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NEW APPROACHES OF FUNCTIONAL EVALUATION OF OXIDATIVE METABOLISM DURING EXERCISE, WITH SPECIAL REFERENCE TO THE EFFECTS OF INACTIVITY – MICROGRAVITY (BED REST)

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ABSTRACT

Oxidative metabolism represents the main energy source for activities related to everyday life and work. Functional evaluation of oxidative metabolism during exercise provides important information on the physiological responses required by the cardiovascular and respiratory systems to meet the skeletal muscle metabolic demand. A better knowledge of the sites of limitations of oxidative metabolism would be of utmost importance from a "basic science" point of view. A refinement of available tools and methods aimed at enhancing oxidative metabolism and at improving aerobic performance would allow to increase the general quality of life of both healthy people and patients.

The two main topics of my PhD research were focused on (i) the effects of aerobic exercise prescription based on heart rate (HR) both in healthy population and in population of patients, and on (ii) the localization of the main site(s) of impairment in oxidative metabolism during exercise in simulated microgravity condition along the O_2 pathway from the ambient air to skeletal muscle mitochondria, with particular interest to the peripheral level.

The first chapter gives a general introduction about oxidative metabolism in terms of energy metabolism during exercise, with a brief overview on the traditional and "more recent" bio-markers utilized for a functional evaluation of this metabolic system. The main adaptive physiological responses induced by exposure to microgravity on the variables taken into consideration in this doctoral research are discussed at the end of the introduction.

Three main studies were conducted in order to evaluate the effects of prescribing aerobic exercise at a specific HR target value, in physiological and pathological conditions and after exposure to environmental stressors. In *Study 1*, carried out on 17 male healthy subjects, the hypothesis was that during constant work rate exercises (CWR) at different intensities the slow component of HR kinetics would occur at lower work rate and would be more pronounced than the slow component of pulmonary oxygen uptake ($\dot{V}O_2$) kinetics. As a consequence, we hypothesized that exercise prescription at a fixed HR value, slightly above the "gas exchange threshold" (GET), as it is often done for training purposes and in clinical studies, would determine a significant decrease in work rate. This would significantly affect exercise evaluation and exercise prescription. In *Study 2*, 16 male obese patients were tested in order to verify the above hypotheses also in a population of patients. In this study measurements were performed before and after a 3-week multidisciplinary programme aimed at reducing body mass. In *Study 3* the same hypotheses were tested in 10 healthy male subjects evaluated before and after a 10-day horizontal bed rest.

The second part of my PhD project intended to identify biomarkers evaluating sites of impairments of oxidative metabolism in a simulated microgravity condition, such as bed rest. A preparatory study (*Study 4*) was conducted in physiological conditions in order to modify and improve a recently proposed method, aimed at assessing mitochondrial function non-invasively by near-infrared spectroscopy (NIRS); this method is based on the measurement of skeletal muscle $\dot{V}O_2$ recovery kinetics following exercise. Finally, in *Study 5* an integrative approach was utilized to determine the sites of impairment of oxidative metabolism during exercise following a 10-day bed rest, spanning from systemic variables to markers more specifically related to peripheral vascular function, skeletal muscle fractional O_2 extraction by NIRS and mitochondrial function. The latter was evaluated by an *ex vivo* approach, that is by high-resolution respirometry (HRR) on permeabilized muscle fibers (in conditions of unlimited O_2 and substrates availability), and by a non-invasive *in vivo* approach, based on the evaluation by NIRS of muscle $\dot{V}O_2$ recovery kinetics following CWR exercise.

The results of *Studies 1, 2* and *3* revealed that both in healthy subjects and obese patients, as well as in microgravity, the "translation" of work rates or percentages of $\dot{V}O_2$ peak into HR values is not straightforward. Indeed, in all these three different scenarios when exercise was performed at a fixed HR value (slightly above that corresponding to GET), both work rate and $\dot{V}O_2$ had to decrease. Surprisingly, in obese patients this phenomenon was not more pronounced compared to that observed in healthy subjects, and it was mitigated after the 3-week structured exercise training programme; this suggests that the work rate decrease is associated with, and can be considered a sign of, exercise intolerance. Finally, the decrease in work rate necessary to keep HR constant was again confirmed in healthy subjects before bed rest, and it was greater following a 10-day bed rest, with obvious implications on exercise evaluation and exercise prescription also in microgravity conditions. Interestingly, the reduction in work rate was more pronounced than that necessary to prevent slow components of $\dot{V}O_2$ and muscle deoxygenation kinetics.

The results of *Study 4* and *Study 5* revealed that the whole-body impairment in oxidative metabolism following a 10-day horizontal bed rest is associated with an impairment of cardiovascular, peripheral vascular and endothelial functions, whereas mitochondrial mass and maximal respiratory functions (both *in vivo* and *ex vivo*) are substantially unaffected (with the possible exception of an improved respiratory response to submaximal ADP stimulation). In other words, after 10 days of bed rest the impairment of oxidative metabolism is mainly "upstream" of mitochondrial function. This concept, besides being of interest from a basic science point of view, may be of interest also for other

pathological conditions characterized by relatively short periods of profound inactivity, and it could affect the definition of countermeasures rather than rehabilitative interventions.

1 CHAPTER I - INTRODUCTION

1.1 TRADITIONAL MARKERS OF OXIDATIVE METABOLISM: $\dot{V}O_2MAX$, GAS EXCHANGE THRESHOLD, CRITICAL POWER, $\dot{V}O_2$ KINETICS AND THE SLOW COMPONENT

The energy to support life and its changing level of physical activity is mainly obtained from oxidative phosphorylation. Oxygen (O_2) is indeed the key that unlocks the energy from the metabolic substrates. When intense and very short exercises (i.e., 10-15 s all out) are done, breakdown of PCr is the main fuel for ATP provision because it requires only one metabolic reaction ($PCr+ADP+H^+$-->ATP + creatine) to provide ATP. The maximal power of this system is achieved in a few seconds but in 15-20 s it reaches a value close to 0. The anaerobic glycolytic capacity is higher and can be depleted in 30-90 s. For oxidative phosphorylation, the maximal power represented by the maximal oxygen uptake ($\dot{V}O_2$ max) is relatively lower, but it can be sustained for much longer periods of time. This mechanism is slower in getting to the action, within 3 minutes of starting exercise maximal power is achieved and maintained for several minutes (see Fig. 1).

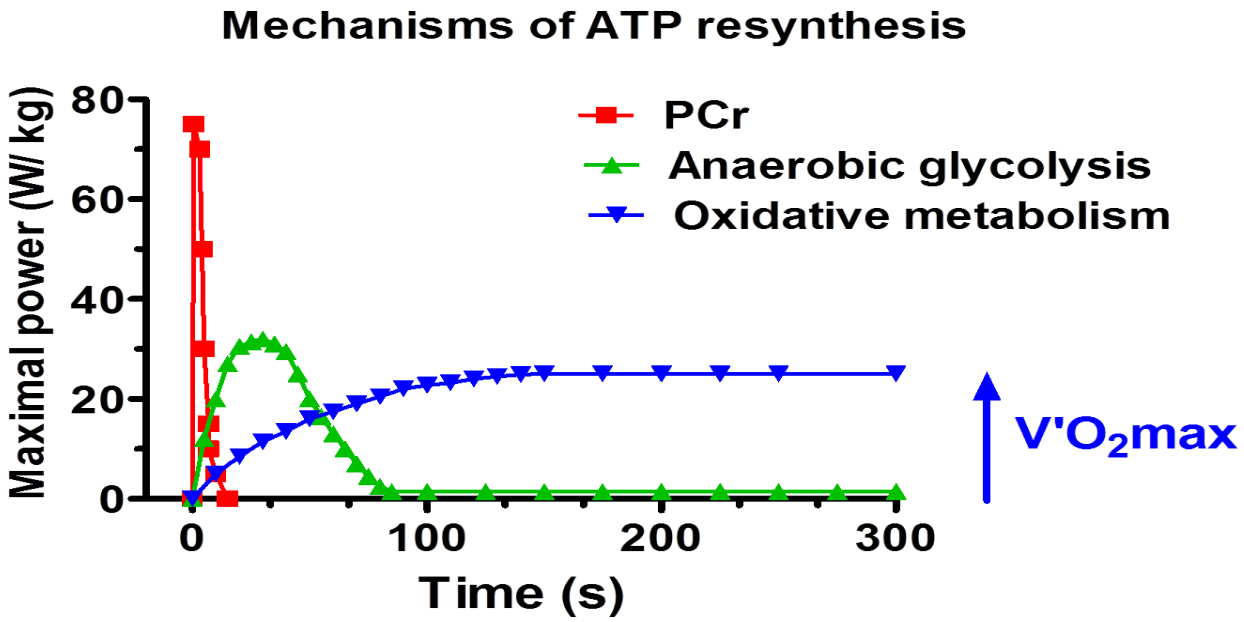

Figure 1. Schematic representation of muscle ATP regeneration during intense exercise (Modified from Grassi 2003). Maximal power output (expressed in watt/kg of body mass) as function of the time of exercise.

Oxidative metabolism has several advantages with respect to the other mechanisms of ATP resynthesis such as phosphocreatine (PCr) hydrolysis and anaerobic glycolysis: (i) the maximal power expressed by oxidative metabolism can be sustained for several minutes (7-10 minutes); (ii) a relatively large fraction of its maximum power can be maintained at steady state without incurring in fatigue; (iii) oxidative metabolism during the recovery period after exercise is the only source for the resynthesis of PCr and ATP.

Physical exercise requires the interaction of different physiological control mechanisms to enable the cardiovascular and ventilatory systems to match the increased respiratory demands of the contracting muscles (i.e., O_2 consumption [$\dot{V}O_2$]), ensuring the delivery of O_2 to contracting muscles and the transport back into mitochondria for ATP resynthesis of the by-products of ATP utilization in the cytoplasm (such as ADP and Pi) (Hargreaves & Spriet, 2020). The reducing equivalents in terms of NADH and FADH2, free ADP, Pi and O_2 are required by the respiratory or electron transport chain (ETC) in the mitochondria in order to produce ATP (see Fig. 2).

