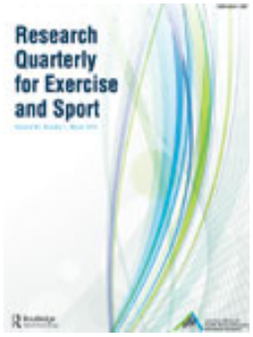

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NIRS-Derived Muscle-Deoxygenation and Microvascular Reactivity During Occlusion–Reperfusion at Rest Are Associated With Whole-Body Aerobic Fitness

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ABSTRACT

Purpose: Near-infrared spectroscopy (NIRS) indices during arterial occlusion–reperfusion maneuver have been used to examine the muscle's oxidative metabolism and microvascular function—important determinants of whole-body aerobic-fitness. The association of NIRS-derived parameters with whole-body VO_2max was previously examined using a method requiring exercise (or electrical stimulation) followed by multiple arterial occlusions. We examined whether NIRS-derived indices of muscle deoxygenation and microvascular reactivity assessed during a single occlusion–reperfusion *at rest* are (a) associated with maximal/submaximal indices of whole-body aerobic-fitness and (b) could discriminate individuals with different VO_2max. We, also, investigated which NIRS-parameter during occlusion–reperfusion correlates best with whole-body aerobic-fitness. **Methods:** Twenty-five young individuals performed an arterial occlusion–reperfusion *at rest*. Changes in oxygenated- and deoxygenated-hemoglobin (O_2Hb and HHb, respectively) in vastus-lateralis were monitored; adipose tissue thickness (ATT) at NIRS-application was assessed. Participants also underwent a maximal incremental exercise test for VO_2max, maximal aerobic velocity (MAV), and ventilatory-thresholds (VTs) assessments. **Results:** The HHbslope and HHbmagnitude of increase (occlusion-phase) and O_2Hbmagnitude of increase (reperfusion-phase) were strongly correlated with VO_2max ($r = .695–.763$, $p < .001$) and moderately with MAV ($r = .468–.530$; $p < .05$). O_2Hbmagnitude was moderately correlated with VTs ($r = .399–.414$; $p < .05$). After controlling for ATT, the correlations remained significant for VO_2max ($r = .672–.704$; $p < .001$) and MAV ($r = .407$; $p < .05$). Individuals in the high percentiles after median and tritile splits for HHbslope and O_2Hbmagnitude had significantly greater VO_2max vs. those in low percentiles ($p < .01–.05$). The HHbslope during occlusion was the best predictor of VO_2max. **Conclusion:** NIRS-derived muscle deoxygenation/reoxygenation indices during a single arterial occlusion–reperfusion maneuver are strongly associated with whole-body maximal indices of aerobic-fitness (VO_2max, MAV) and may discriminate individuals with different VO_2max.

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Maximal oxygen consumption; microvascular reactivity; muscle oxidative metabolism; near-infrared spectroscopy

Introduction

Aerobic fitness is strongly associated with longevity and is considered an important predictor of a wide range of conditions across the spectrum of health and disease (Lee et al., 2010; Ross et al., 2016). Maximal oxygen uptake (VO_2max) has been accepted as the gold standard for assessing aerobic fitness. Direct VO_2max testing requires an open-circuit respirometry, induces high physiological stress (Ferguson, 2014), and is influenced by the individual's motivation to exert maximal effort (Grant et al., 1995; Sartor et al., 2013), which may not be always feasible.

The muscle's ability to use/consume oxygen is one of the main determinants of whole-body VO_2max and aerobic fitness. The positive correlation between the muscle's oxidative capacity and VO_2max is well established (Jacobs & Lundby, 2013; Larson-Meyer et al., 2000; Tonkonogi & Sahlin, 1997; Zoll et al., 2002). Muscle oxidative metabolism is primarily assessed using traditionally accepted *in vitro* and *in situ* techniques (e.g. high-resolution respirometry, bioluminescence, and mitochondrial enzyme content/activity) or costly and not readily available *in vivo* methods (^{31}P-MRS; Grassi & Quaresima, 2016; Ryan et al., 2013, 2014; Willingham & McCully, 2017). Despite providing valuable information, the applicability and utility of such approaches are limited by their invasive nature, the inability to reflect *in vivo* muscle oxidative capacity (except for ^{31}P-MRS), the need for a high level of technical expertise and cost, and unavailability for onsite measurement (Grassi & Quaresima, 2016; Willingham & McCully, 2017).

Near-infrared spectroscopy (NIRS) offers a less expensive, portable, noninvasive and *in vivo* evaluation of the muscle's ability to use/consume oxygen (Grassi & Quaresima, 2016; Jones et al., 2016; Willingham & McCully, 2017). Recent studies highlighted the correlation between NIRS-derived muscle mitochondrial oxidative capacity and VO_2max (Beever et al., 2020; Guzman et al., 2020; Lagerwaard et al., 2019, 2021), supporting the physiological relevance of NIRS measurements (Lagerwaard et al., 2019). The approach to assess mitochondrial oxidative capacity and its correlation with VO_2max (Beever et al., 2020; Guzman et al., 2020; Lagerwaard et al., 2019, 2021) or endurance performance (Batterson et al., 2020) used in these studies is based on the recovery kinetics of oxyhemoglobin (O_2Hb) by

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using multiple arterial occlusions, following electrical stimulation or voluntary contractions. The method has a good agreement with well-established *in situ* and *in vivo* techniques (Ryan et al., 2013, 2014). The numerous repeated occlusions (typically 2–3 trials of 18–36 occlusions per trial) and length of protocol (30–45 min) may not be tolerated by some individuals (Sumner et al., 2020) and, the relatively complicated measuring (Lagerwaard et al., 2020) and analyzing procedures (Beever et al., 2020) accompanying this method could limit its wide use. Also, some of the assumptions underlying this technique may not necessarily apply for populations with impaired microcirculation.

An arterial occlusion/reperfusion maneuver at rest with NIRS monitoring enables the noninvasive evaluation (a) of the muscle's oxidative metabolism (occlusion phase; Barstow, 2019; Grassi et al., 2007; Hamaoka et al., 1996; Sako et al., 2001; Soares et al., 2019; Van Beekvelt et al., 2001) and (b) the muscle's microvascular reactivity (reperfusion phase) that has been used as a proxy measure of the downstream microvascular function within the skeletal muscle (De Backer et al., 2012; Gayda et al., 2015; Manetos et al., 2011; McLay et al., 2016a). The single occlusion–reperfusion maneuver is more time efficient, less costly, simpler (with no advance analysis and calculations), and has been consistently used in individuals with vascular problems to assess microvascular function (Dipla, Triantafyllou, Koletsos et al., 2017; Gayda et al., 2015; Manetos et al., 2011; Soares & Murias, 2018). Most importantly, the procedure does not require any type of exercise or electrical stimulation, which is required for methodology used in previous studies, to assess muscle oxidative metabolism (see references above). The underlying theory is that in the absence of blood flow during occlusion, the oxygen content of the muscle tissue diminishes as oxygen is consumed by the mitochondria (Barstow, 2019; Grassi & Quaresima, 2016; Hamaoka et al., 1996; Sako et al., 2001; Van Beekvelt et al., 2001); whereas, during reperfusion the rate of muscle reoxygenation shows the ability of the microvasculature to accommodate the increase in blood flow (an index of microvascular function; Dipla, Triantafyllou, Koletsos et al., 2017; McLay et al., 2016a, McLay et al., 2016). The latter primarily reflects the function of capillaries, as these micro-vessels compose the largest portion of vascular volume in skeletal muscle (>90%; Jones et al., 2016; Jonk et al., 2007) and they function mainly to supply oxygen to muscle fibers playing an important role in whole-body VO_2 during exercise.

