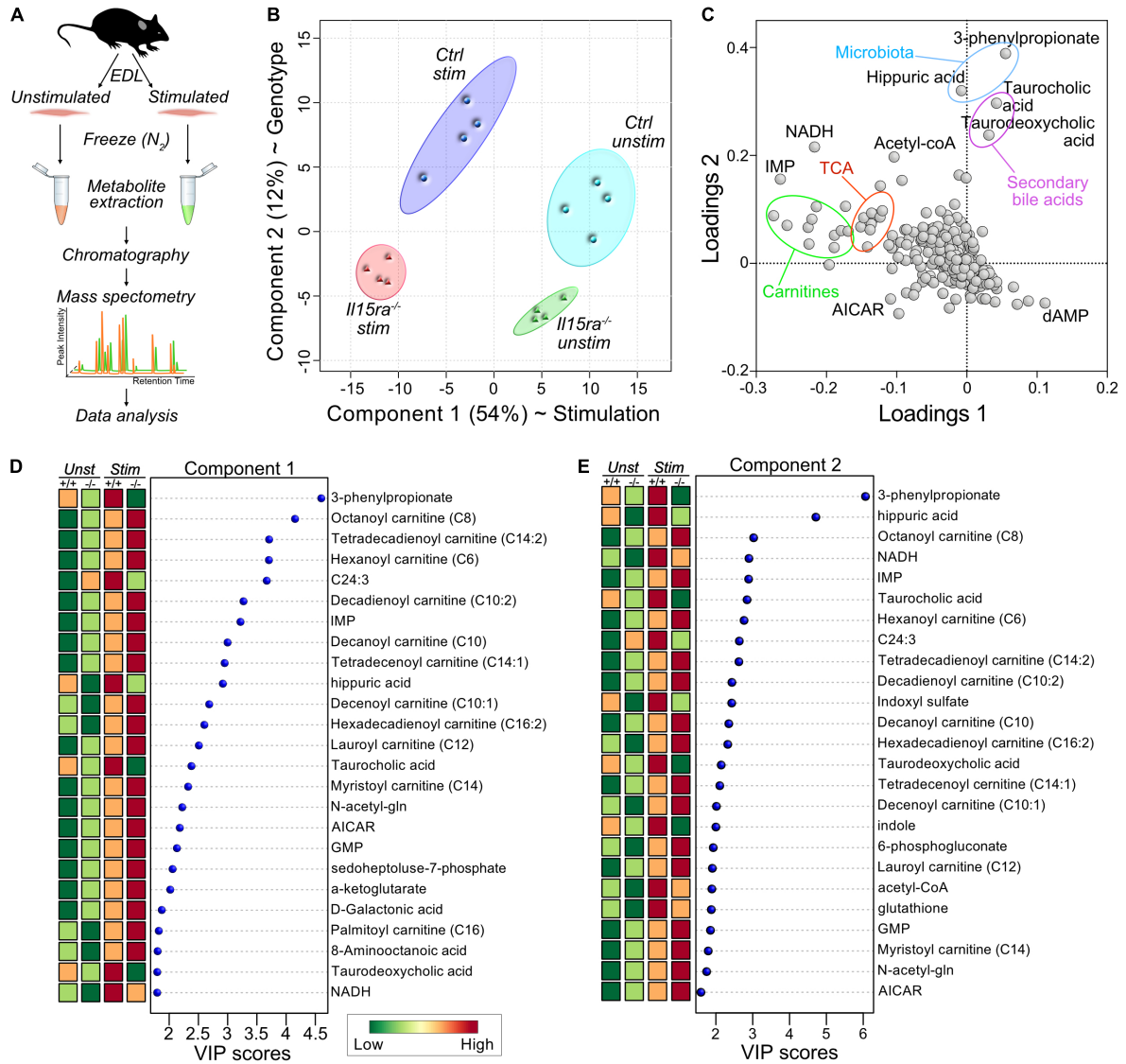

FIGURE 3 | Supervised multivariate analysis. **(A)** Diagram of the experimental pipeline adopted for the study. **(B)** Partial Least Square Discriminant Analysis (PLS-DA) plot of the metabolomics data. **(C)** Loadings plot for the principal components selected by PLS-DA. **(D,E)** Ranked representation of Variable Importance in Projection (VIP) scores for the features contributing to the variance of component 1 **(D)** and 2 **(E)**, according to PLS-DA.

of fumarate (Blatnik et al., 2008) (7.48×, P = 1.48E-09). The application of our *in vivo* stimulation protocol allowed us to map the metabolic consequences of a single bout of moderate intensity exercise, highlighting the important contribution of fatty acid metabolism and anaplerotic reactions to muscle energetics.