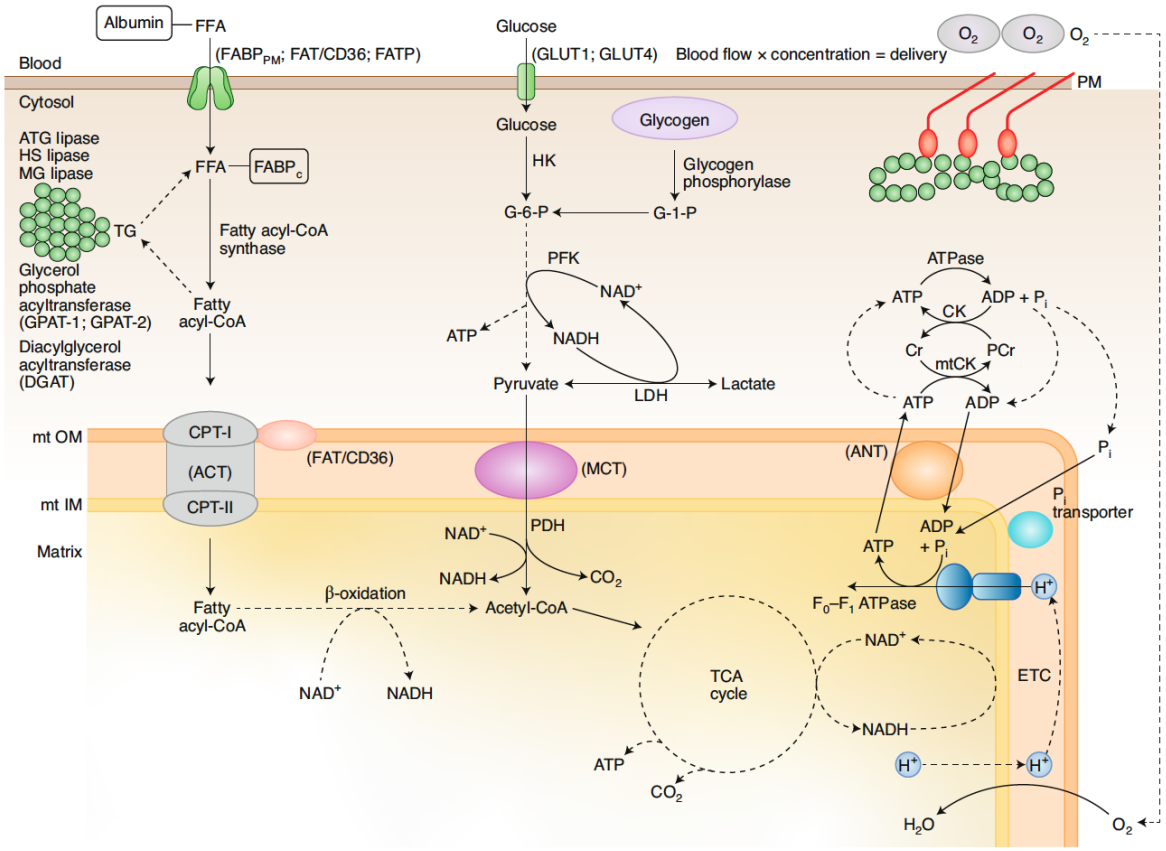

Figure 2. Schematic representation of metabolic pathways during exercise in skeletal muscle (From Hargreaves & Spriet, 2020).

The dynamics of the O_2 transport systems upstream of the contracting myocytes - pulmonary, cardiovascular, and muscle microvascular - are such that ventilation, cardiac output, and skeletal muscle vasomotor control ensure that O_2 is delivered to the exercising muscle (see Fig. 3). Each of these systems has a crucial role in terms of O_2 transport and utilization, and adequate interactions among these systems are essential to maintain homeostasis.

The respiratory system moves oxygen from the ambient air to the alveoli and carbon dioxide (CO_2) from the alveoli to the atmosphere, and exchanges gases across the alveolar-capillary barrier. The circulation increases at the rates at which O_2 is supplied to the cells and CO_2 is removed from the cells. Cardiac output increases in proportion to the metabolic requirements in healthy subjects in order to pump oxygenated blood to the active muscles and to return blood poor of oxygen and rich of CO_2 to the alveoli. Finally, the O_2 utilization from the muscles is obtained by increased extraction of O_2 from the blood perfusing the muscles, and ATP is generated in the mitochondria.

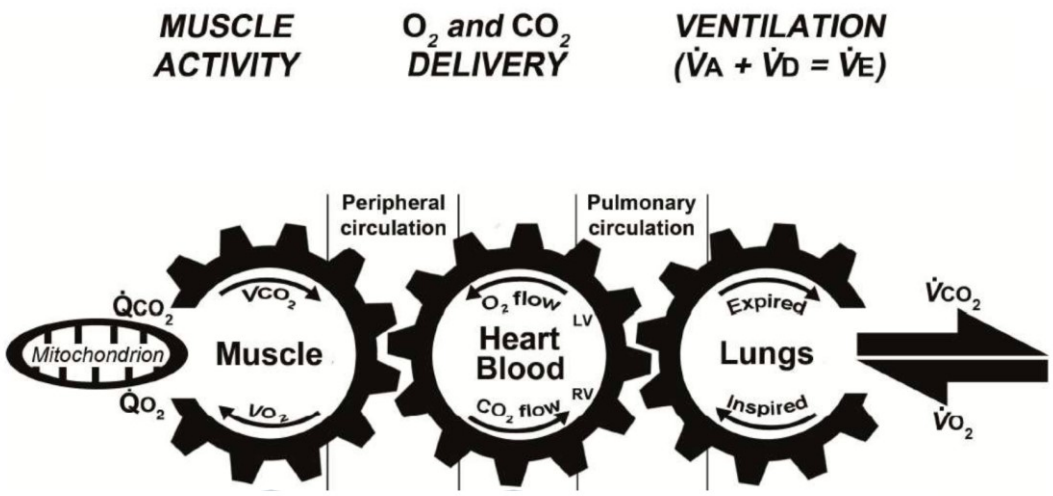

Figure 3. Schematic representation of gas transport mechanism of coupling of external to cellular respiration (modified from Wasserman 1996).

All these systems are highly coordinated and they work together in proportion to the amount of work being performed. Traditional markers for the functional evaluation of oxidative metabolism are: $\dot{V}O_2$ max, gas exchange threshold and the respiratory compensation point, critical power, $\dot{V}O_2$ kinetics and its slow component.

$\dot{V}O_2max$. The maximal flux of O_2 from the ambient air to the mitochondria is defined as maximal O_2 uptake ($\dot{V}O_2max$), corresponding to the maximal mechanical power sustainable by oxidative phosphorylation (see Fig 1, see below). The relevant point in terms of exercise tolerance both in

physiological and pathological conditions lies in the fact that the mechanical power corresponding to $\dot{V}O_2max$ can be maintained for relatively longer periods of time (7-10 minutes).
$\dot{V}O_2max$ is the product of maximal cardiac output and the maximal arterio-mixed venous oxygen concentration difference (Fick equation). In other words, it is the maximal amount of O_2 here and elsewhere utilized by the whole body per unit of time (minute).

$$\dot{V}O_2max = \dot{Q}max\ (CaO_2\text{-}C\bar{v}O_2)max$$

where $\dot{Q}$ is cardiac output, CaO_2 is the arterial oxygen content, and $C\bar{v}O_2$ is the mixed venous oxygen content.

A multifactorial model for $\dot{V}O_2max$ limitation was proposed by di Prampero & Ferretti (1990). This model was based on the concept that each of the multiple steps of the O_2 cascade from the ambient air to skeletal muscle mitochondria can provide a given fraction of the overall $\dot{V}O_2max$ limitation.
In healthy non-athletic subjects, during whole body exercise performed in normoxia, the cardiovascular O_2 transport system (i.e., cardiac output and stroke volume) resulted being responsible for about 70% of the overall limitation in $\dot{V}O_2max$ whereas the remainder was divided between peripheral O_2 diffusion and mitochondrial capacity.
Commonly, an easy and widely utilized way to express oxidative energy expenditure resides in the Metabolic Equivalent of Task (MET). This parameter represents the physiological concept of expressing energy expenditure of physical activities as multiples of resting metabolic rate. One MET indeed corresponds to the resting oxidative energy expenditure (i.e., $\dot{V}O_2$ at rest).

$\dot{V}O_2max$ represents one of the most important variables of functional evaluation of oxidative metabolism during exercise, and it is usually assessed by the cardiopulmonary exercise test (CPET). It provides non-invasive measurements of the cardiorespiratory fitness of the subject in response to exercise. $\dot{V}O_2max$ is usually determined by measuring $\dot{V}O_2$ at the mouth of the subject with an indirect calorimetry during an incremental test. Different modalities of tests have been proposed over the time (Martin-Rincon & Calbet, 2020). Nowadays, one of the most used tests is the incremental test (with 1-3 minutes step or ramp increases) in which the subject is asked to deal with an increasing resistance (speed on the treadmill or work rate on the cycle ergometer) over the time while pulmonary ventilation, $\dot{V}O_2$ and $\dot{V}CO_2$ are monitored non-invasively breath-by-breath. CPET also involves measurements of heart rate, oxygen saturation, muscle deoxygenation by near-infrared spectroscopy, electrocardiogram (ECG) and blood pressure. $\dot{V}O_2$ increases linearly during an incremental exercise

in relation to the workload up to the $\dot{V}O_2max$. $\dot{V}O_2$ can increase from a resting value of ≈3.5 $ml \cdot kg^{-1} \cdot min^{-1}$ to a peak of about 15-20 times the resting values (30-80 $ml \cdot kg^{-1} \cdot min^{-1}$).

Gas exchange threshold. While a work rate corresponding to $\dot{V}O_2max$ can be kept for maximum 7-10 minutes, exercises of even longer duration (corresponding to most work, recreational, or everyday activities) can only be sustained at a power corresponding to a fraction of $\dot{V}O_2max$. Defining this fraction, is the objective of the variables introduced below.