Previous studies showed that muscle deoxygenation achieved during an arterial occlusion *at rest* were similar or even greater compared to that achieved during maximal incremental or severe intensity aerobic exercise (Inglis et al., 2017, 2019; Spencer et al., 2014). Furthermore, NIRS studies using the single occlusion and/or reperfusion maneuver have reported a greater deoxygenation rate (Boone et al., 2014; Dipla, Triantafyllou, Koletsos et al., 2017, Dipla, Triantafyllou, Grigoriadou et al., 2017; Manetos et al., 2011) and reoxygenation (Dipla, Triantafyllou, Koletsos et al., 2017; Gayda et al., 2015; Manetos et al., 2011; Soares & Murias, 2018) in healthy compared to individuals with chronic diseases. The NIRS-derived parameters of muscle's deoxygenation (McLay et al., 2016; Rasica et al., 2022; Van Thienen & Hespel, 2016) and reoxygenation (McLay et al., 2016; Soares et al., 2018) were also higher in trained compared to untrained individuals. These studies, however, do not show whether the changes in NIRS (magnitude/rate of muscle deoxygenation/reoxygenation) during an arterial occlusion–reperfusion maneuver are associated with various whole-body indices of aerobic fitness and which is the magnitude of this association, as well as whether NIRS-changes during this simple test may discriminate individuals with different VO_2max. That is, to what extent changes in NIRS parameters of muscle deoxygenation or reoxygenation during an arterial occlusion–reperfusion may explain VO_2max differences among individuals and act as markers (predictors) of whole-body aerobic fitness. Furthermore, the association of muscle microvascular reactivity with whole-body aerobic capacity is understudied (Rasica et al., 2022). Such association would increase the physiological relevance and utility of the NIRS measurements in assessing aerobic capacity.

Thus, the novel aims of this study were to examine whether during a *single* leg arterial occlusion–reperfusion maneuver *at rest*, the vastus lateralis muscle deoxygenation and reoxygenation NIRS-parameters: (a) would correlate with whole-body VO_2max, maximal aerobic velocity (MAV), and first and second ventilatory thresholds (VT_1, and VT_2); (b) would discriminate individuals based on their VO_2max values; and (c) which of the most commonly assessed NIRS measurement during a simple to perform occlusion–reperfusion maneuver is best associated with VO_2max and other indices of whole-body aerobic fitness.

Materials and methods

Participants

Twenty-five healthy, young, adult individuals (20 males and 5 females) with moderate-to-high aerobic capacity (VO_2 max: 57.7 ± 6.7 ml·kg^{-1}·min^{-1}; age: 21.8 ± 4.3 years; body weight: 69.2 ± 9.5 kg; body height: 173.1 ± 10.0 cm) participated in this study. All participants were free of cardiac, respiratory, and metabolic diseases and none of them used nutritional supplements or medication prior to or during the experimental procedures. The study was approved by the institutional review board committee and was conducted in accordance with the Declaration of Helsinki (1983 revision). Prior to enrollment, the participants were informed for all testing procedures, potential risks and benefits associated with the study and signed the informed consent form.

Study design

Each individual participated in two experimental sessions performed in a random order; the two sessions were separated by 3–4 days. In one visit, the participants performed a maximal incremental exercise test on a treadmill, for the assessment of VO_2max, maximal aerobic velocity (MAV), and first and second ventilatory thresholds (VT_1 and VT_2, respectively).

In the other experimental visit, the participants underwent an arterial occlusion–reperfusion maneuver at rest, to examine the muscle's oxidative metabolism (magnitude and rate of deoxygenation) and microvascular reactivity/function (magnitude and rate of reoxygenation) using near-infrared spectroscopy (NIRS). The two sessions were conducted at similar morning hours. Participants were requested to refrain from consuming caffeinated and tobacco products during the 12-hr period preceding the tests, and to abstain from alcohol drinks and exercise for at least 24 hr before tests.

Maximal incremental exercise test

Each participant performed a maximal incremental test on a treadmill (h/p/cosmos pulsar 3p, Nussdorf-Traunstin, Germany) for the assessment of VO_2max, MAV, VT_1, and VT_2. The initial velocity was set at 8 km·hr^{-1} (1% slope) and was increased by 1.5 km·hr^{-1} every 3 min until volitional exhaustion. Respiratory gas exchange was measured breath by breath (Viasys Encore 229) and heart rate was recorded continuously (Polar RS400, Polar Electro, Finland) during the incremental test. The O_2 and CO_2 analyzers were calibrated prior to and after each test using ambient air and gas of known composition (15.8% and 5.0%, respectively). It was considered that the participants attained maximal effort when at least three of the following criteria were achieved: (1) a plateau in oxygen consumption (<2 ml·kg^{-1}·min^{-1} despite an increase in running velocity), (2) visual exhaustion of the participants and inability to sustain exercise despite continuous verbal encouragement, (3) respiratory exchange ratio of at least 1.15 and (4) maximal heart rate higher than 90% of the predicted maximum (220-age; Kraemer et al., 2011; Marciniuk et al., 2013).

All the cardiorespiratory data of the maximal incremental test were averaged over 30-s periods. VO_2max was defined as the highest 30-s VO_2 value recorded. MAV was calculated using the following formula: MAV (km/h) = Velocity of last completed stage + (sec run at the last stage/180; Kuipers et al., 1985; Smilios et al., 2018).

The ventilatory equivalents (VE/VO_2 and VE/VCO_2) and the end tidal pressures ($P_{ET}O_2$ and $P_{ET}CO_2$) of VO_2 and VCO_2 were used for the identification of the first and second ventilatory thresholds (VT_1 and VT_2, respectively). VT_1 was determined as the VO_2 value (ml/min) where increases in both VE/VO_2 and $P_{ET}O_2$ were observed with no concomitant increase in VE/VCO_2, whereas VT_2 as the VO_2 value (ml/min) where increases in both the VE/VO_2 and VE/VCO_2 and a decrease in $P_{ET}CO_2$ were detected (Keir et al., 2022; Santos & Giannella-Neto, 2004). The running speeds corresponding to these VO_2 values were used as velocities at VT_1 (vVT_1) and VT_2 (vVT_2).

Occlusion–reperfusion maneuver at rest

The muscle oxidative metabolism and microvascular reactivity were assessed during the arterial occlusion–reperfusion maneuver using near-infrared spectroscopy (NIRS; Oxymon III, Artinis, The Netherlands). The NIRS device consists of two probes, a light emitter and a detector, and continuously measures the relative changes in O_2Hb, HHb, and tHb in the microvasculature of the skeletal muscle (Boushel et al., 2001). Participants adopted a supine position with their legs extended and the NIRS device (a pair of probes) was placed on the shaved and cleaned skin surface over the belly of the vastus lateralis (VL) muscle (~12 cm above the patella and 5 cm lateral to the midline). The probes were housed in a plastic holder and separated by 4.5 cm resulting in a penetration depth of approximately 22.5 mm. The plastic holder was secured with Velcro straps and a black bandage stabilized and covered the optode holder to reduce the intrusion of external light. NIRS parameters (O_2Hb, HHb, and tHb) were recorded during rest for 5 min, arterial occlusion (350 mmHg) for 3 min, and a subsequent 2-min reperfusion phase. The NIRS data were collected at a frequency of 10 Hz and stored for subsequent analysis. All NIRS signals were adjusted to zero just prior to the initiation of occlusion–reperfusion maneuvers and thus present changes from baseline value. Adipose tissue thickness (ATT) at the site of NIRS application was measured in triplicate with skin-fold caliper to the nearest 0.5 mm (Harpenden, UK). The skinfold data for each site were averaged and then halved to provide an ATT measurement at the site of NIRS-device application. These data were subsequently used for controlling for the associations among whole-body indices of aerobic fitness and NIRS parameters.