Differential Metabolic Activation in Il15ra$^{-/-}$ Mice

Il15ra$^{-/-}$ mice perform significantly better than controls when tested for muscle fatigability and recovery capacity. We, therefore, focused on the differences associated with genotype in unstimulated and stimulated muscles. With this approach, the number of significantly different metabolites between control and Il15ra$^{-/-}$ muscles were 8 before and 11 after stimulation (**Figure 6**). Consistent with the PLS-DA model, the metabolites showing the most significant variations between genotypes were the microbiota metabolites 3-phenylpropionate, hippuric acid, and indoxyl sulfate, suggesting an impact of IL15RA deficiency on gut immune system and microbiota. Some fatty acids, as well as intermediates of the glucuronic acid pathway (part of the pentose phosphate pathway), were significantly higher in Il15ra$^{-/-}$ than controls. In line with previous reports showing that lack of IL15RA has a role in bone homeostasis (Djaafar et al., 2010; Loro et al., 2017), glucosamine, a metabolite important for joint health, was induced 2.2-fold by stimulation and was 1.8 times higher in the stimulated Il15ra$^{-/-}$ than stimulated controls (**Figure 6A**). The AMPK activator ZMP trended to be higher

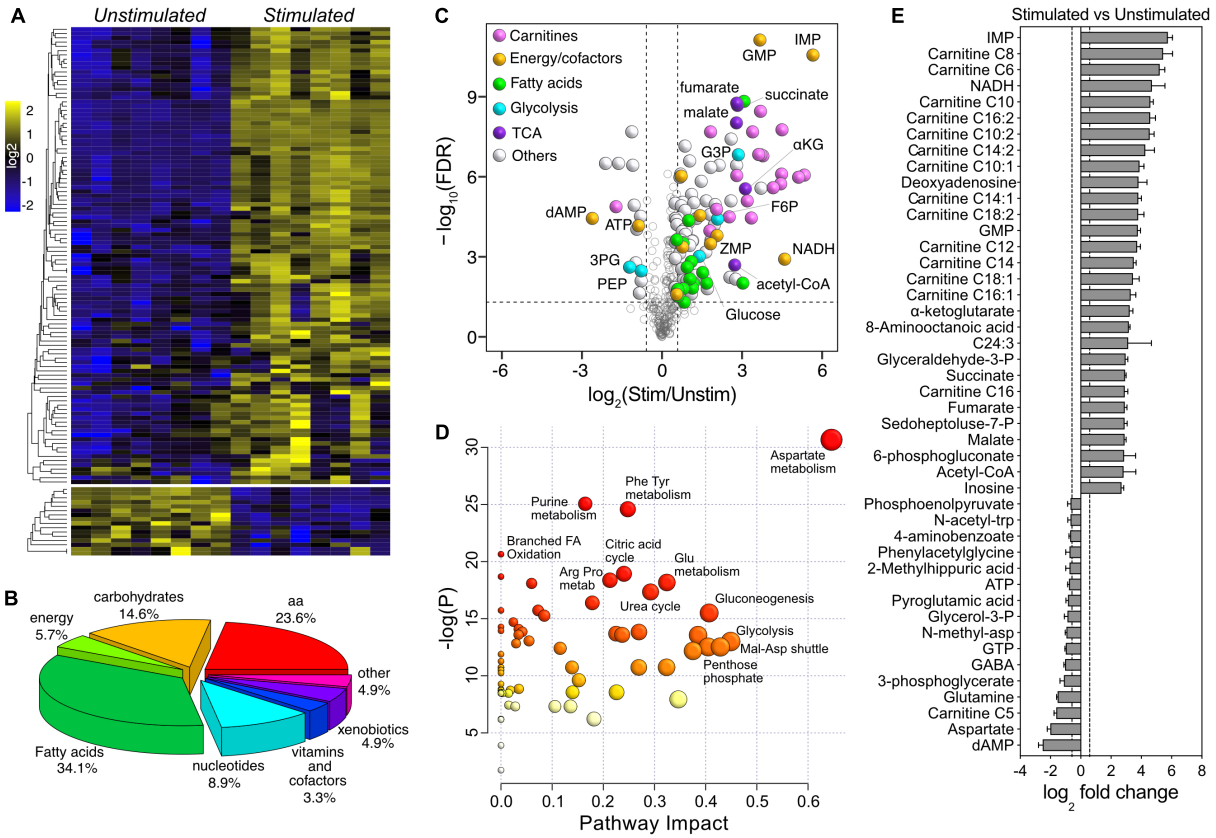

FIGURE 4 | Univariate analysis of the effect of moderate exercise. **(A)** Heatmap of $\log_2$-transformed, row-wise normalized intensities for metabolites with FDR-adjusted $P < 0.05$ and fold change $> \pm 1.5$ (123 of 321). **(B)** Pie chart representation of the metabolite superclasses significantly affected by stimulation. **(C)** Volcano plot showing the effect of stimulation. The dotted horizontal line represents the significance threshold (ANOVA, FDR-adjusted $P \leq 0.05$, $n = 8$), while the dotted vertical lines represent the fold change cut-off ($\pm$ 1.5). Significant data points are color-coded according to the different superclasses. **(D)** Pathway impact analysis of metabolites differentially regulated upon stimulation. The node color is based on the *P*-values from pathway enrichment (*y*-axis), while the size is proportional to the pathway impact value (*x*-axis). **(E)** Ranked representation of the 45 most differentially regulated metabolites upon stimulation ($n = 8$).

in Il15ra$^{-/-}$ than in control muscles (1.88× in unstimulated and 2.36× in stimulated muscles). Of 48 detected fatty acids, 24 were significantly higher after stimulation in control muscles (**Figure 6B**). In Il15ra$^{-/-}$ unstimulated muscles, the basal pool size of long-chain fatty acids (LC-FA) was higher than in controls, but did not change after stimulation (**Figures 6B,C**). Given the previously described enhancement of FA uptake into Il15ra$^{-/-}$ isolated muscle mitochondria (Loro et al., 2015), we considered whether the pool of acyl-carnitines was significantly elevated in Il15ra$^{-/-}$ compared to control muscles. The levels of 19 detected acyl-carnitines (C2–C20) increased significantly upon stimulation and were higher in Il15ra$^{-/-}$ compared to control muscles (**Figure 6D**).