During incremental exercise, pulmonary ventilation increases to deliver O_2 to the alveoli and to eliminate CO_2. $\dot{V}CO_2$ output, in addition to the linear increases associated with a given $\dot{V}O_2$, changes its slope with a disproportionate increase in respect to $\dot{V}O_2$. This excess in $\dot{V}CO_2$ is a reflection of the buffering by bicarbonate of H+ resulting from the dissociation of lactic acid deriving from anaerobic glycolysis. The "ventilatory threshold" (VT) or "gas exchange threshold" (GET) is defined as the metabolic rate at which excess CO_2 increases proportional to the rate at which muscle and blood bicarbonate concentrations decrease as consequence of a metabolic acidosis (Beaver et al., 1986). Increases in blood lactate concentration appears proximal to GET. After the onset of the anaerobic glycolysis, if the work rate keeps increasing, a second threshold named respiratory compensation point (RCP) can be identified. A second increase in $\dot{V}CO_2$ is a consequence of the hyperventilation due to the fact that the bicarbonate buffer system becomes insufficient: the working muscles become progressively acid and the system responds to the metabolic acidosis by increasing pulmonary ventilation and $\dot{V}CO_2$.

The identification of these thresholds is fundamental in terms of performance and control of the physiological systems under investigation (Poole et al., 2020).

For young, healthy and physically active subjects GET occurs at as early as about 60% of $\dot{V}O_2max$. This percentage could be lower in untrained subjects or in patients, and it can be quite higher in athletes.

Critical power. Critical power (CP) is defined as the asymptote of the power-duration curve for high intensity exercises. When the time to the limit of tolerance is plotted against speeds or power outputs, the relation is not linear, but it follows a hyperbolic profile with the ability to tolerate exercise decreasing more suddenly at higher power compared with lower exercise power (Hill 1925; Poole et al., 2016). When exercise tolerance is considered, the power asymptote represents CP and the curvature constant represents W' (measured in unites of work done (J)), which reflects the rate of the finite work capacity available above CP. The limit of tolerance will coincide with the depletion of W' and with the coincident achievement of $\dot{V}O_2max$. This curvilinear profile defines exercise tolerance for locomotory activities in different animal species and in different exercise modalities (Poole et al.,

1998; Whipp et al., 1999; Lauderdale & Hinchcliff, 1999; Fukuba & Hill, 2003; Billat et al., 2005). CP is therefore the highest metabolic rate for which $\dot{V}O_2$ can be stabilized below $\dot{V}O_2max$ and it is a cardinal feature for the development of exercise intolerance and metabolic instability. Disproportionate changes in metabolic variables associated with fatigue and exercise intolerance, such as muscle pH, muscle lactate, neuromuscular excitability and rates of changes in PCr concentration, are reported following exercises above CP compared to exercises performed below CP (Burnley wt al., 2012; Black et al., 2017;).

CP therefore represents an important physiological threshold indicating a boundary above which exercise results in the attainment of $\dot{V}O_2max$. CP separates two domains of exercise which have distinct physiological profiles (see below in "$\dot{V}O_2$ kinetics"). In healthy subjects CP occurs at 50% of the difference between GET and $\dot{V}O_2max$ (ΔGET- $\dot{V}O_2max$) obtained from the incremental exercise (Poole & Jones, 2012).

The maximum speed of progression during a locomotion activity of relatively long duration can also be tackled from another point of view: the maximum rate of progression (Smax) can in fact be calculated, as a first approximation, using the following equation (Di Prampero et al., 1986)

$$\mathrm{Smax} = \frac{\mathrm{Fx}\dot{\mathrm{V}}\mathrm{O}_2\mathrm{max}}{\mathrm{C}}$$

in which F indicates the fraction of $\dot{V}O_2max$ utilizable in the exercise test in question, while C indicates the energy cost of the type of locomotion in question, (i.e., the amount of energy necessary to transport the body over a given distance). C is generally expressed in kJ/km (1 kcal = 4.2 kJ; a liter of O_2 consumed in the human body develops about 5 kcal or 21 kJ). In practice, the equation indicates that the maximum speed that can be developed in a given mode of locomotion, for a given (relatively prolonged) duration, is defined by the fraction of the maximum aerobic power that can be developed during an activity of that duration, divided by the energy cost of the locomotion in question. Since endurance athletes of different specialties have relatively similar values of $\dot{V}O_2max$ and F, it follows that the very different values of Smax obtainable during different modes of locomotion (which can vary between about 23 km/h during 5 km of running and about 51 km/h during 5 km of track cycling) depend on substantial differences in the value of C.

In locomotion on land, the energy expended by the subject per unit of travel is used, in part, to overcome the resistance of the air (proportional to the square of the speed) and, in part, against gravitational and inertial forces, respectively to lift and lower, accelerate and decelerate the center of mass of the body and limbs, and, in part, for the work of the heart and respiratory muscles.

$\dot{V}O_2$ kinetics. At the onset of physical and dynamic activities, upon a stepwise or sudden increase in work rate, a coincident stepwise or sudden increase in ATP turnover occurs. However, ATP resynthesis deriving from oxidative phosphorylation is slower than the total ATP demand and the level of oxidative phosphorylation needed to couple the work rate is achieved in 2-3 minutes (Cerretelli and Di Prampero 1987). As it is shown in Fig. 4 pulmonary $\dot{V}O_2$ as a function of time follows increases as an exponential process in response to constant work-rate exercises (CWR). More specifically, at the onset of CWR, there is an early rapid increase (Phase I or cardiodynamic component, normally 15-20 seconds) due to the instantaneous increase in cardiac output and pulmonary blood flow. This initial phase is followed by a second and fast exponential increase (Phase II or primary component) which drives to the actual steady state (Phase III or steady state) within 2-3 minutes. Phase II largely reflects the kinetics of O_2 consumption in the exercising muscles although with a temporal leg (time delay, TD, normally 15-20 s). The rate of $\dot{V}O_2$ increases is quantified by the time constant (τ, time required to reach 63% of the difference between the steady state and the baseline) of the exponential which may span from 10 s in athletes to >100 s in patients (Rossiter, 2011).

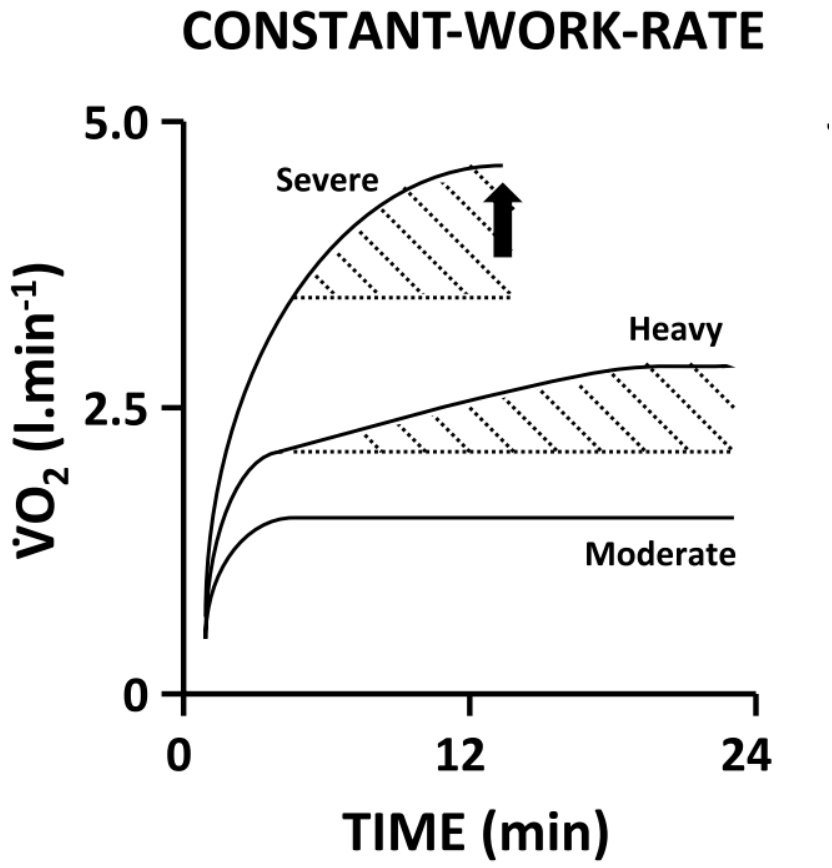

Figure 4. Schematic representation of $\dot{V}O_2$ responses following the onset of moderate, heavy and severe constant work rate exercises (From Jones at et al., Med Sci Sports Exerc, 2011).

The profile of $\dot{V}O_2$ in response to the onset of CWR may be defined with respect to the exercise intensity domain in which the exercise is performed because of its specific responses. More specifically, for moderate intensity exercise (work rates <GET), after the first increase in $\dot{V}O_2$ within the first breaths (phase I), there is an achievement of the steady state after the phase II within about 2-3 min in a healthy young subject. For cycle ergometry the gain (G) is approximately 10 $ml \cdot min^{-1} \cdot W^{-1}$ (Rossiter, 2011). In the heavy intensity domain (work rates between GET and CP) instead, a further increase in $\dot{V}O_2$ becomes apparent after 90-120 s and it is superimposed on the primary component. This further and progressive increase in $\dot{V}O_2$ is commonly known as slow component (see paragraph slow component). This represents an additional O_2 cost which increases G to >11 $ml \cdot min^{-1} \cdot W^{-1}$ thus reducing work efficiency and delaying the achievement of the steady state. For the severe intensities (work rates >CP) $\dot{V}O_2$ slow component drives $\dot{V}O_2$ to $\dot{V}O_2$peak: it raises rapidly and exponentially directly to $\dot{V}O_2$peak. Both profiles heralding imminent fatigue when $\dot{V}O_2$peak is attained (Poole et al., 1988).