The muscle oxidative metabolism was assessed using the linear slope and the magnitude of increase in HHb (HHbslope and HHbmagnitude, respectively). This technique is based on the premise that obstruction of both arterial inflow and venous outflow results in a static compartment. As a consequence, the skeletal muscle relies only on O_2 available within the capillary bed and the increases in HHb (or reductions in O_2Hb) per unit of time, reflect the ability of muscle to extract/utilize O_2 (Barstow, 2019; Grassi & Quaresima, 2016; Hamaoka & McCully, 2019; Ryan et al., 2012; Van Beekvelt et al., 2001). The cuff was inflated to supra-systolic pressure 350 mmHg to ensure a closed circulatory compartment (Barstow, 2019; Van Beekvelt et al., 2001). Despite the circulatory occlusion, we corrected the changes in O_2Hb and HHb during the occlusion phase for possible blood volume fluctuations (tHb) as previously described (Ryan et al., 2012). After blood volume (tHb) corrections, the regressions for O_2Hb and HHb were highly linear and the changes of O_2Hb and HHb mirrored each other. Representative traces for changes in O_2Hb, HHb, and tHb before and after correction for changes in blood volume (tHb) are depicted in Figure 1. The HHbslope and HHbmagnitude of increase were calculated during the 180-s of arterial occlusion.

Microvascular reactivity and responsiveness were assessed by monitoring the changes in muscle oxygenation (O_2Hb) during the reperfusion phase following cuff release (reactive hyperemia). This measure shows the ability of microvasculature to accommodate the increase in blood flow in response to a physiologic stimulus and has been used as an index of microvascular function (Dipla, Triantafyllou, Grigoriadou et al., 2017; McLay et al., 2016; Papadopoulos et al., 2018; Soares et al., 2019). This NIRS approach has shown high inter- and intraday reliability (McLay et al., 2016a, McLay et al., 2016) and it has emerged as a robust tool for the in vivo evaluation of

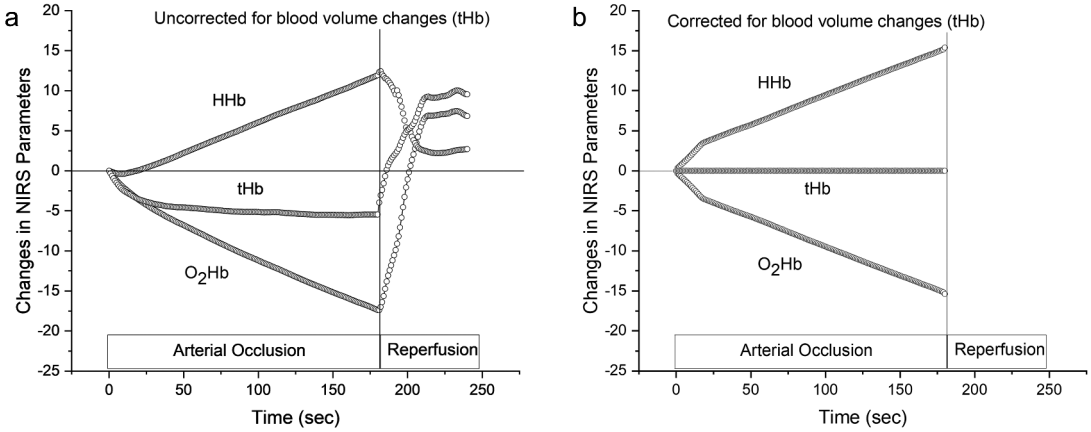

Figure 1. Representative traces for changes in O_2Hb, HHb, and tHb (1-s averages) during occlusion before (a) and after (b) correction for blood volume changes (tHb). NIRS-signals were corrected for changes in blood volume only during arterial occlusion.

muscle microvascular function and/or microcirculation in healthy individuals and in patients with chronic disease (Dipla, Triantafyllou, Koletsos et al., 2017, Dipla, Triantafyllou, Grigoriadou et al., 2017; McLay et al., 2016; Papadopoulos et al., 2018; Soares et al., 2019). The magnitude of O_2Hb increase (O_2Hbmagnitude) during reperfusion (difference between the lowest and the highest value) and the linear slopes for O_2Hb increase to peak (O_2Hbslope-to-peak) and for O_2Hb increase during the initial 10 s (O_2Hbslope10s) after cuff release were used as measures of microvascular reactivity.

Statistical analysis

Data are reported as mean ±SD and were analyzed using SPSS software (Version 25, IBM, Armonk, NY). The normality of data distribution was checked by the skewness and kurtosis statistics. The muscle HHbmagnitude during the occlusion and O_2Hbmagnitude during the reperfusion phases were calculated by the difference between the maximal and minimal values in the respective phases of the occlusion–reperfusion maneuver. A linear regression analysis was applied to the occlusion and reperfusion data for calculating the slope (rate) of deoxygenation and reoxygenation (muscle microvascular reactivity). Pearson (r) and Spearman (r_s) correlations for normally and not normally distributed data, respectively, were used to examine the relationship between NIRS variables during occlusion and reperfusion phases with the indices of aerobic fitness (VO_2max, MAV, VT_1, and VT_2). Partial correlations were also performed for identifying significant correlations among whole-body indices of aerobic fitness and NIRS-derived parameters after controlling for ATT at the site of NIRS-device parametric and non-parametric correlations.

The NIRS-derived variables with the highest correlation with VO_2max during the occlusion (HHbslope) and reperfusion (O_2Hbmagnitude) phases were selected to examine the ability of NIRS to distinguish individuals with different VO_2 max values. Three approaches were used: (a) the participants were divided into two groups using the 50th percentile value; (b) we divided the sample in tritiles dropping the middle tritile. This practice (excluding the middle values) has been recommended (DeCoster et al., 2011; Rucker et al., 2015) as one gains additional confidence that those in the "low" and "high" categories are distinct from one another; (c) two groups of seven participants were formed after matching for ATT (within 0–0.25 mm) the participants from the higher and the lower 50th percentile groups. Student's t-tests and ANCOVA when appropriate (using ATT as covariate) were performed to assess the ability of NIRS-derived parameters during the occlusion–reperfusion maneuver to distinguish individuals with different VO_2max. Cohen's d statistic was used to determine the effect size for comparisons between groups. The small, medium, and large effects would be reflected for d values greater than 0.2, 0.5, and 0.8, respectively. The 95% confidence intervals for the between-group differences (CI_{95}) were also calculated. The level of significance for all statistical tests was set at a p value of .05.

Results

Aerobic performance, muscle oxidative metabolism, and muscle microvascular reactivity

Participants' characteristics during the assessment of whole-body aerobic capacity (VO_2max, MAV, VT_1, and VT_2, and ATT), as well as the HHbmagnitude and HHbslope of increase during 180-s of occlusion and the O_2Hbmagnitude of increase during reperfusion are presented in Table 1.

Correlations of NIRS-derived muscle deoxygenation with whole-body aerobic fitness

As expected, the changes in HHb mirrored those of O_2Hb after correction for blood volume (tHb) during arterial occlusion; hence, only the results for HHb are presented during the occlusion. Skeletal muscle oxidative metabolism was assessed by evaluating the increase in HHb signal during the 3 min of arterial occlusion. The HHbslope and HHbmagnitude of increase during the 180-s arterial occlusion showed the highest correlation with whole-body relative VO_2max among all NIRS-derived variables included in this study (r = .763 and r = .717,

Table 1. Participants' whole-body parameters of aerobic capacity and of NIRS-derived parameters from vastus lateralis muscle during the arterial occlusion and reperfusion.