To obtain a visual indication of whether IL15RA ablation can function as exercise mimetic, we then calculated the Euclidean distance between the levels of each metabolite in Il15ra$^{-/-}$ muscles and in the unstimulated and stimulated control conditions. We found that approximately 30% of the metabolites detected in Il15ra$^{-/-}$ unstimulated muscle, including fatty acids, amino acids, and nucleotides, were closer to the levels found in stimulated control muscles (**Figure 7A**, distance closer to 1).

Taken together, these results demonstrate that lack of IL15RA primes basal muscle energy metabolism to a state consistent with a moderate exercise condition. In such a state, the previously described activation of lipases and the AMPK pathway (Loro et al., 2015) cooperates with the increased availability of fatty acids to promote reliance on fatty acids and support the higher energy expenditure and exercise capacity (**Figure 7B**).

DISCUSSION

In this study, we examined the metabolic responses to a single bout of moderate isometric muscle contraction in normal mice and in mice with increased spontaneous activity and fatigue-resistance induced by the ablation of IL15RA. Our primary goal was to draw a network of the metabolic reactions activated by muscle contraction, and then identify the pathways contributing to the beneficial effects of lack of IL15RA on exercise capacity and energy metabolism. We found that (1) moderate muscle contraction activates oxidative and anaplerotic reactions; (2) consistent with the proposed exercise-mimetic effects, lack of