The rates of adjustment of oxidative phosphorylation to increases in metabolic requirements are directly related to exercise (in)tolerance (Grassi et al., 2011). $\dot{V}O_2$ kinetics are correlated with the size of O_2 deficit that is the amount of energy that has to be borrowed either from the stored PCr or from the ongoing substrate level phosphorylation (i.e., glycolysis) (Cerretelli and Di Prampero 1987). Slower adjustments would result in a greater O_2 deficit and thus in a greater metabolic acidosis and depletion of PCr and glycogen stores, impairing exercise tolerance. On the contrary, rapid response kinetics are associated with a lower O_2 deficit, greater metabolic homeostasis and they represent a sign of the effective integrated function of the pulmonary, circulatory and skeletal muscle systems (Koppo et al., 2004; Allen et al., 2008).

Slow component. As mentioned before, for work rates above GET the attainment of a steady state is delayed or even not reached for the emergence of the supplementary and slowly developing component of $\dot{V}O_2$ response, the so-called "slow component". For exercise intensities below CP, the eventual $\dot{V}O_2$ steady state is greater than the predicted value derived from the sub GET-$\dot{V}O_2$ work rate relationship. When the work rate is above CP, $\dot{V}O_2$ continues to rise with time until reaching the $\dot{V}O_2$peak, heralding the cessation of the exercise. This elevation in $\dot{V}O_2$ above the predicted value can account for as much as 1.5 $l \cdot min^{-1}$, representing then >25% of the total increase in $\dot{V}O_2$ with respect to the pre-exercise baseline (Poole et al., 1994).

From a more functional point of view, the $\dot{V}O_2$ slow component is of significance because it seems to be closely related to progressive loss of muscle homeostasis and associated with the development of fatigue that occurs typically during exercises performed above GET (Rossiter et al, 2002; Burney & Jones, 2007). During high intensity dynamic constant work rate exercises, the $\dot{V}O_2$ slow component

is associated with a progressive recruitment of additional type II muscle fibers and their low efficiency might contribute to the increased O_2 cost (Krustrup et al., 2008). Fatigued fibers might also require either an increased ATP turnover (unchanged P/O) or an increased O_2 cost of ATP turnover (decreased P/O) (Barclay, J Physiol, 1996; Woledge, 1998). However, using the isolated dog gastrocnemius preparation *in situ* during a fatiguing high intensity exercise in which fibers are maximally activated with tetanic contractions by direct electrical stimulation of the motor nerve, the classic increase in $\dot{V}O_2$ with the time above the expected steady was not present, but there was a significant decrease (by 15-35 %) in force output (Zoladz et al, 2008). Interestingly, the $\dot{V}O_2$ data normalized by unit of force showed a clear slow component with an amplitude of 20% with respect to the total response. In other words, in exercising humans during CWR exercise, the external power output is conserved (probably by recruiting additional fibers) at the expense of an increase in $\dot{V}O_2$. On the contrary, in the isolated muscle *in situ* model, the muscle cannot recruit more fibers, the force decreases as a consequence of fatigue and $\dot{V}O_2$ remains constant. This phenomenon was named as *mirror image* of the $\dot{V}O_2$ slow component. The common point for these two different scenarios is a reduced efficiency of muscle contraction: constant mechanical power output with an increase in $\dot{V}O_2$ or on the contrary, a constant $\dot{V}O_2$ with a decreasing force. Thus there was the demonstration that the reduced efficiency of muscle contraction and therefore the putative mechanism responsible for the $\dot{V}O_2$ slow component is not necessarily related to a progressive recruitment of muscle fibers.

1.2 NEW APPROACHES

1.2.1 Heart rate (HR) kinetics: work rate decrease in order to keep a constant HR

In the past, exercise prescription for aerobic exercise was often given in terms of work rates corresponding to specific percentages of $\dot{V}O_2$peak (Franklin et al., 2000; Powers & Howley 2004). This was based on the concept of a linear relationship between $\dot{V}O_2$ and work rate (see, e.g., Astrand et al., 1986). However, it has been demonstrated that a disproportionate increase in $\dot{V}O_2$, usually termed ‘‘slow component’’ of the $\dot{V}O_2$ kinetics, is present during constant work rate (CWR) exercise above GET, and even more markedly above the CP (Rossiter, 2011; Poole & Jones 2012). As a consequence of this, exercise intensities and exercise prescriptions are nowadays usually not expressed as a percent of $\dot{V}O_2$peak, but with respect to GET or CP (Poole et al., 2016; Iannetta et al., 2020). The same type of problem would be present also in terms of HR. Textbook and guidelines (Franklin et al., 2000; Powers & Howley 2004) suggest indeed to prescribe exercise at an intensity identified as a percentage of HRpeak, mainly on the basis of the facility to measure and record HR. However, a slow component is also present for the HR kinetics (Wasserman et al., 1967; Linnarsson 1974; Orizio et al., 1988; Grassi et al., 1997; Hebestreit et al., 1998; Engelen et al., 1996; Bearden & Moffatt 2001), although its amplitude (particularly with respect to the amplitude of the $\dot{V}O_2$ kinetics) and the work rate above which this slow component seems to appear have not been formally analyzed. According to anecdotal observations (Orizio et al., 1988; Engelen et al., 1996), a slow component of HR kinetics may be present also during CWR exercise below GET.

In the present thesis, we hypothesized that a slow component of HR kinetics would be present also during CWR < GET, whereas during CWR > GET, the amplitude of the slow component of the HR kinetics would be greater than the amplitude of the slow component of the $\dot{V}O_2$ kinetics and therefore in order to keep a constant an HR target value, both work rate and $\dot{V}O_2$ had to decrease. We also hypothesized that this phenomenon will also be present also in populations of patients and microgravity (simulated by bed rest).

By negating the presence of a linear relationship between HR and $\dot{V}O_2$ during CWR, these findings, if confirmed, would have profound implications on exercise prescription and tolerance. In short, also for HR (and possibly more markedly for HR than for $\dot{V}O_2$), the concept of a value corresponding to a specific CWR would not hold true, at least above a certain CWR. Exercise prescriptions at specific HR values, when carried out for periods longer than a few minutes, could lead to premature fatigue and to exercise termination.

1.2.2 Near infrared spectroscopy: kinetics of muscle $\dot{V}O_2$ recovery

Non-invasive near-infrared spectroscopy (NIRS) has been extensively used in exercise physiology mostly to study skeletal muscle oxygenation in vivo both in health and disease in exercise physiology (Quaresima and Grassi 2016). The main variable evaluated by NIRS is the skeletal muscle fractional O_2 extraction, which reflects the balance between O_2 delivery and O_2 utilization (the fraction of haemoglobin that is bound to oxygen). NIRS measures light attenuation in media to determine the concentration or the relative values of light absorbing chromophores. The main absorber of NIR light is heme and it has O_2-dependent absorption features. The physical principle of NIRS is based on the absorption of the NIR light by haemoglobin (Hb) in small arteries, arterioles, capillaries, venules and small veins so the attenuation of the light in media is used to determine the concentration of light absorbing chromophores. A NIRS probe is applied on the skin overlying the muscle of interest and NIR light penetrates skin and subcutaneous fat to finally reach the underlying skeletal muscle tissue. The parameters commonly derived from NIRS measurements are: micromolar (μM) changes of deoxygenated haemoglobin (Hb) + myoglobin (Mb) concentrations (Δ[deoxy(Hb + Mb)]) and of oxygenated (Hb + Mb) (Δ[oxy(Hb + Mb)]). The sum between the two variables (Δ[deoxy(Hb + Mb)+ oxy(Hb + Mb)]) is related to changes in the total Hb volume (blood volume in the investigated tissue). An increased Δ[deoxy(Hb + Mb)] or a decreased Δ[oxy(Hb + Mb)], would indicate an increased fractional O_2 extraction only when Δ[deoxy(Hb + Mb)+ oxy(Hb + Mb)] is constant. This is unlikely in exercising muscles. Normally the problem is circumvented, at least in part, by taking as an index of deoxygenation the Δ[deoxy(Hb + Mb)] variable, which is relatively insensitive to blood volume changes, and has been demonstrated to nicely correlate with other variables related to fractional O_2 extraction (Grassi & Quaresima, 2016).

As mentioned before, skeletal muscle fractional O_2 extraction is the balance between O_2 delivery (DO_2) and O_2 utilization ($\dot{V}O_2m$). Although this parameter can yield to relevant information pertinent to exercise performance and exercise tolerance, it does not specifically reflect $\dot{V}O_2m$. More recently, a new approach to dissociate $\dot{V}O_2m$ from DO_2 using NIRS has been proposed: the repeated arterial occlusion method following exercise in association with NIRS measurements. With the transient arterial occlusions provided by a rapid pneumatic cuff during the recovery phase it is possible to interrupt the DO_2 to the investigated muscle. Based on a concept originally developed by Hamaoka et al. (1996) and by Van Beekvelt et al. (2001): in ischemic conditions, the linear rate of increase in deoxy-(haemoglobin-myoglobin), or the linear rate of decrease of oxy-(haemoglobin+myoglobin), as determined by NIRS (Grassi & Quaresima, 2016; Barstow, 2019), represents an index of $\dot{V}O_2m$. By performing a series of repeated short ischemia (blood flow occlusions induced by rapid inflation and subsequent deflation of a pneumatic cuff with suprasystolic pressure) during the recovery from

exercise, $\dot{V}O_2m$ measurements have been obtained with a temporal resolution allowing performance of a reliable $\dot{V}O_2$m off-kinetics analysis (Ryan et al., 2012; Adami and Rossiter, 2017). The method has been validated against other approaches of functional evaluation of skeletal muscle oxidative metabolism, such as [PCr] (squared brackets denote concentrations) recovery kinetics (Ryan et al., 2013) and high-resolution respirometry of permeabilized skeletal muscle fibers (Ryan et al., 2014). This NIRS technique has already been used to investigate differences in oxidative function across a wide range of muscles, ages and disease states (Adami & Rossiter, 2017).

1.2.3 Peripheral vascular adaptations: blood flow response to passive leg movement

Exercise achieved without voluntary activation and contraction has a long history in vascular medicine (Trinity & Richardson, 2019). The passive exercise evokes little increase in O_2 consumption, allowing for the evaluation of exercise-induced hyperaemia without an increase in metabolism.