Whole-body aerobic capacity (assessed by maximal exercise testing)	
VO_2max ($ml \cdot kg^{-1} \cdot min^{-1}$)	57.7 ± 6.7
MAV ($km \cdot hr^{-1}$)	15.4 ± 1.9
VT_1 ($km \cdot hr^{-1}$)	10.4 ± 1.9
VT_2 ($km \cdot hr^{-1}$)	12.4 ± 1.8
Adipose tissue thickness at NIRS application (mm)	5.50 ± 1.7
NIRS-derived parameters from the vastus lateralis muscle	
Whole period (180 s) of arterial occlusion	
Magnitude of increase in HHb (μM)	17.35 ± 6.50
Slope of increase in HHb from baseline to peak values ($\mu M \cdot s^{-1}$)	0.088 ± 0.033
Reperfusion following 180-min of occlusion	
Magnitude of increase in O_2Hb (μM)	31.82 ± 9.99
Slope of increase in O_2Hb to peak values ($\mu M \cdot s^{-1}$)	0.993 ± 0.581
Slope of increase in O_2Hb from cuff release to 10 s ($\mu M \cdot s^{-1}$)	1.232 ± 0.581

Note. Values are means ±SD (n = 25); VO_2max = maximal oxygen consumption; MAV = maximal aerobic velocity; VT_1 = first ventilatory threshold; VT_2 = second ventilatory threshold; HHb = deoxygenated hemoglobin; O_2Hb = oxygenated hemoglobin; NIRS = near infrared spectroscopy.

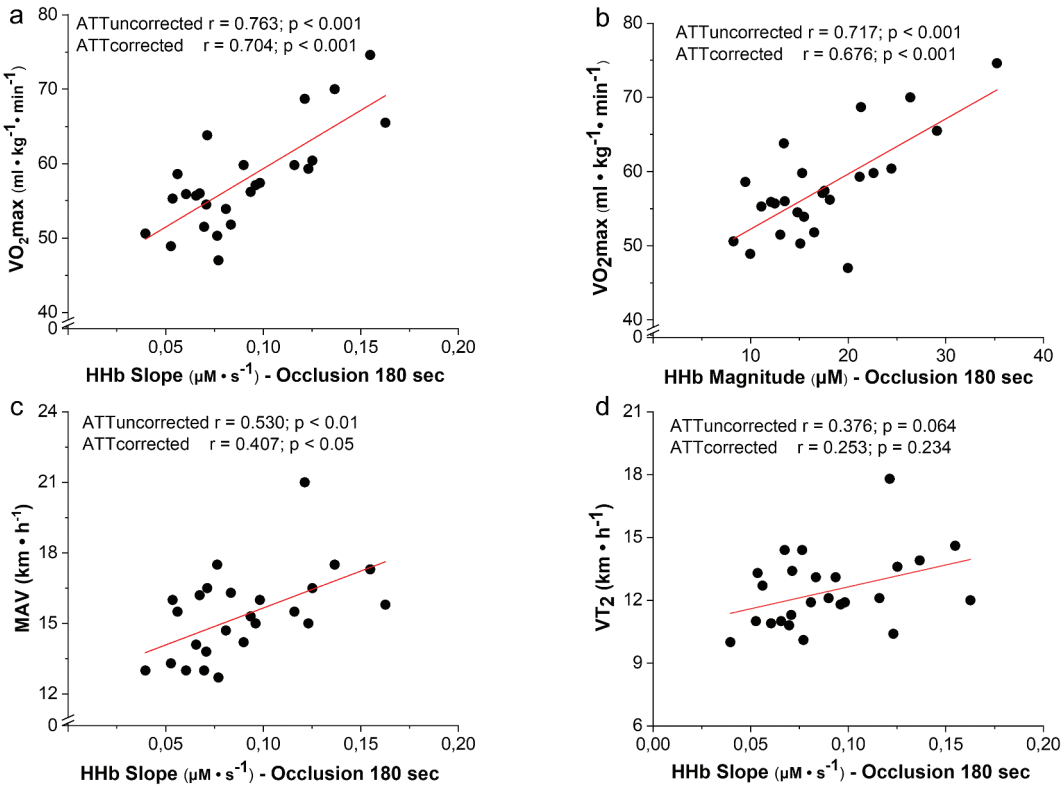

Figure 2. Correlation of VO_2max with HHbslope (a) and HHbmagnitude (b) after 180 s of arterial occlusion; correlation of HHbslope after 180 s of arterial occlusion with MAV (c) and VT_2 with (d); ATT = adipose tissue thickness at NIRS application.

respectively; $p < .001$; Figure 2(a–b). ATT was moderately correlated with HHbslope ($r = .463$; $p < .05$), but not with HHbmagnitude ($r = .327$; $p = .111$). After controlling for ATT, significant correlations remained between the NIRS occlusion indices with VO_2max ($r = .704$ and $r = .676$, for slope and magnitude respectively; $p < .001$). The above correlations were similar for absolute VO_2max as well.

The NIRS-derived variables of muscle deoxygenation moderately correlated with MAV (HHbslope: $r = .530$, $p < .01$, Figure 2c; HHbmagnitude: $r = .468$, $p < .05$). There were no statistically significant relationships for muscle deoxygenation indices during arterial occlusion with vVT_1 (HHbslope: $rs = .370$; $p = .069$ and HHbmagnitude: $rs = .354$; $p = .082$) and absolute VO_2 at VT_1 (HHbslope: $r = .254$; $p = .220$ and HHbmagnitude: $r = .260$; $p = .209$). No significant correlations were also observed for NIRS parameters with vVT_2 (HHbslope: $r = .376$; $p = .064$; HHbmagnitude: $r = .356$, $p = .081$, Figure 1d and absolute VO_2 at VT_2 (HHbslope: $r = .318$; $p = .122$ and HHbmagnitude: $r = .342$; $p = .095$). After ATT adjustment, a significant correlation remained between the HHbslope and MAV (HHbslope: $r = .407$, $p < .05$; HHbmagnitude: $r = .381$, $p = .066$), but the associations were not significant for NIRS occlusion indices with

vVT_1 (HHbslope: $rs = .279$; $p = .187$ and HHbmagnitude: $rs = .282$; $p = .182$), absolute VO_2 at VT_1 (HHbslope: $r = .082$, $p = .704$ and HHbmagnitude: $r = .132$, $p = .537$), vVT_2 (HHbslope: $r = .253$, $p = .234$ and HHbmagnitude: $r = .270$, $p = .203$), and absolute VO_2 at VT_2 (HHbslope: $r = .128$, $p = .551$ and HHbmagnitude: $r = .209$, $p = .327$).

VO_2max values were also significantly correlated with MAV ($r = .652$, $p < .001$), vVT_1 ($rs = .415$, $p < .05$), vVT_2 ($r = .578$, $p < .01$); a high correlation was observed between MAV and ventilatory thresholds (vVT_1: $rs = .911$, $p < .001$) and vVT_2 ($r = .936$, $p < .001$).