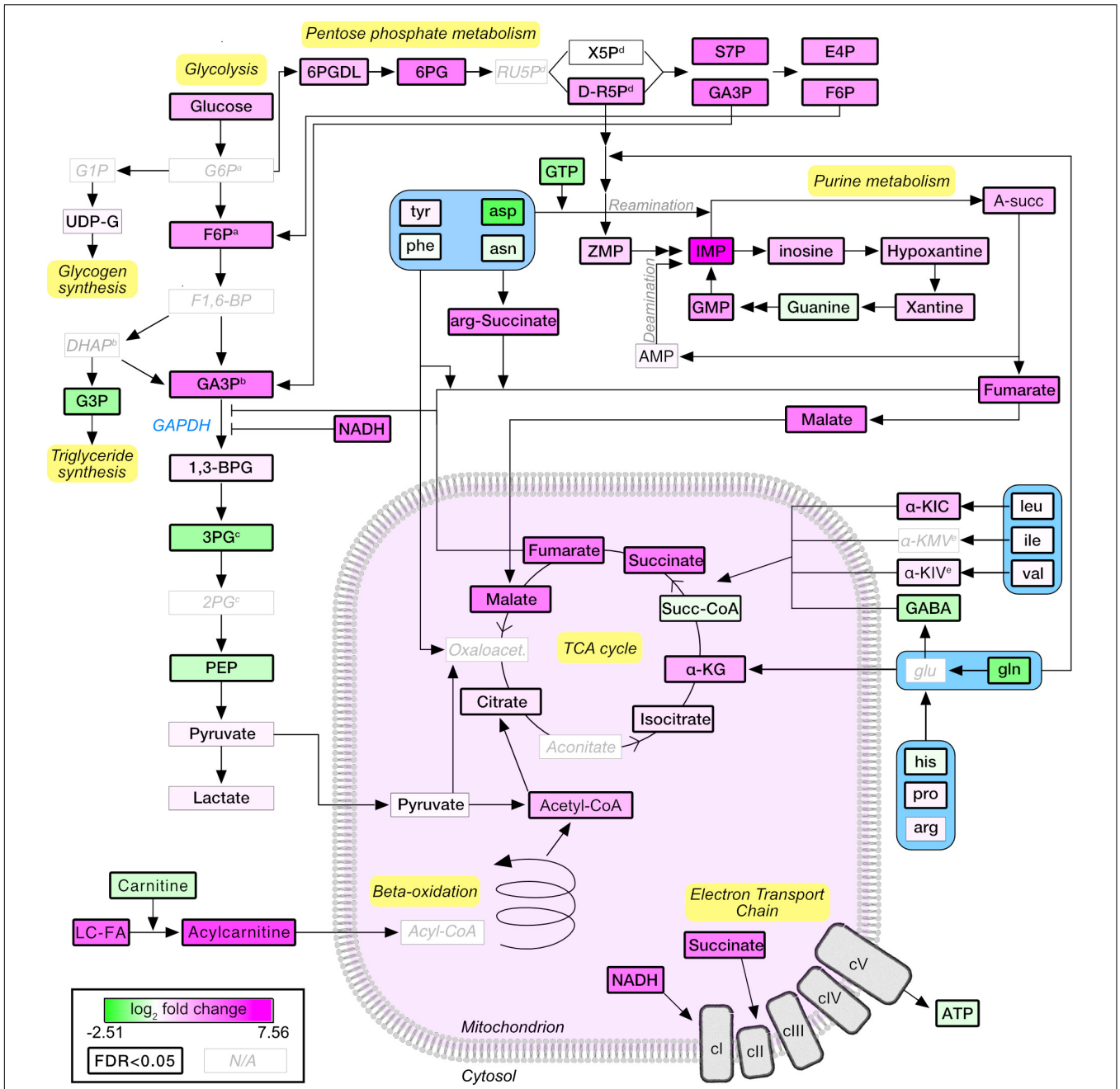

FIGURE 5 | Schematic representation of the metabolic pathways regulated upon moderate exercise. Node colors are proportional to the $\log_2$-normalized fold change (stimulated vs. unstimulated). Statistically significant nodes (FDR-adjusted *P*-value ≤0.05) have thick borders. Metabolites that were not detected are labeled in light gray. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6-BP, fructose-1,6-bisphosphate; GA3P, glyceraldehyde-3-phosphate; 1,3-BPG, 1,3-biphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphor-enol pyruvate; DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; LC-FA, long-chain fatty acids; α-KG, α-ketoglutarate; α-KIC, α-ketoisocaproate; α-KMV, α-ketomethylvalerate; α-KIV, α-ketoisovalerate; 6PGDL, 6-phosphoglucono-delta-lactone; 6PG, 6-Phosphogluconate; RU5P, ribulose-5-phosphate; X5p, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate; A-succ, adenylosuccinate; IMP, inositol-monophosphate; GMP, guanosine monophosphate. Superscript a,b,c,d,e indicate groups of metabolites which cannot be separated by the LC-MS approach used.

IL15RA promotes the reliance on FAO independently from muscle activity.

The application of stimulation-evoked muscle contraction has some limitations, as it only partially recapitulates the complexity of tissue-specific and systemic reactions taking place during exercise. However, it offers superior control of frequency and intensity of the evoked contraction, therefore eliminating sources of variance such as anxiety and other behavioral factors, typically