The blood flow increase detected in the common femoral artery, by Eco-Doppler, during 1 minute of passive leg extension has been identified, in recent years, as a tool of functional evaluation of peripheral and muscle blood flow (Gifford & Richardson 2017). The increase in leg blood flow elicited by the passive leg movement (PLM) is primarily a product of changes in peripheral arterial diameter or tone. Trinity at al. (2012) and Mortensen et al., (2012) have independently demonstrated that up to 80% of the overall increase in leg blood flow during PLM is nitric oxide (NO) dependent in healthy young subjects. By inhibiting nitric oxide synthase (NOS) via an intra-arterial infusion of N^G -monomethyl-$_L$-arginine ($_L$-NMMA) the hyperaemic and vasodilatatory response to PLM was attenuated by almost 80% (Trinity et al., 2012; Mortensen et al., 2012). It appears that when the leg is moving, the release of NO and other dilator mechanisms are initiated in response to the mechanical deformation of the leg (Jufri et al., 2015) which produces the dilation of the vascular bed. Thus, the blood flow increase, observed by this method, is directly related to nitric oxide (NO)-mediated vasodilation, and is therefore considered an index of peripheral endothelial and vascular function *in vivo*. Therefore, the PLM-induced hyperaemic response appeared to be directly related to exercise training, to have an age-related attenuation and emerged to be impaired in patient populations, such as patients with chronic heart failure (Gifford & Richardson 2017).

1.2.4 Mitochondrial respiration in permeabilized skeletal muscle fibers

The last step along the O_2 pathway of oxidative metabolism during exercise is represented by oxidative phosphorylation at the mitochondrial level in skeletal muscle fibers. A "state of the art" method for the functional evaluation of mitochondrial respiration is high-resolution respirometry (HRR) (Pesta & Gneiger 2012), in which permeabilized skeletal muscle fibers obtained by a biopsy are exposed, in a chamber of the instrument, to increasing concentrations of ADP and to a sequence of saturating levels of substrates, in the presence of saturating levels of O_2. Several variables can be determined by HRR, such as: "leak respiration"; maximal ADP-stimulated mitochondrial respiration, supported by respiratory complex I or by respiratory complexes I and II; maximal uncoupled respiration; oxidative phosphorylation coupling; and others more depending on the adopted protocol (Pesta & Gneiger 2012). Mitochondrial respiration variables are usually "normalized" with respect to mitochondrial mass, estimated by citrate synthase protein content or activity. Compared to similar measurements carried out in isolated mitochondria, HRR presents the advantage of substantially preserving the cellular architecture of the muscle fiber (Picard et al. 2011). In recent years, our group has extensively utilized HRR, on skeletal muscle fibers obtained from subjects undergoing resistance training (Salvadego et al. 2013), subjects exposed to chronic hypoxia (Tam et al. 2016), subjects undergoing short-term bed rest (Zuccarelli et al. 2020) or hypoxic bed rest (Salvadego et al. 2016, Salvadego et al. 2018), transgenic mice with heart failure (Grassi et al. 2017) and mice undergoing hindlimb suspension (Cannavino et al. 2011).

1.3 EFFECTS OF INACTIVITY – MICROGRAVITY (BED REST) ON OXIDATIVE METABOLISM DURING EXERCISE

Horizontal or head-down bed rest interventions have been utilized over the years as analogues for studying the adaptive responses of astronauts exposed to microgravity. As mentioned before, oxidative metabolism represents the main energy source for activities related to everyday life and work. This applies to activities carried out on Earth, but also to activities performed in microgravity conditions. Maximal oxygen consumption ($\dot{V}O_2max$), traditionally considered an index evaluating the maximal performance of the integrated respiratory, cardiovascular and muscular factors governing oxidative metabolism during exercise, has been shown to be reduced immediately after short-duration Space Shuttle missions (8-14 days) (Levine et al., 1996; Moore et al., 2001) and after long-duration International Space Station (ISS) missions (typically 6 months) (Moore at al., 2014; Ade et al., 2017). Moreover, the decline in $\dot{V}O_2max$ and the physiological mechanisms mediating its decrease seem to be dependent on the duration of microgravity exposure (Ferretti & Capelli 2009; Ade et al., 2015; Salvadego et al., 2018).
According to the review and meta-analysis by Reid-Larsen et al. (2017) $\dot{V}O_2max$ during normoxic bed rest declines linearly as a function of the bed rest duration (in the range from a few hours up to 90 days), at a rate of about 0.3-0.4 % per day. The rate of $\dot{V}O_2max$ decrease seems also to be inversely related with the level of $\dot{V}O_2max$: higher pre bed rest values are associated with greater declines in $\dot{V}O_2max$ (Reid-Larsen et al. 2017). The decrease in $\dot{V}O_2max$ during prolonged situations of muscle inactivity would translate into a significantly reduced exercise tolerance, but also (if it had not been transitory) in a significantly increased mortality risk (Myers et al. 2002).

During bed rest, changes in cardiac function occurred immediately upon reclining. Within the first 24-48 h, stroke volume (SV) and cardiac output ($\dot{Q}$) decrease in response to the diuresis-induced hypovolemia. As the bed rest continues without physical countermeasures, SV and thus $\dot{Q}$ continue to decrease below the pre-bed rest value in response to the reduced blood volume and oxygen demand, loss of active muscle mass, decreased cross-sectional area and contractile strength (Fortney at al., 1996).

During exercise after bed rest, exaggerated cardiovascular responses at a given oxygen uptake are reported, such as: increases in heart rate values with a reduced increase in stroke volume, increases in $\dot{V}E/\dot{V}O_2$, increases in blood lactate concentrations and in respiratory exchange ratio (Fortney at al., 1996). $\dot{V}O_2$ kinetics are also reported to be slower after bed rest (Convertino et al., 1984; Capelli et al., 2009).

The mechanisms responsible for the decline in exercise tolerance observed during bed rest involve changes at different levels of the O_2 cascade from the ambient air to the skeletal muscle mitochondria: from the pulmonary system and the cardiovascular function to the reduced active muscle mass and to the altered neuromuscular function (Fortney at al., 1996). Altered function in the pulmonary system is thought not to play a great role in limiting $\dot{V}O_2max$, since minimal changes were found in arterial saturation and arterial partial pressure of O_2 after simulated microgravity up to 90 days of bed rest (Capelli et al., 2006; Prisk et al., 2000). As for the cardiovascular system, data from bed rest studies as well as from spaceflight missions reported similar decreases in SV and $\dot{Q}$ during rest and during submaximal and maximal exercises (Buderer et al., 1976; Levine et al., 1996; Porcelli et al., 2010). A reduction in cardiac mass of 1% per week has also been reported after exposure to microgravity (Dorfman et al., 2007).

In addition to these well-known alterations in cardiovascular functions during microgravity condition, functional limitations in oxidative metabolism also at peripheral level, localizable at the intramuscular level, have been demonstrated.

The elegant study of Ade et al., (2015) examined the determinants of $\dot{V}O_2max$ decrease detected following microgravity exposure by retrospectively modelling adaptation within the O_2 transport system. During a short spaceflight mission with the duration of about 11 days, $\dot{V}O_2max$ decreased by about 20% (Levine et al., 1996). It has been hypothesized that for this relatively short duration spaceflight a decreased plasma volume and subsequent reductions in $\dot{Q}max$ and haemoglobin concentration would lead to impair convective O_2 transport (about 23%) with less decreases in diffusive O_2 transport (about 13%). Instead, the retrospective analysis of prolonged periods of muscle disuse such as 90 days of bed rest, revealed that the additional 10% decrease in $\dot{V}O_2max$ (Capelli et al., 2006) is mediated by about 40% decrease in diffusive O_2 transport with very similar decrease to short-term microgravity exposure in convective O_2 delivery (Ade et al., 2015).

Some recent studies conducted by our group, using bed rest models, have suggested the presence of different sites of intramuscular limitations, also depending on the duration of exposure to microgravity. Specifically, while after a 10-day exposure to microgravity the main limitation would be “upstream” of the mitochondria (Porcelli et al., 2010; Salvadego et al., 2016; Ade et al., 2017), such as at the level of microvascular supply of O_2, intramuscular matching between O_2 delivery and O_2 uptake, and peripheral O_2 diffusion, after a 20-day bed rest also the mitochondrial function would be affected (Salvadego et al., 2018). The effects of short periods of bed rest on maximal ADP-stimulated mitochondrial respiration (as evaluated by high-resolution respirometry [HRR] of isolated

and permeabilized fibers obtained by biopsy) are somehow controversial. Whereas Miotto et al. (2019) and Dirks et al. (2020) described an impaired mitochondrial function following bed rest periods of 3 and 7 days, respectively, other authors (Larsen et al. 2018, Salvadego et al. 2016) did not see impairments following 4 and 10 days of bed rest exposure. An impaired mitochondrial respiration was seen by HRR after 21 days of bed rest (Salvadego et al. 2018), confirming the impairment of skeletal muscle oxidative function described in that study by other methods. Another aspect which has been recently investigated by HRR deals with the sensitivity of mitochondrial respiration to submaximal (and physiological) ADP concentrations (Holloway et al. 2018). Only one study investigated this variable following a very short (7 days) bed rest exposure, describing a decreased mitochondrial respiration at submaximal [ADP] (Dirks et al. 2020).

2 CHAPTER II - RESEARCH AIM

The overall objectives of my PhD thesis deal with the implementation of new methods for the functional evaluation of oxidative metabolism during exercise allowing the identification of biomarkers of functional impairment.

The two main topics were focused on (i) the effects of aerobic exercise prescription based on heart rate (HR) both in healthy population and in population of patients, and on (ii) the localization of the main site(s) of impairment in oxidative metabolism during exercise in simulated microgravity condition along the O_2 pathway from the ambient air to skeletal muscle mitochondria, with particular interest to the peripheral level.