Correlations of NIRS-derived muscle oxygenation during reperfusion with aerobic parameters

The muscle microvascular reactivity was evaluated during the reperfusion phase using (a) the magnitude of O_2Hb increase and (b) the rates of the O_2Hb increase to peak value and during the initial 10 s of reactive hyperemia. All of these reperfusion indices (proxy measures of microvascular function) were associated with VO_2max (O_2Hbmagnitude: $r = .695$, O_2Hbslope10s: $r = .653$, Figure 3(a–b); O_2Hbslope-to-peak: $r = .606$; $p < .001$ for all). ATT was not correlated with reperfusion indices ($r = -.202$ - $-.340$; $p = .100–.333$). After controlling for ATT, the significant correlations remained between the NIRS reperfusion indices with VO_2max (O_2Hbmagnitude: $r = .672$; O_2Hbslope10s: $r = .641$; O_2Hbslope-to-peak: $r = .541$; $p < .01–.001$). The correlations were similar for absolute VO_2max.

All NIRS-derived muscle oxygenation indices during reperfusion were moderately associated with MAV (O_2Hbmagnitude: $r = .495$, Figure 3c; O_2Hbslope-to-peak: $r = .433$; O_2Hbslope10s: $r = .443$; $p < .05$ for all). vVTs were moderately correlated only with O_2Hbmagnitude (VT_1: $r_s = .399$, $p < .05$; VT_2: $r = .414$, $p < .05$, Figure 3d. When associations of NIRS reperfusion parameters with absolute VO_2 at VT_1 and VT_2 were examined, significant moderate correlations were found for O_2Hbmagnitude and O_2Hbslope-to-peak with absolute VT_2 ($r = .410$ and $r = .431$, $p < .05$); the correlations of NIRS reperfusion parameters with absolute VO_2 at VT_1 were not significant ($r = .271–.358$, $p = .079–.190$). After ATT adjustment, significant correlations remained between the NIRS reperfusion indices and MAV (O_2Hbmagnitude: $r = .442$ and O_2Hbslope10s: $r = .402$; $p < .05$), but not for vVTs ($r/r_s = .209–.358$, $p = .086–.327$) and absolute VO_2 at VTs ($r = .213–.350$, $p = .094–.317$).

Ability of NIRS-derived muscle deoxygenation to distinguish individuals based on their VO_2max

The results showed that after the median split of HHbslope and O_2Hbmagnitude data, the "high" and "low" groups differed by design (for HHbslope: 0.114 ± 0.026 vs. 0.063 ± 0.011 $\mu M \cdot s^{-1}$, $d = 2.5$, $CI_{95} = 0.03–0.07$, and for O_2Hbmagnitude: 40.2 ± 7.5 vs. 24.1 ± 3.5 μM, $d = 2.8$, $CI_{95} = 11.32–20.88$; $p < .001$ for both). ATT was different between the "high" and "low" groups with HHbslope split (4.7 ± 1.2 vs. 6.5 ± 1.8 mm, $d = 1.2$, $CI_{95} = -3.06$ to -0.54; $p < .01$) but not with the

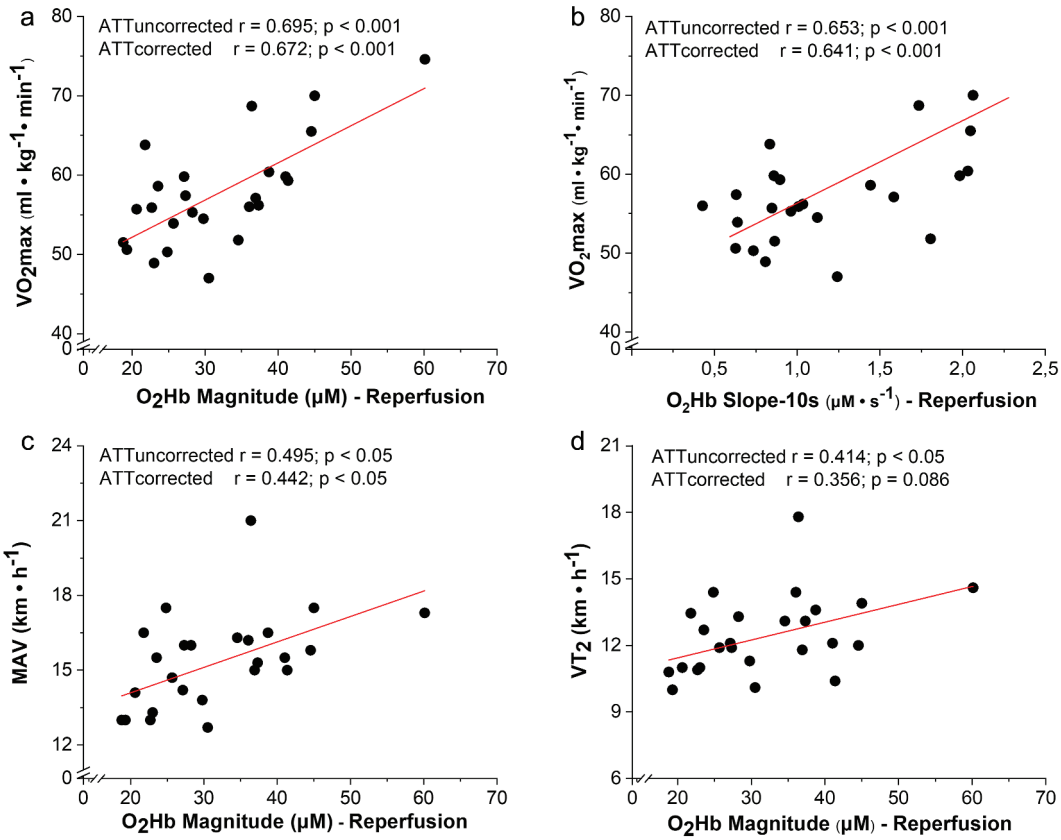

Figure 3. Correlation of VO_2max with the magnitude of O_2Hb increase during reperfusion (a) and the rate of O_2Hb increase (slope) during the initial 10 s of reperfusion (b); correlation of the magnitude of O_2Hb increase during reperfusion with MAV (c) and VT_2 (d); ATT = adipose tissue thickness at NIRS application.