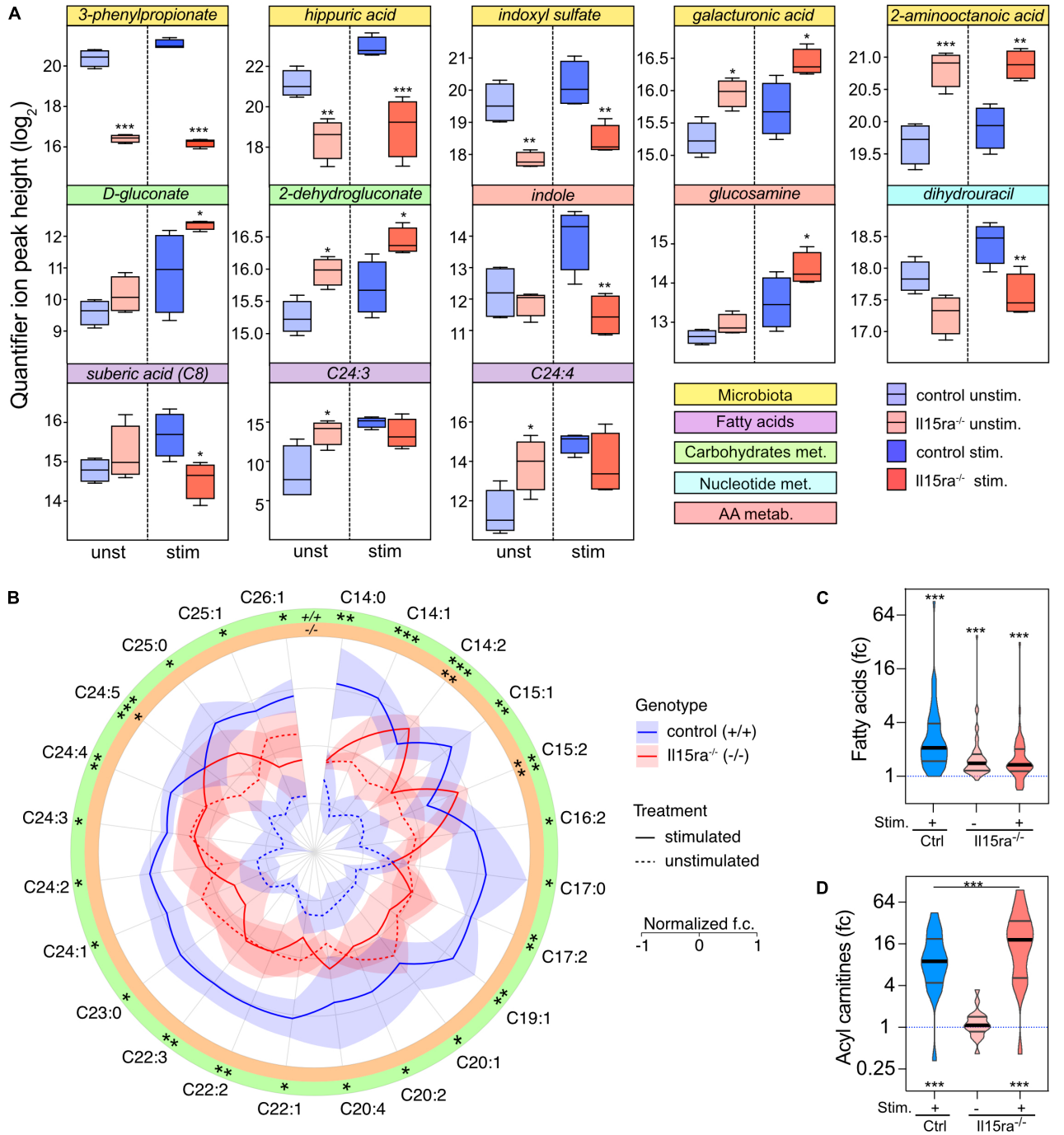

FIGURE 6 | Univariate analysis of the effect of genotype. **(A)** 2-way ANOVA with multiple comparisons of metabolites with significant interaction between stimulation and genotype. Data are represented as mean ± SEM. **(B)** Radar chart representation of the levels of fatty acids significantly mobilized upon stimulation in control (blue lines) and Il15ra$^{-/-}$ (red lines) EDL muscles. The fold changes of each fatty acid have been normalized (mean 0 and variance 1) for visualization purposes. Violin plots of mobilization of the total fatty acids **(C)** and carnitine **(D)** pools detected by the assay. In panels **(C)** and **(D)**, the dashed line represents controls unstimulated. Statistical significance was calculated vs. control unstimulated using one-way ANOVA on $\log_2$-transformed intensities before conversion to fold change. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

present during other *in vivo* protocols (e.g., treadmill, forced-swim test). In addition, it preserves systemic circulation of metabolites from other tissues (e.g., adipose, liver, gut). While the role of IL15 in adipose tissue lipolysis to support muscle activity was previously described (Quinn et al., 2009; Pierce et al., 2015), the differential regulation of gut microbiota-derived metabolites

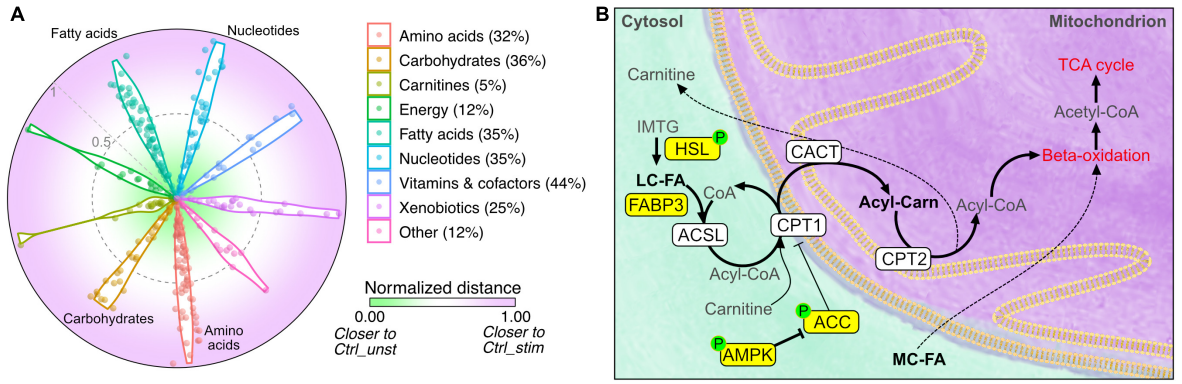

FIGURE 7 | Comparison between unstimulated Il15ra$^{-/-}$ and stimulated controls. **(A)** Radar chart representation of the Euclidean distance between unstimulated Il15ra$^{-/-}$, unstimulated control muscle and stimulated control muscle for the metabolites in each superclass. Values were rescaled to a range from 0 (close to unstimulated control) and 1 (close to stimulated control). Percentage number indicate the ratio of metabolites in Il15ra$^{-/-}$ muscle that were similar to stimulated control. **(B)** Diagram summarizing the effects of lack of IL15RA on muscle fatty acid metabolism. Yellow nodes indicate enzymes significantly upregulated/activated in Il15ra$^{-/-}$ muscle, according to previous publications by our group (Loro et al., 2015, 2018). Thick arrows represent metabolic reactions that are enhanced in Il15ra$^{-/-}$ muscle.

in stimulated IL15RA muscles is a novel finding originated from our approach. It would be interesting to use additional knockouts models to address how tissue-specific IL15RA deletion alters the response to muscle activity in adipose tissue, gut microbiome and liver. It should be considered, however, that the presence of circulating IL15RA (Bergamaschi et al., 2012) can challenge the interpretation of such studies.

The effects of lack of IL15RA are more evident in fast muscles, where it promotes fatigue-resistance (Pistilli et al., 2011; Loro et al., 2015), mitochondrial biogenesis and cristae complexity (Loro et al., 2018). We, therefore, focused on the metabolome of the EDL, a fast muscle which is highly recruited during ambulatory and exercise activity (Slawinska and Kasicki, 2002), and suitable for *ex vivo* evaluation with routine muscle contractility assessment protocols. From a translational perspective, future studies would be required to evaluate how our findings apply to human muscles, in which slow fibers are generally more abundant.

Effect of the *in vivo* Moderate Muscle Stimulation

The predominantly fast EDL muscle, composed of approximately 80% type 2B-2X glycolytic fibers, responded to stimulation with a strong elevation of intermediates of oxidative metabolism. Upon stimulation, 40% of the detected metabolites were significantly changed. 13% of them were down-regulated compared to unstimulated condition. Among the downregulated metabolites were energy substrates such as ATP, GTP and amino acids. Amino acids such as aspartate and glutamine are substrates for the anaplerotic generation of TCA intermediates (e.g., succinate). Consistent with a moderate-intensity exercise protocol, the majority (87%) of the up-regulated metabolites belonged to FAO, TCA cycle, purine nucleotide metabolism, and pentose phosphate pathways. Similarly to our study, exercise in the form of spontaneous wheel running or treadmill increase lipolysis during the resting phase (Monleon et al., 2014; Ezagouri et al., 2019; Sato et al., 2019). Despite higher stimulated glucose levels (2.83×, P = 8.2E-04) from uptake and/or glycogenolysis), glycolytic intermediates accumulated upstream of GAPDH, suggesting that its enzymatic activity could be reduced by the accumulation of NADH (25.95×, P = 1.05E-03) (Mohr et al., 1996) or fumarate (7.48×, P = 1.48E-09) (Blatnik et al., 2008).