More specific, the projects carried out during the three years of this PhD aimed to:

- Test in 17 healthy male subjects the hypothesis that during constant work rate exercises (CWR) at different intensities the slow component of HR kinetics would occur at lower work rate and would be more pronounced than the slow component of pulmonary oxygen uptake ($\dot{V}O_2$) kinetics. As a consequence, we hypothesized that exercise prescription at a fixed HR value, slightly above the "gas exchange threshold" (GET), as it is often done for training purposes and in clinical studies, would determine a significant decrease in work rate (*Study 1*)
- Verify the above hypotheses also in a population of patients. 16 male obese patients were tested before and after a 3-week multidisciplinary programme aimed at reducing body mass (*Study 2*).
- Investigate the same hypotheses in 10 healthy male subjects evaluated before and after 10-day of horizontal bed rest (*Study 3*).
- Modify and improve a recently proposed method, aimed at assessing mitochondrial function non-invasively by near-infrared spectroscopy (NIRS). This method is based on the measurement of skeletal muscle $\dot{V}O_2$ recovery kinetics following exercise. (*Study 4*).
- Determine, with an integrative approach, the sites of impairment of oxidative metabolism during exercise following a 10-day bed rest, spanning from systemic variables to markers more specifically related to peripheral vascular function, skeletal muscle fractional O_2 extraction by NIRS and mitochondrial function. The latter was evaluated by an *ex vivo* approach, that is by high-resolution respirometry (HRR) on permeabilized muscle fibers (in conditions of unlimited O_2 and substrates availability), and by a non-invasive *in vivo* approach, based on the evaluation by NIRS of muscle $\dot{V}O_2$ recovery kinetics following CWR exercise (*Study 5*)

A better knowledge of the site(s) of impairment in oxidative metabolism and of new parameters for the evaluation of exercise (in)tolerance could potentially provide a background for improving exercise prescription and therapeutic interventions.

3 CHAPTER III - EXPERIMENTAL STUDIES

3.1 COMPARISON BETWEEN SLOW COMPONENTS OF HR AND $\dot{V}O_2$ KINETICS: FUNCTIONAL SIGNIFICANCE – *STUDY 1*

This article has been published in "Medicine and Science in Sport and Exercise" 50(8): 1649-1657 (2018) as "Comparison between slow components of HR and $\dot{V}O_2$ kinetics: functional significance" by Lucrezia Zuccarelli, Simone Porcelli, Letizia Rasica, Mauro Marzorati, Bruno Grassi.

ABSTRACT

Purpose: Aerobic exercise prescription is often based on a linear relationship between pulmonary oxygen consumption ($\dot{V}O_2$) and heart rate (HR). The aim of the present study was to test the hypothesis that during constant work rate (CWR) exercises at different intensities, the slow component of HR kinetics occurs at lower work rate and is more pronounced that the slow component of $\dot{V}O_2$ kinetics. Methods: Seventeen male (age, 27 ± 4 yr) subjects performed on a cycle ergometer an incremental exercise to voluntary exhaustion and several CWR exercises: 1) moderate CWR exercises, below gas exchange threshold (GET); 2) heavy CWR exercise, at 45% of the difference between GET and $\dot{V}O_2$ peak (Δ); 3) severe CWR exercise, at 95% of Δ; 4) ‘‘HRCLAMPED’’ exercise in which work rate was continuously adjusted to maintain a constant HR, slightly higher than that determined at GET. Breath-by-breath $\dot{V}O_2$, HR, and other variables were determined. Results: In moderate CWR exercises, no slow component of $\dot{V}O_2$ kinetics was observed, whereas a slow component with a relative amplitude (with respect to the total response) of 24.8 ± 11.0% was observed for HR kinetics. During heavy CWR exercise, the relative amplitude of the HR slow component was more pronounced than that for $\dot{V}O_2$ (31.6 ± 11.2% and 23.3 ± 9.0%, respectively). During HRCLAMPED, the decrease in work rate (~14%) needed to maintain a constant HR was associated with a decreased $\dot{V}O_2$ (~10%). Conclusions: The HR slow component occurred at a lower work rate and was more pronounced than the $\dot{V}O_2$ slow component. Exercise prescriptions at specific HR values, when carried out for periods longer than a few minutes, could lead to premature fatigue.

Key Words: SLOW COMPONENT, CONSTANT WORK RATE EXERCISE, $\dot{V}O_2$ KINETICS, HR KINETICS, EXERCISE PRESCRIPTION.

INTRODUCTION

In healthy subjects, in older adults, and in populations of patients, exercise prescription needs to be specifically performed in terms of exercise intensity and duration (Franklin et al., 2000). Although health benefits can be gained from any amount of exercise, several bodies of evidence suggest that an individualized exercise prescription is more effective in improving subjects' health status and physical performance. In the past, exercise prescription for aerobic exercise was often given in terms of work rates corresponding to specific percentages of peak O_2 uptake ($\dot{V}O_2$peak) (Franklin et al., 200; Powers & Howley 2004). This was based on the concept of a linear relationship between pulmonary oxygen consumption ($\dot{V}O_2$) and work rate (see, e.g., Ref. [Astrand et al., 1986]). However, it has been demonstrated that a disproportionate increase in $\dot{V}O_2$ is present during constant work rate (CWR) exercise above the ''gas exchange threshold'' (GET), and even more markedly so above the ''critical power'' (CP) (Rossiter, 2011; Poole & Jones 2012). Because it is slowly developing during CWR exercise, this excess $\dot{V}O_2$ is usually termed ''slow component'' of the $\dot{V}O_2$ kinetics. The mechanistic bases of this phenomenon have been discussed in recent reviews (Rossiter, 2011; Poole & Jones 2012; Grassi et al., 2015; Jones et al., 2011). To prevent the occurrence of the $\dot{V}O_2$ slow component, which heralds a loss of efficiency and fatigue, and to maintain the prescribed $\dot{V}O_2$, the subject/athlete/patient is forced to decrease exercise intensity (Grassi et al., 2015; Gaesser & Poole 1996). As a consequence of this, exercise intensities and exercise prescriptions are nowadays usually not expressed as a percent of $\dot{V}O_2$peak, but with respect to GET or CP (Franklin et al., 2000; Rossiter, 2011; Poole & Jones 2012). The same type of problem would be present also in terms of heart rate (HR). Textbook and guidelines (Franklin et al., 2000; Powers & Howley 2004) suggest indeed to prescribe exercise at an intensity identified as a percentage of HRpeak, mainly on the basis of the facility to measure and record HR. Whereas the $\dot{V}O_2$ response to different CWR exercise intensities has been extensively studied (Rossiter, 2011; Poole & Jones 2012; Grassi et al., 2015; Jones et al., 2011), the HR response has not received the same attention. However, a slow component is present also for the HR kinetics (Wasserman et al., 1967; Linnarsson 1974; Orizio et al., 1988; Grassi et al., 1997; Hebestreit et al., 1998; Engelen et al., 1996; Bearden & Moffatt 2001), although its amplitude (particularly in respect to the amplitude of the $\dot{V}O_2$ kinetics) and the work rate above which this slow component seems to have not been formally analyzed. According to anecdotal observations (Orizio et al., 1988; Grassi et al., 1997; Hebestreit et al., 1998), a slow component of HR kinetics may be present also during CWR exercise below GET. In the present study, we hypothesized that a slow component of HR kinetics would be present also during CWR < GET, whereas during CWR > GET, the amplitude of the slow component of the HR kinetics would be greater than the amplitude of the slow component of the $\dot{V}O_2$ kinetics. By negating the presence of a linear relationship between HR

and $\dot{V}O_2$ during CWR, these findings, if confirmed, would have profound implications on exercise prescription and tolerance. In short, also for HR (and possibly more markedly so for HR than for $\dot{V}O_2$), the concept of a value corresponding to a specific CWR would not hold true, at least above a certain CWR. Exercise prescriptions at specific HR values, when carried out for periods longer than a few minutes, could lead to premature fatigue and to exercise termination.

METHODS

Subjects

Seventeen young healthy male volunteers (age, 27 ± 4 years; height, 181 ± 5 cm; weight, 77 ± 10 kg; body mass index, 23.3 ± 2.8 $kg \cdot m^{-2}$) participated in this study. All participants were moderately trained (training sessions = 5.9 ± 4.3 $hours \cdot week^{-1}$), non-smokers, normotensive and were not taking any drugs. The procedures used in this study were approved by the local ethics committee. All subjects gave their written informed consent after they received a detailed explanation of the experimental procedures before commencement of the study.

Experimental design

Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state and to avoid strenuous exercise in the 24 hours preceding each testing session. In addition, they were told to avoid alcohol and caffeine intake 48 hours before the exercise test and to refrain from food ingestion 3 hours before each testing session. Exercise tests were carried out in a well-ventilated laboratory at 19–21 °C, under continuous medical supervision and 12-lead electrocardiography (ECG; Custo Med GmbH). Each subject completed the entire experimental protocol within two weeks from enrollment. On their first visit anthropometric measurements were performed, and the subjects completed an incremental exercise up to voluntary exhaustion on an electronically braked cycle ergometer (Corival; Lode BV). Pedaling frequency was digitally displayed to the subjects, who were asked to keep a constant cadence throughout the tests between 70 and 80 rpm. Voluntary exhaustion was defined as the incapacity to maintain the imposed load and pedaling frequency despite vigorous encouragement by the researchers. The protocol began with a power output of 120 W for 5 min, and then the power output was increased by 20 W every minute. The peak values of the main cardiovascular, respiratory and metabolic parameters were taken as the highest 30-s mean values attained prior to the subject's voluntary exhaustion. The $\dot{V}O_2$ at the gas exchange threshold (GET) was determined by two independent investigators by utilizing the "V-slope" methods and the "secondary criteria" (Beaver et al., 1986). In order to identify the work rate corresponding to the $\dot{V}O_2$ at GET, the effect of the delayed $\dot{V}O_2$ adjustment to the increased work rate during the incremental test was corrected by shifting the

linear $\dot{V}O_2$ *vs.* time (and work rate) relationship to the left, by an amount corresponding to the mean response time of the $\dot{V}O_2$ kinetics determined in each subject (Whipp et al., 1981).

After the incremental exercise, the subjects completed five repetitions of CWR cycle ergometer exercises in a randomized order. Three 20-min bouts were at a moderate intensity (MODERATE) corresponding to 70% of GET. The fourth CWR exercise was performed in the heavy intensity domain (HEAVY), at 45% of the difference between GET and $\dot{V}O_2$ peak (Δ), and its maximum duration was set at 15 minutes (or until exhaustion). The fifth CWR exercise was performed in the severe intensity domain (SEVERE), at 95% of Δ until exhaustion.