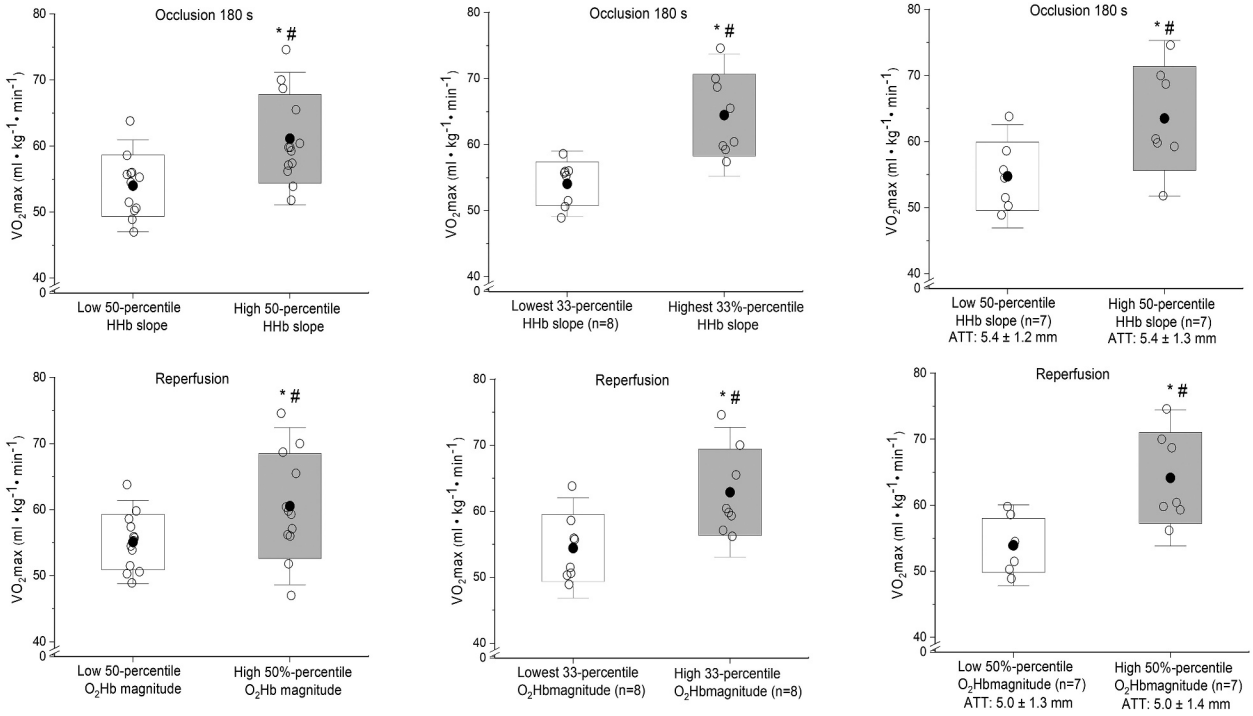

Figure 4. Box plots depicting 95% confidence interval, mean values (black circle), SDs (whiskers) and individual VO_2max values after (a) a median split (50th percentile) for HHbslope and O_2Hbmagnitude (left upper and lower graphs, respectively), (b) tritile split (33.3th percentile - excluding the "middle values") for HHbslope and O_2 Hbmagnitude (middle upper and lower graphs, respectively) and (c) matching for ATT the participants in the higher and lower HHbslope and O_2Hbmagnitude median split groups (right upper and lower graphs, respectively); * denotes $p < .05$ between groups using an independent *t*-tests; # denotes $p < .05$ between groups with ANCOVA (using ATT as covariate; the adjusted means are presented in the text).

O_2Hbmagnitude split and 5.4 ± 1.5 vs. 5.7 ± 2.0 mm, $d = 0.17$, $CI_{95} = -1.77–1.17$). Individuals within the higher vs. those in lower 50th percentile HHbslope and O_2Hbmagnitude had higher VO_2max (61.1 ± 6.7 vs. 54.0 ± 4.6 ml·kg^{-1}·min^{-1}, $d = 1.2$, $CI_{95} = 2.30–11.90$ and 60.5 ± 7.9 vs. 55.1 ± 4.2 ml·kg^{-1}·min^{-1}, $d = 0.9$, $CI_{95} = 0.25–10.58$, respectively; $p < .01–0.05$, Figure 4). The comparison of the two groups with ANCOVA, using ATT as covariate, also revealed that the high vs. the low HHbslope and O_2Hbmagnitude groups had significantly higher VO_2max (adjusted means: 60.5 ± 6.3 vs. 54.7 ± 6.4 ml·kg^{-1}·min^{-1}, $d = 1.0$, $CI_{95} = 0.54–11.06$, and 60.3 ± 5.8 vs. 55.3 ± 5.8 ml·kg^{-1}·min^{-1}, $d = 0.90$, $CI_{95} = 0.22–9.80$; $p < .05$ for both).

The highest (n = 8) and lowest (n = 8) tritiles differed, by design, in HHbslope and O_2Hbmagnitude (0.130 ± 0.021 vs. 0.058 ± 0.010 μM·s^{-1}, $d = 4.4$, $CI_{95} = 0.05–0.09$, and 43.1 ± 7.5 vs. 21.8 ± 2.1 μM, $d = 3.9$, $CI_{95} = 15.39–27.21$. ATT differed only after the HHbslope split (4.9 ± 1.3 vs. 6.9 ± 1.8 mm, $d = 1.3$, $CI_{95} = -3.68$ to -0.32, $p < .05$) but not after the O_2 Hbmagnitude split (5.0 ± 1.3 vs. 6.1 ± 2.1 mm, $d = 0.68$, $CI_{95} = -2.97–0.77$). VO_2max was higher in the highest vs. lowest HHbslope and O_2Hbmagnitude tritile groups (64.5 ± 6.2 vs. 54.1 ± 3.3 ml·kg^{-1}·min^{-1}, $d = 2.1$, $CI_{95} = 5.07–15.73$, and 62.9 ± 6.6 vs. 54.4 ± 5.1 ml·kg^{-1}·min^{-1}, $d = 1.4$, $CI_{95} = 2.18–14.82$; $p < .001–.01$, Figure 4). ANCOVA analysis (using ATT as covariate), also showed significantly higher VO_2max in highest vs. lowest HHbslope and O_2Hbmagnitude tritile groups (adjusted means: 64.1 ± 5.7 vs. 54.5 ± 5.7 ml·kg^{-1}·min^{-1}, $d = 1.7$, $CI_{95} = 3.49–15.71$, and 62.4 ± 6.1 vs. 54.8 ± 6.1 ml·kg^{-1}·min^{-1}, $d = 1.3$, $CI_{95} = 1.06–14.14$; $p < .01–.05$).

After matching for ATT the participants in the high (n = 7) and low (n = 7) HHbslope and O_2Hbmagnitude median split groups, they had (by design) different HHbslope and O_2 Hbmagnitude (0.123 ± 0.022 vs. 0.066 ± 0.010 μM·s^{-1}, $d = 3.4$, $CI_{95} = 0.04–0.08$, and 42.9 ± 8.1 vs. 24.7 ± 3.5 μM, $d = 2.9$, $CI_{95} = 10.93–25.47$, for HHbslope and O_2Hbmagnitude, respectively; $p < .001$ for both) but similar ATT values (5.4 ± 1.2 vs. 5.4 ± 1.3 mm and 5.0 ± 1.3 vs. 5.0 ± 1.4 mm). VO_2max was greater in higher vs. the ATT-matched lower HHbslope and O_2Hbmagnitude median split groups (63.5 ± 7.9 vs. 54.8 ± 5.2 ml·kg^{-1}·min^{-1}, $d = 1.3$, $CI_{95} = 0.91–16.49$, and 64.1 ± 6.9 vs. 53.9 ± 4.1 ml·kg^{-1}·min^{-1}, $d = 1.8$, $CI_{95} = 3.59–16.81$, $p < .01–.05$, Figure 4).

Discussion

In this study, we used a novel approach to examine whether NIRS-derived parameters of muscle oxidative metabolism and microvascular reactivity assessed at resting condition are associated with maximal and submaximal indices of whole-body aerobic fitness. More specifically, we adopted an easily applicable and time-efficient arterial occlusion *at rest*, which may be practical for those who cannot tolerate multiple occlusions and/or not be able to perform exercise. The main new findings of this study were that the oxidative metabolism and microvascular reactivity of the *vastus lateralis* muscle, as assessed by NIRS during a single arterial occlusion–reperfusion at resting condition, were highly correlated with whole-body VO_2max and moderately with maximal aerobic velocity. We, also, report for the first time that the

NIRS-derived muscle deoxygenation and reoxygenation parameters during this test are able to discriminate individuals based on their VO_2max values. Among the most commonly assessed NIRS variables, the HHbslope and HHbmagnitude of increase during the 3-min arterial occlusion and the O_2 Hbmagnitude of increase during reperfusion appear as best predictors for VO_2max and MAV. Collectively, our findings reinforce the utility of NIRS-derived parameters as markers of whole-body aerobic fitness and in describing differences in whole-body aerobic fitness. We show that this may be achieved with a simple arterial occlusion–reperfusion maneuver that does not require exercise and complex measuring and analyzing procedures.