The most up-regulated metabolite upon stimulation was the nucleoside IMP (inosine monophosphate, 54.1×, P = 2.34E-11). The deamination of AMP to IMP occurs during moderately intense muscle activity and is catalyzed by the enzyme adenosine deaminase (AMPD), the first and rate-limiting step of the purine nucleotide cycle. The conversion of AMP to IMP is advantageous for the muscle, as it maintains the ATP:AMP ratio and spares adenine nucleotides from degradation to purine bases, allowing their quick reamination during muscle recovery (Sabina et al., 1980). Supporting the role of IMP and purine metabolism during muscle activity, AMPD activity was recently found to be elevated in EDL muscles of mice lacking HDAC3, accounting for their increased fatigue resistance (Hong et al., 2017). Another source of IMP derives from the conversion of glucose-6-phosphate to ribose-5-phosphate in the pentose phosphate pathway, which is then converted to ZMP (3.78×, P = 2.77E-4 induction by stimulation) and enters the purine metabolism cycle. During this process, the concomitant generation of NADPH may support the exercise-induced activation of antioxidant enzymes (Frasier et al., 2013). ZMP is also a potent activator of AMPK, which in turn regulates energy metabolism and exercise capacity by promoting glucose uptake and FAO (Narkar et al., 2008). ZMP-mediated AMPK activation could therefore participate in the regulation of the network of kinase reactions recently identified by phosphoproteomic investigations of exercised muscles (Hoffman et al., 2015; Potts et al., 2017).

In summary, *in vivo* muscle stimulation allowed us to map the metabolic reaction (oxidative and anaplerotic) activated to ensure the replenishment of energy substrates and the recovery

from fatigue. We then compared such map with the metabolic reactions activated in Il15ra$^{-/-}$ muscles.

Role of IL15RA in the Metabolic Response to Stimulation

The enhanced fatigue resistance and energy expenditure (**Figure 2**) of Il15ra$^{-/-}$ mice is accompanied by a significant reprogramming of muscle metabolism, with increased basal IMTG and higher AMPK activation. Such changes are independent of the exercise state and lead to higher basal mitochondrial uptake and oxidation of fatty acids in isolated mitochondria (Loro et al., 2015).

Here, we hypothesized that if IL15RA blockade has exercise mimetic effects, it would cause a shift in basal energy metabolism toward that of stimulated control muscles. Indeed, the levels of several metabolites in the unstimulated Il15ra$^{-/-}$ muscles were intermediate between unstimulated and stimulated controls (**Figure 7A**). A significant effect of genotype was found in microbiota metabolites, amino acid metabolism intermediates, and in the pool sizes of fatty acids and carnitines (**Figure 6**). Glycolytic intermediates were similar between Il15ra$^{-/-}$ and controls, suggesting normal muscle glucose utilization during exercise.

The higher energy output required for sustaining Il15ra$^{-/-}$ exercise capacity is therefore due to enhanced IMTG utilization, fatty acids mobilization, higher mitochondria content (Pistilli et al., 2011, 2013) and cristae complexity (Loro et al., 2018), similarly to what has been described in the muscles of highly trained athletes (Nielsen et al., 2017). In contrast to other experimental models (e.g., overexpression of PGC1-alpha), ablation of IL15RA exerts such effects without changing muscle composition (Loro et al., 2018). It is noteworthy that such small but significant metabolic enhancements translate into clear benefits in terms of muscle contractility, fatigability, and energy expenditure during the mostly inactive light phase (ZT4-8). The magnitude of these differences will be likely bigger during the active dark phase.

The mechanisms responsible for the increased FAO in Il15ra$^{-/-}$ could be mediated by AMPK and CPT1B activity. AMPK activation in Il15ra$^{-/-}$ muscle was not accompanied by substantial changes in the AMP/ATP ratio, suggesting that other factors such as ZMP (elevated by 1.88× and 2.36× in unstimulated and stimulated Il15ra$^{-/-}$) or calcium-dependent signaling could be causing AMPK phosphorylation (Funai et al., 2013). Together with changes in metabolites, protein phosphorylation is an important mediator of the circadian control of metabolism and physiology (Robles et al., 2017) and its kinetics are consistent with the timescale of events happening during a short bout of exercise (Hoffman et al., 2015; Potts et al., 2017). Hence, this study can serve as a starting point for future investigations correlating multiple parameters such as phosphoproteomic and metabolomic responses to different stimulation intensities, different phases of the day, or multiple rodent disease models.

In summary, our data show that IL15RA ablation elevates basal metabolic rates to levels consistent with moderate exercise, in turn favoring FAO reactions as the primary fuel. Together with our previously published work on exercise capacity and resistance to diet-induced obesity (Pistilli et al., 2011; Loro et al., 2015), these findings strengthen the rationale for the future development of new pharmacological exercise mimetic strategies by blocking IL15RA.