The subjects performed also an "HR controlled" exercise ($HR_{CLAMPED}$) in which a target HR slightly above GET was identified as the HR corresponding to GET +10%. During $HR_{CLAMPED}$ the work rate was kept constant for the first 3 minutes, or until HR reached its target value, and then it was adjusted by the operator every 5 seconds in order to maintain a constant HR at the target value. Before performing $HR_{CLAMPED}$ trial, the subjects familiarized with the protocol in practice runs.

Measurements

Pulmonary ventilation ($\dot{V}E$, in BTPS), O_2 consumption ($\dot{V}O_2$), and CO_2 output ($\dot{V}CO_2$), both in STPD, were determined breath-by-breath by a metabolic cart (Vmax29c; SensorMedics). Expiratory flow was determined by a mass flow sensor (hot wire anemometer). $\dot{V}O_2$ and $\dot{V}CO_2$ were determined by continuously monitoring PO_2 and PCO_2 at the mouth throughout the respiratory cycle and from established mass balance equations. Gas exchange ratio (R) was calculated as $\dot{V}CO_2/\dot{V}O_2$. HR was determined from the ECG signal.

Stroke volume (SV) was estimated beat-by-beat by means of transthoracic impedance cardiography (Physio Flow; Manatec Biomedical) and averaged every 10 beats during all exercise tests. The accuracy of this device has been previously evaluated during incremental exercise in healthy subjects against the direct Fick method (Richard et al., 2001). A detailed description of the method has been provided elsewhere (Lamarra et al., 1987). Briefly, the Physio Flow emits a 75 kHz 1.8 mA alternating electrical current via two sets of electrodes (two "transmitting" and two "sensing" electrodes) applied above the supraclavicular fossa at the left base of the neck and next to the spine corresponding to the xiphoid process of the subject, respectively. Another set of two electrodes is used to monitor a single ECG lead. Verification of the correct signal quality is accomplished by visualization of the ECG signal, the impedance waveform, and their first derivatives. Then, the subject stands or sits still and relaxed for at least 5 minutes, during which the auto-calibration procedure is performed in order to obtain reference curves and data necessary to measure SV variations. HR was obtained from the R-R interval determined on the ECG first lead. Cardiac output (CO) was then calculated by multiplying SV and HR.

At 1, 3, and 5 minutes of recovery, 20 μL of capillary blood were obtained from a preheated earlobe for the determination of blood lactate concentration ($[La]_b$) by an enzymatic method (Biosen C-line; EKF).

Kinetics analysis

$\dot{V}O_2$ kinetics were mathematically evaluated during transitions from rest to MODERATE, HEAVY and SEVERE intensity CWR exercises. Breath-by-breath $\dot{V}O_2$ values obtained during exercise were time aligned and then superimposed for each subject (Lamarra et al., 1987). Average $\dot{V}O_2$ values every 10 s were calculated. Data obtained during the first 20 s of the transition ("cardiodynamic" phase [Whipp et al., 2002]) were excluded from analysis. Thus, $\dot{V}O_2$ kinetics analysis dealt mainly with the "phase 2" (or "fundamental" component) of the response. To evaluate mathematically the $\dot{V}O_2$ kinetics, data were fitted by the function:

$$y(t) = y_{BAS} + A_f [1 - e^{(t - TD_f) / \tau_f}] \qquad (1)$$

and parameter values (TDf, τf) were determined that yielded the lowest sum of squared residuals. In *equation 1*, t is time, *y*BAS indicates the baseline, Af is the amplitude between the *y*BAS and the steady state during the fundamental component, TDf is the time delay and τf the time constant of the function for the fundamental component. To check the presence of a slow component (Whipp et al., 2002) of the kinetics, data were also fitted by the function:

$$y(t) = y_{BAS} + A_f [1 - e^{(t - TD_f) / \tau_f}] + A_s [1 - e^{(t - TD_s) / \tau_s}] \qquad (2)$$

In equation 2, As, TDs, and τs indicate the amplitude, the time delay, and the time constant of the slow component, respectively. The equation that best fitted the experimental data was determined by the F-test (see Statistical analysis). That is to say, when Eq. 2 provided a better fit of the data, a slow component of $\dot{V}O_2$ kinetics was present, superimposed on the fundamental component. The slow component, however, did not always follow an exponential function, being sometimes linearly related to the time of exercise; moreover, its τ_f and A_s values were devoid of physiological significance. In these cases, a third equation (3) was also utilized, with an exponential function for the fundamental component and a linear function for the slow component (exponential + linear fitting) (Linnarsson et al., 1974):

$$y(t) = y_{BAS} + A_f [1 - e^{(t - TD_f) / \tau_f}] + S [t - TD_s] \qquad (3)$$

where S (slope) is the angular coefficient of the linear regression of $\dot{V}O_2$ vs. time t. The actual amplitude (A_s') of the slow component was calculated as the difference between the average $\dot{V}O_2$ value obtained during the last 20–30 s of CWR exercise and the asymptotic value of the fundamental component. The percentage contribution of the slow component to the total amplitude of the response (A_s'/A_{tot}) was also calculated.

To confirm the presence/absence of an increase in $\dot{V}O_2$ as a function of time, average $\dot{V}O_2$ values were also calculated for each subject every 30 seconds, from the 3rd to the 15th minute of exercise (or until exhaustion), and linear regression lines were drawn. The absence of a significantly positive slope would indicate that the variable has reached a steady state.

As for HR, beat-by-beat values obtained during exercise were time aligned and then superimposed for each subject (Engelen et al., 1996). Average HR values every 10 beats were calculated. HR kinetics were analyzed by applying the same equations described above for $\dot{V}O_2$.

Statistical analysis

Results are expressed as mean ± SD values. Data fitting by exponential functions was performed by the least-squared residuals method. Comparisons between fitting with different models were carried out by the *F*-test. Statistical significance of differences between HR and $\dot{V}O_2$ slow component amplitudes was checked by two-tailed Student's *t*-test for paired data. The effects of intensity domains (MODERATE, HEAVY and SEVERE) on the main respiratory, cardiovascular, and metabolic end-exercise values were tested using a one-way repeated measures ANOVA. When significant differences were found, a Tukey's post hoc test was used to determine the exact location of the difference. The level of significance was set at $P < 0.05$. Statistical analyses were carried out by a commercially available software package (Prism 6.0; GraphPad).

RESULTS

All subjects concluded the entire experimental protocol except one, who did not complete the SEVERE and the $HR_{CLAMPED}$ exercises for personal reasons. Peak values of the main respiratory, cardiovascular, and metabolic variables are shown in **Table 1**. Mean values of the main variables determined during the last 30 s of the three CWR exercises are also given in **Table 1**.

Table 1. Main respiratory, cardiovascular, and metabolic end-exercise values determined during incremental and constant work rate exercises.

	INCR	MODERATE	HEAVY	SEVERE
Work rate, W	288±53*#§	123±12^#§	237±49^*§	269±50^*#
$\dot{V}O_2$, l·min^{-1}	3.664±0.52*	1.936±0.10^#§	3.667±0.66*	3.733±0.70*
$\dot{V}O_2$, ml·kg·min^{-1}	48.5±8.6*	26.0±3.8^#§	48.5±10.4*	49.0±9.5*
$\dot{V}CO_2$, l·min^{-1}	4.324 ± 0.55*#	1.744 ± 0.12^#§	3.694±0.59^*§	4.201±0.53*#
$\dot{V}E$, l·min^{-1}	145.7±21.2*	49.7±4.9^#§	132.6±28.7*§	141.5±32.5*#
R	1.19±0.08*#	0.89±0.04^#§	1.04±0.06^*§	1.19±0.10*#
HR, beats·min^{-1}	188±10*§	138±15^#§	184±11*	180±11^*
SV, ml	134±27	124±19	129±23	124±20
CO, l·min^{-1}	24.9±4.7*	17.0±3.5^#§	23.6±4.4*	21.9±3.3*
$[La]_b$, mM	11.7±1.6*	2.5±1.5^#§	11.0±2.0*	12.3±1.8*
RPE, (6-20)	18±1*§	12±2^#§	18±1*§	19±1^*#

Mean values ± SD. $\dot{V}O_2$, oxygen uptake; $\dot{V}CO_2$, CO_2 output; $\dot{V}E$, pulmonary ventilation; R, gas exchange ratio; HR, heart rate; SV, stroke volume; CO, cardiac output; $[La]_b$, blood lactate concentration; RPE, rate of perceived exertion. INCR, incremental exercise; MODERATE, moderate CWR exercise; HEAVY, heavy CWR exercise and SEVERE, severe CWR exercise. ^P<0.05 vs INCR; *P<0.05 vs MODERATE; #P<0.05 vs HEAVY; §P<0.05 vs SEVERE.

All subjects attained peak HR values around 97% of the age predicted maximum (calculated as 208 - 0.7 x age). Taking into account also R peak, $[La]_b$ peak and RPE peak values, it can be assumed that exhaustion was indeed reached. GET occurred at a $\dot{V}O_2$ of 2.84 ± 0.50 l·min^{-1}, corresponding to 77% of $\dot{V}O_2$peak. HR at GET was 163 ± 12 beats·min^{-1}, corresponding to 88% of HRpeak. The relationship between CO and $\dot{V}O_2$ was linear and the slope of the regression line was 5.4 ± 0.2.

Work rate for MODERATE, HEAVY and SEVERE was 44 ± 7 %, 83 ± 6 % and 92 ± 4 % of Wpeak, respectively. All subjects completed the three 20 minutes of MODERATE. Only 6 subjects completed the 15 minutes of HEAVY; for the remaining 11 subjects the time to exhaustion was about 9 minutes (ranging from 7.5 to 14 minutes). No subjects completed the 15 minutes of SEVERE; the time to exhaustion was 5.0 ± 1.2 minutes (ranging from 2.4 to 7.3 minutes). As a consequence of the

increasing work rate, $\dot{V}CO_2$, $\dot{V}E$, R, $[La]_b$ and HR increased significantly across the different exercise intensity domains. $\dot{V}O_2$ was higher in HEAVY and SEVERE *vs.* MODERATE. $\dot{V}O_2$ was not different between HEAVY and SEVERE, and in both conditions values were not significantly different from $\dot{V}O_2$peak. The same behavior was observed for HR, CO, $[La]_b$ and RPE. SV increased significantly from rest to MODERATE and it did not further increase during HEAVY and SEVERE.