Correlations of NIRS-derived muscle oxidative metabolism with aerobic fitness

The muscle oxidative metabolism during arterial occlusion *at rest* demonstrated positive and high correlations with VO_2max ($r = .704$ and .763 with and without controlling for ATT; $p < .001$). These results support previous findings using well-established *in situ* and *in vitro* techniques that the muscle's ability to utilize/consume oxygen dictates independently, and to a large extent ($R^2 = 50\%$), the whole-body VO_2max (Jacobs & Lundby, 2013; Larson-Meyer et al., 2000; Tonkonogi & Sahlin, 1997; Zoll et al., 2002) and highlight the utility of NIRS to discriminate individuals with different aerobic fitness levels. Consistent with our results, recent NIRS studies have also reported significant associations between VO_2max and mitochondrial oxidative capacity of leg muscles assessed by post-exercise recovery kinetics of mVO_2 following brief, multiple arterial occlusions (Beever et al., 2020; Guzman et al., 2020; Lagerwaard et al., 2019, 2021). Furthermore, other studies implementing NIRS during maximal incremental exercise also support an association between peak oxygen extraction of the vastus lateralis muscle and VO_2max in young, physically active individuals (Caen et al., 2019; Okushima et al., 2016).

The significant correlation between NIRS-derived muscle deoxygenation parameters during arterial occlusion with VO_2 max partially support previous studies documenting greater rate and magnitude of muscle deoxygenation (O_2 extraction) during arterial occlusion in trained vs. untrained individuals (McLay et al., 2016; Rasica et al., 2022; Van Thienen & Hespel, 2016). In Van Thienen and Hespel (2016), the greater muscle's O_2 extraction rate in highly trained endurance athletes was evident from the first minute of arterial occlusion at rest and was approximately double compared to less-trained controls (Van Thienen & Hespel, 2016). The authors suggested that during the arterial occlusion, blood flow is cut to and from the tissue but as mitochondrial oxidative metabolism in the occluded tissue persists and O_2Hb is continuously transformed into HHb, which is proportional to O_2 extraction and muscle O_2 consumption. Factors that may also affect O_2 extraction include capillary density/volume, diffusion distance from the capillary to the cell, the size of the capillary bed to cellular PO_2 gradient, and the rate of use of oxygen by cells (Beer & Yonce, 1972; Curtis et al., 1995; Leach & Treacher, 1998); in particular, the latter is among the factors that is needed to be maintained for prevention of hypoxia and tissue injury (Leach & Treacher, 1998). It has been shown that ischemia may result to additional capillary recruitment increasing the diffusive O_2 efficiency and extraction, and offsetting, somewhat, the detrimental effect of reduced blood flow (Beer & Yonce, 1972; Curtis et al., 1995). Based on the above, the increased muscle O_2 extraction (deoxygenation) during an arterial occlusion in individuals with higher VO_2 max may be related to their greater mitochondrial content (and ability to utilize O_2; Hoppeler et al., 1985; MacInnis et al., 2017) as well as intramuscular factors that increase capillary O_2 diffusive conductance such as enhanced capillarity density/volume, smaller diffusing distances and greater intramyocellular myoglobin (Andersen & Henriksson, 1977; Egan & Zierath, 2013; Leinonen et al., 1978; Poole et al., 2021). Arterial occlusion causes ischemia, oxygen deprivation, and an increase in reactive oxygen species, that accumulate rapidly at the beginning of the ischemic period (Clanton, 2007; Sack, 2006), placing a risk to cell/tissue integrity. Cellular use of oxygen is important in maintaining tissue function and overall survival (Leach & Treacher, 1998). Mitochondria, apart from being energy-producing organelles, monitor the condition of the cell and modify their metabolic activity in response to various insults (such as ischemia and the resultant oxidative stress) to protect the cell/tissue from injury or endure its survival (Galluzzi et al., 2012; Mookerjee et al., 2010). Mitochondrial uncoupling of oxidative and phosphorylation processes (reduction in P/O ratio), has been widely identified as a cytoprotective strategy under conditions of oxidative stress by reducing the production of reactive oxygen species (Brand, 2000; Mookerjee et al., 2010; Sack, 2006; Salin et al., 2015). Thus, the extra oxygen consumed by the muscle in individuals with higher VO_2max (and conceivably greater mitochondrial content) was possibly used to support tissue integrity by diminishing the production of reactive oxygen species and not for energy (ATP) production (Brand, 2000; Mookerjee et al., 2010; Sack, 2006; Salin et al., 2015). In line, Befroy et al. (2008) showed that the basal mitochondrial oxidative function (oxygen consumption) in the muscle is higher in endurance trained individuals compared to untrained, yet the energy production (ATP synthesis) is not altered, leading to an increased uncoupling of oxidative phosphorylation at rest (Befroy et al., 2008). Thus, although the mitochondrial uncoupling of oxidative phosphorylation (reduction in the P/O ratio) is energetically costly (by ~20–30%), it may be associated with advantages in terms of cellular/somatic maintenance and protection by reducing ROS production (Mookerjee et al., 2010; Sack, 2006; Salin et al., 2015). Nevertheless, the exact physiological mechanisms that support the correlation of NIRS-derived muscle deoxygenation parameters during arterial occlusion and VO_2max should be further examined.

From a methodological viewpoint, the technique we used (a 3-min arterial occlusion at rest) seems to be advantageous over the forenamed *in vitro* techniques and other NIRS techniques. It permits a noninvasive, onsite measurement of *in vivo* skeletal muscle oxidative metabolism (compared *to in situ* and *in vitro* approaches (Grassi & Quaresima, 2016; Willingham & McCully, 2017), it is simple, relatively low-cost, easily accessible and less operator-dependent compared to ^{31}P-MRS approach (Grassi & Quaresima, 2016; Willingham &

McCully, 2017). Importantly, the technique used in this study, is independent of the participant's motivation, it does not require exercise modalities and complex measuring and analyzing procedures compared to previous NIRS approaches (Beever et al., 2020; Lagerwaard et al., 2020; Sumner et al., 2020; Zuccarelli et al., 2020). Of note, the technique applied in our study has been used to assess the muscle oxidative metabolism at rest, whereas the one used by previous NIRS studies (multiple occlusions following a brief exercise or electrical stimulation) is a cross validated technique that is targeted to examine mitochondrial oxidative capacity. Thus, the NIRS-derived muscle deoxygenation during a 3-min arterial occlusion of vastus lateralis muscle *at rest* appears as a promising marker of whole-body aerobic fitness, particularly when the precise assessment of VO_2max is not essential. This, however, should be further investigated.

Among all NIRS-derived variables assessed in this study, HHbslope (depicting the rate of deoxygenation) during the arterial occlusion showed the highest correlation with VO_2 max ($r = .704$). It is tempting to compare this finding with the corresponding associations of maximal and submaximal exercise tests which are largely used for the estimation of cardiorespiratory fitness. For example, in studies with healthy, young participants, several predictive field tests using maximal incremental exercise have revealed a wide range of correlations with VO_2max ($r = 0.40–.90$; Castagna et al., 2006; Grant et al., 1999; McNaughton et al., 1998); while methods using submaximal exercise have, nearly systematically, showed much lower correlation values than the one observed in our study (Evans et al., 2015; Grant et al., 1995; Marsh, 2012). Notably, the NIRS deoxygenation values during resting arterial occlusion showed a higher association with VO_2max ($r = 0.704$) than the maximal aerobic velocity (MAV, $r = 0.407$), a commonly used parameter for the prediction of performance in endurance events and VO_2max.