MATERIALS AND METHODS

Mice

All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Mice were housed at 22°C under a 12:12-h light-dark cycle (7:00 AM – 7:00 PM) with food and water provided *ad libitum*. All founder mouse lines were purchased from Jackson Laboratories. C57BL/6-Il15ratm2.1Ama/J (IL15RA-flox, stock number 022365) and B6.FVB-Tg(EIIa-cre)C5379Lmgd/J (EIIA-cre, stock number 003724) were crossed to obtain a progeny with ubiquitous deletion of the floxed alleles in all tissues. The correct genotype was confirmed by PCR of floxed alleles (according to Jackson protocol for strain 022365), Cre-recombinase (according to Jackson protocol for strain 003966), *Il15ra* exon 2 (included in the region deleted by EIIa-Cre, Forward 5′-ATTGAGCATGCTGACATCCG-3′, Reverse 5′- TCCAGTGGGCAACATTTGTG-3′, product size 145 bp) and *Il15ra* exon 4 (outside the region deleted by EIIa-Cre, Forward 5′-TGCAGCAACAATGACCTTGG-3′, Reverse 5′- TATGTGGGAAACTGGCCTGTC-3′, product size 96 bp). These mice were then backcrossed to C57BL/6J (stock number 000664) mice to obtain heterozygous Il15ra$^{\pm}$ on a C57BL/6J background, used for the experimental breeding. Ten-week-old male control (Control) and Il15ra$^{-/-}$ littermates were used for the experiments. Spontaneous wheel running (Columbus Instruments) was monitored for approximately 7 days during which mice were single-housed in cages with *ad libitum* access to food and water. Energy expenditure measurements were performed on a CLAMS setup (Columbus Instruments) at the Mouse Phenotyping, Physiology and Metabolism Core of the University of Pennsylvania. In line with previous studies, all *in vivo* experiments were performed between ZT4 and ZT8.

In vivo Sciatic Nerve Stimulation

For *in vivo* stimulation, mice (4 controls and 4 Il15ra$^{-/-}$) were anesthetized with 1% isoflurane. Depth of anesthesia was confirmed by toe pinch and by monitoring HR and SpO_2 with a pulse oximeter (PhysioSuite, Kent Scientific). The sciatic nerve was exposed at both sides, crushed distally to the muscle terminations to prevent retrograde propagation of the stimuli, and connected to a custom-made bipolar stimulating electrode driven by a Grass S48 stimulator. Two recording platinum-iridium (bare 0.13 mm, coated 0.02 mm) needle-electrodes were applied at fixed distance (5 mm), one in the gastrocnemius and the other near the Achilles tendon. The ground electrode was connected to the skin of the back of

the mouse. The recording electrodes were then connected to a Warner Instruments DP-311 differential amplifier and finally to an A/D converter (ADInstruments). Traces were acquired with LabChart 8 (ADInstruments). One leg of each mouse was electrically stimulated, the other was used as sham-operated unstimulated control and dissected prior to the start of the stimulation, paying attention to minimize bleeding. The EMG signal was used to monitor the efficiency and consistency of the high-intensity stimulation protocol. The sequence of stimuli was based on the protocol originally described by Baar and Esser (1999) and was previously used to investigate phosphoproteomic changes in exercised muscle of mice (O'Neil et al., 2009; Potts et al., 2017). Throughout the experiment, vital parameters were monitored with a pulse oximeter (**Figure 1A**). The stimulation protocol used for the study consisted of 3-s trains of 0.1 ms pulses repeating at a frequency of 100 Hz (**Figure 1B**, – block A). Each 3-s train was repeated 6 times, alternating with 10 s of rest (**Figure 1B** – block B). This stimulation pattern was repeated 10 times with 1 min of rest between each repetition, for a total duration of 22 min (**Figure 1B**, – block C).

Stimulated muscles were collected immediately at the end of the protocol. EDL muscles were quickly flash frozen after dissection and processed for metabolite extraction. An independent set of two control and two Il15ra$^{-/-}$ mice (not included in the current study) was used to validate the efficacy of the stimulation protocol and the capacity of our metabolomics approach to detect changes associated with muscle activity.

Ex vivo Muscle Physiology

Muscle physiological analysis was performed on isolated EDL muscles using an Aurora Mouse 1200A System equipped with Dynamic Muscle Control v.5.415 software. EDL muscles were dissected and analyzed in constantly oxygenated Ringer's solution (100 mM NaCl, 4.7 mM KCl, 3.4 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES, 5.5 mM D-glucose) at 24°C. The twitch stimulation protocol applied was a single stimulus with a duration of 0.2 ms. Muscle length was adjusted to obtain the maximal twitch response and this length was measured and recorded as optimal length (L_0). For measuring tetanic maximal force generation, the stimulus was repeated at a frequency of 120 Hz for 500 ms. 5 min were allowed between two tetanic contractions to ensure muscle recovery. For induction of fatigue, 5 min after the last maximal tetanic contraction, muscles were stimulated every second for 8 min using 40-Hz pulses lasting 330 ms. Following the fatigue protocol, a burst of 50 maximal tetanic contractions (120 Hz for 500 ms) was applied to ensure complete fatigue across all samples. The recovery protocol started 1 s after the last burst contraction. A maximal tetanic stimulation (120 Hz for 500 ms) was given every 5 min for 30 min, and the force recovery was expressed as the percentage of the maximal isometric tetanic force. Muscle cross-sectional area (CSA) of EDL muscles was calculated dividing the muscle mass by the product of the muscle density coefficient (1.06 g/cm^3), muscle L_0, and the fiber length coefficient (0.45 for EDL). Specific force was determined by normalizing maximum isometric tetanic force to CSA.

Metabolites Extraction

Metabolomics was performed at Penn DRC Metabolomics Core. To extract metabolites from tissue samples, frozen tissue samples were ground at liquid nitrogen temperature with a Cryomill (Retsch, Newtown, PA, United States). The resulting tissue powder (∼20 mg) was weighed and then extracted by adding −20°C extraction solvent (40:40:20 methanol:acetonitrile:water), vortexed, and immediately centrifuged at 16,000 × *g* for 10 min at 4°C. The volume of the extraction solution (μL) was 40× the weight of tissue (mg) to make an extract of 25 mg tissue per mL solvent. The supernatant was collected for LC-MS analysis.

Metabolite Measurement by LC-MS

A quadrupole-orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific, San Jose, CA, United States) operating in negative or positive ion mode was coupled to hydrophilic interaction chromatography via electrospray ionization and used to scan from m/z 70 to 1000 at 1 Hz and 75,000 resolution. LC separation was on a XBridge BEH Amide column (2.1 mm × 150 mm, 2.5 μm particle size, 130 Å pore size; Waters, Milford, MA, United States) using a gradient of solvent A (20 mM ammonium acetate, 20 mM ammonium hydroxide in 95:5 water: acetonitrile, pH 9.45) and solvent B (acetonitrile). Flow rate was 150 μL/min. The LC gradient was: 0 min, 85% B; 2 min, 85% B; 3 min, 80% B; 5 min, 80% B; 6 min, 75% B; 7 min, 75% B; 8 min, 70% B; 9 min, 70% B; 10 min, 50% B; 12 min, 50% B; 13 min, 25% B; 16 min, 25% B; 18 min, 0% B; 23 min, 0% B; 24 min, 85% B; 30 min, 85% B. Autosampler temperature was 5°C, and injection volume was 3 μL. Data were analyzed using the MAVEN software.

Metabolomics Data Analysis

Missing or equal to zero values (11/5136) were replaced with a small value, calculated as the half of the minimum detection limit (minimum positive values in the original data). The assumption of this approach is that most missing values are caused by low abundance metabolites (i.e., below the detection limit). The values were for the following metabolites: 6-phosphogluconate (1), C24:3 (2), NADH (2), acetyl-CoA (1), deoxyadenosine (4 = 2 control unstimulated and 2 control stimulated), Tetradecadienoyl carnitine C14:2 (1).

For all the analyses, metabolomics data were $\log_2$-transformed. Heatmap of $\log_2$-transformed, row-wise normalized (mean of 0 and variance of 1) values was generated with the ComplexHeatmap R package (Gu et al., 2016) considering euclidean distance and average linkage. PLS-DA and pathway analysis were performed using the Metaboanalyst online platform (Chong et al., 2018) with standard settings. PLS-DA is a supervised method that uses multivariate regression techniques to maximize the covariance between exploratory and categorical codependent variables. The significance of class discrimination was assessed using a permutation test (2000 permutations,

P = 0.007). The variance on each component in relation to metabolites can be graphically represented as loading plots. The PLS-DA analysis also returned a VIP score. The VIP score describes the weighted contribution of each metabolite to the variation of each component. The 25 most important features in each component, as identified by PLS-DA, were plotted. Pathway representation in **Figure 5**, was obtained with Cytoscape 3.7.1. Euclidean distance for each detected metabolite in **Figure 7A**, was calculated as the absolute value of the difference between the $\log_2$-transformed mean values at the different conditions. The obtained distances were then rescaled to a range from 0 (unstimulated control) and 1 (stimulated control).

Data Analysis and Statistics

Data are presented as mean ± standard error of the mean. All statistical analyses were performed in R 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria[1]) using the packages lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2017). Data visualization was performed with the ggplot2 R package (Gómez-Rubio, 2017) or GraphPad Prism 8 (GraphPad Software, La Jolla, CA, United States). All statistical tests were considered significant at $\alpha \leq 0.05$, unless stated otherwise. Univariate analysis of the metabolomics data was performed by 2-way ANOVA (Genotype × Stimulation) followed by the Benjamini-Hochberg FDR adjustment. To compare the effects of stimulation in the two genotypes, pairwise multiple comparisons were performed using the R package emmeans and adjusting the raw P-values using the Sidak method. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

[1] http://www.r-project.org/

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC of the University of Pennsylvania.

AUTHOR CONTRIBUTIONS

EL contributed to the conceptualization, the formal analysis, and the writing of the original draft. EL and CJ contributed to the methodology. EL, CJ, and WQ contributed to the investigation. EL, CJ, JB, ZA, and TK contributed to the reviewing, editing of the article writing. TK contributed to the funding acquisition and supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01439/full#supplementary-material

TABLE S1 | Contractile and morphometric measurements of EDL muscles.

TABLE S2 | Metabolomics dataset.

TABLE S3 | Partial least squares discriminant analysis loadings.

TABLE S4 | Partial least squares discriminant analysis VIP scores.

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