Muscle deoxygenation showed significant moderate correlations with MAV ($r = .407$ and .530 with and without controlling for ATT; $p < .05$). We are unaware of any studies examining such a relationship. The markedly lower associations than to VO_2max could be explained by the anaerobic energetic contribution to MAV measurement (Billat & Koralsztein, 1996). We did not observe statistically significant correlations between muscle deoxygenation indices with VTs. The possible explanations to this finding is that the continuous arterial occlusion at rest is not able to detect (a) the metabolic events (transition to a greater anaerobic contribution to energy production) that are expressed by VTs and/or (b) changes in other factors that are important to determine these thresholds such as lactate removal/clearance, buffering capacity and oxygen transport to the exercising muscles (Rusko et al., 1980). For example, it has been shown that during an arterial occlusion at rest the tissue metabolic demand is met by oxidative metabolism, as muscle PCr, and pH do not change (Hamaoka et al., 1996; Sako et al., 2001).

Our data also show that NIRS-derived muscle deoxygenation during a 3-min arterial occlusion at rest may discriminate individuals based on their VO_2max. Specifically, when the participants were divided in percentiles based on the rate of muscle deoxygenation during arterial occlusion, the individuals in the highest percentiles had a significantly higher VO_2max value compared to those at the lower. These outcomes are in strict line with previous NIRS studies which reported greater muscle deoxygenation during an arterial occlusion in endurance athletes vs. fit healthy (Van Thienen & Hespel, 2016) and in trained vs. untrained individuals (McLay et al., 2016). In summary, our results reinforce the application of the NIRS method for the *in vivo* examination of skeletal muscle oxidative metabolism and highlight the usefulness and practicality of the arterial occlusion at rest as a tool for evaluating differences in whole-body aerobic fitness among individuals.

Correlations of NIRS-derived muscle microvascular function with aerobic fitness

NIRS-derived measurements of microvascular reactivity (O_2Hb 10 s reperfusion slope) and responsiveness (magnitude and slope of O_2Hb increase) during the reperfusion provide a reliable, noninvasive, *in vivo* assessment of microvascular function within the skeletal muscle microcirculation (Dipla, Triantafyllou, Grigoriadou et al., 2017; Iannetta et al., 2019; McLay et al., 2016a, McLay et al., 2016). In our study, all NIRS-indices of microvascular reactivity of the lower limb that we examined (i.e. $O_2Hb_{magnitude}$, $O_2Hb_{slopeto\text{-}to\text{-}peak}$, $O_2Hb_{slope\ to\ 10s}$) were significantly correlated with VO_2max. The highest correlation was observed between the peak magnitude of O_2Hb achieved during the 2-min of the re-oxygenation phase ($r = .672–.695$; $p < .001$). In line, a recent study reported a relatively similar ($r = .589$) correlation between VO_2max and reactive hyperemia (10-s slope reperfusion; Rasica et al., 2022). We also show, for the first time, the magnitude of reoxygenation during reperfusion using NIRS is capable of differentiating individuals based on their VO_2max.

Our significant correlations establish a robust association between muscle microvascular function and whole-body aerobic fitness. Although we cannot infer to a direct causal relationship, these data are likely to suggest that a better microvascular function of lower limbs contributes, substantially, to an enhanced aerobic capacity. Support to this notion comes from previous cross-sectional (George et al., 2018; McLay et al., 2016; Schroeder et al., 2019; Soares et al., 2018) and longitudinal (Ihsan et al., 2020) NIRS studies which reported that trained, healthy young or elderly individuals, exhibited a higher microvascular responsiveness compared to untrained counterparts. A recent study (Gifford et al., 2020) also observed that indices of resistance artery function in the leg using a *non-NIRS approach* were independently related to VO_2max and accounted for ~30% of the variance in its values. In the same context, Poole et al. (2021) pointed out that exercise training improves endothelial function in the vasculature which results in elevated perfusive and diffusive O_2 delivery. Together these effects raise the QO_2-to-VO_2 ratio and microvascular O_2 partial pressure thereby accelerating the VO_2 kinetics and increasing VO_2max (Poole et al., 2021).

The relationship between microvascular function at skeletal muscle and various maximal and submaximal indices of aerobic fitness (i.e. MAV, VT_1 and VT_2) is an underexamined field. We noted a significant association with MAV,

an independent predictor of endurance performance and of VO_2max; fortifying the proposed link of muscle microvascular function with performance in endurance events and aerobic fitness. Unexpectedly, the correlations of vascular reactivity/responsiveness (assessed at rest) and MAV (assessed with maximal incremental test) with VO_2max were comparable. Finally, our indices of muscle reoxygenation were not associated with submaximal indices of aerobic endurance capacity (i.e. VT_1 and VT_2) after adjusting the NIRS parameters for ATT. This finding contrasts previous studies that examined vascular responses in large conduit arteries (Palmieri et al., 2005) and in skin microcirculation (McKune et al., 2015).

Limitations

Despite the novel findings of our study, there are some limitations should be considered. First, we performed a 3-min arterial occlusion at rest to examine the muscle oxidative metabolism and microvascular function. A 5-min occlusion is often used to achieve a higher oxygen desaturation of the muscles under the area of NIRS probes. However, studies have shown that the 3-min ischemic challenge represents an adequate time to detect such differences between high-fitness and low-fitness young, healthy individuals (McLay et al., 2016), producing highly reliable outcomes (Iannetta et al., 2019). The shorter occlusion time has the advantage of reducing the participants' discomfort and burden derived from the suprasystolic pressures (350 mmHg) in the vastus lateralis muscle (Gerovasili et al., 2010). Second, we recruited individuals from both sexes. A potential limitation in including both sexes might be that men and women differ in body composition and men may appear fitter when normalizing VO_2max to body mass. We believe, however, that this should not influenced our conclusions, as the absolute VO_2max values were also correlated to NIRS-parameters (see results) and previous studies have shown that NIRS-derived skeletal muscle oxidative capacity and its correlation with the VO_2peak are not affected by sex (Beever et al., 2020; Guzman et al., 2020). Another potential limitation might be that MAV may be dependent on the incremental test (Keir et al., 2018). Nevertheless, all our participants performed the same test; thus, it is unlikely that this may pose a serious threat to our results. Finally, we have accounted for most factors that may *confound* the NIRS signal, such as the adipose/skin tissue thickness at the site of measurement and the muscle/cutaneous blood flow (arterial occlusion; Barstow, 2019). It is possible, however, that other confounding factors below the subcutaneous adipose layer might have affected our results.

In conclusion, the vastus lateralis oxidative metabolism and microvascular reactivity as assessed with NIRS during a 3-min arterial occlusion–reperfusion maneuver at resting condition show positive and strong correlations with whole-body VO_2max and moderate correlations with MAV. We also observed that NIRS-derived indices of muscle deoxygenation and microvascular function during an occlusion–reperfusion may discriminate the individuals based on their VO_2max. Our data suggest that the rate and magnitude of deoxygenation during a single arterial occlusion and the magnitude of reoxygenation during subsequent reperfusion appear as good predictors of whole-body aerobic fitness and highlight the ability of NIRS to detect differences in whole-body aerobic fitness. The results of the present study, in conjunction with previous research, suggest that NIRS-derived parameters of muscle deoxygenation (an index of muscle oxidative metabolism) and reoxygenation (an index of microvascular function) during occlusion–reperfusion maneuver, and mainly the deoxygenation slope, may be used as markers of whole-body aerobic fitness. These indices may also be considered (as markers) to identify groups with different VO_2max, especially when precise assessment of VO_2max is not essential. Our data suggest that changes in NIRS parameters during arterial occlusion and reperfusion at rest may be used to study questions related to whole-body aerobic capacity. Future studies, however, should examine the utility and practicality of this method in older and immobilized individuals, and in clinical populations.

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Data availability statement

The data that support the findings of this study are available on reasonable request from the corresponding author.

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