In **Figure 1** $\dot{V}O_2$ and HR kinetics obtained in a typical subject during CWR exercise at the three investigated intensity domains are shown. For $\dot{V}O_2$ no slow component was observed in MODERATE, whereas a clear slow component (see Equation 2 in Methods) was observed in HEAVY. For HR a slow component was already present during MODERATE.

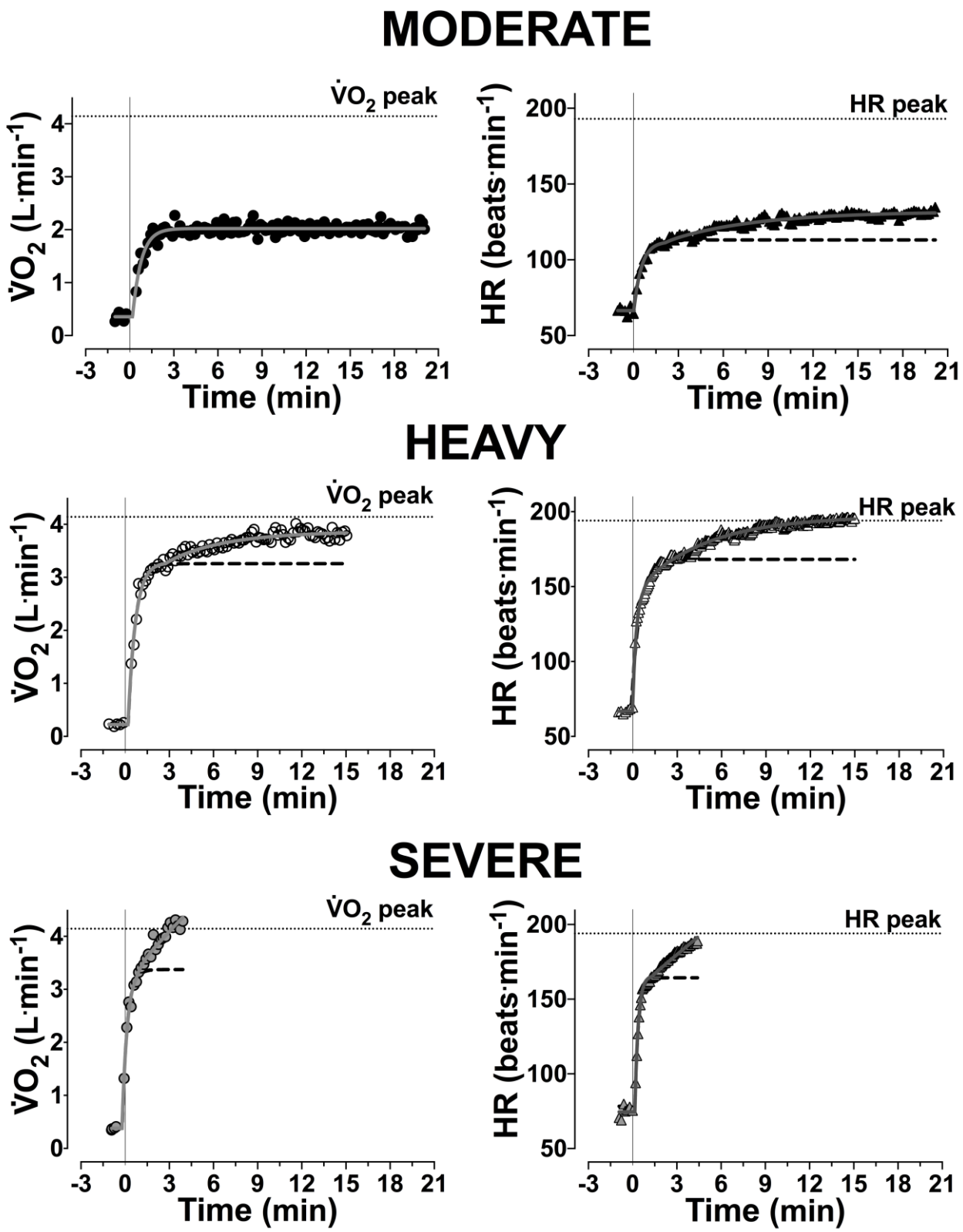

FIGURE 1. Pulmonary O_2 uptake ($\dot{V}O_2$) and heart rate (HR) kinetics for a representative subject during constant work rate (CWR) exercise at three investigated intensity domains MODERATE, HEAVY, SEVERE. Each data point indicates breath-by-breath or beat-to-beat values averaged every 10 s. The dashed curves indicate the asymptotes of the fundamental component.

Pulmonary $\dot{V}O_2$ and HR mean values obtained during MODERATE, HEAVY and SEVERE are plotted as a function of time in the upper panels of **Figure 2**. To obtain this figure (as well as **Figures 3**, see below), individual values were grouped for discrete work rate intervals, which were determined in order to have, in each interval, each subject represented by one data point. When the subject had more than one "original" data point in the interval, mean individual values were calculated, both for the x and the y variable, and were taken into consideration to obtain the figure. The general pattern confirms what was mentioned above for the typical example, and $\dot{V}O_2$ reached at exhaustion values which were substantially identical to the $\dot{V}O_2$peak values determined during the incremental exercise. HR slightly but significantly increased as the time of exercise progressed also during MODERATE; during HEAVY and SEVERE the increase as a function of time was more pronounced, and values close to HR peak were eventually reached when exhaustion ensued.

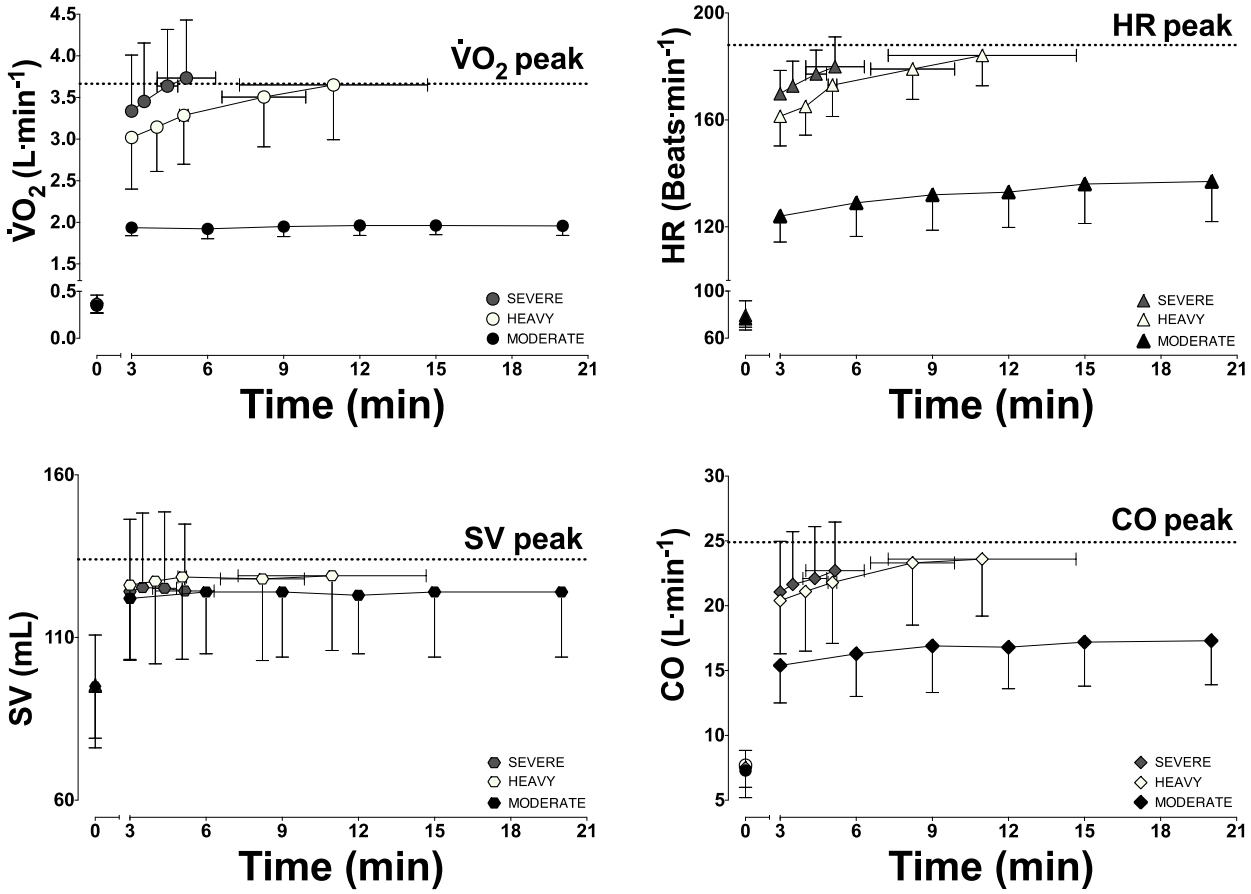

FIGURE 2. Group mean (±SD) pulmonary O_2 uptake ($\dot{V}O_2$), heart rate (HR), stroke volume (SV) and cardiac output (CO) grouped for discrete intervals at three investigated intensity domains corresponding to 50% of $\dot{V}O_2$peak (MODERATE), 45% of the difference between GET and $\dot{V}O_2$peak (Δ) (HEAVY) and 95% of Δ (SEVERE).

Some parameters deriving from the fitting of the $\dot{V}O_2$ and HR kinetics are presented in **Table 2**.

TABLE 2. Pulmonary $\dot{V}O_2$ and HR kinetics parameters determined during CWR exercises.

Intensity Domain	Work Rate, W	$\dot{V}O_{2BAS}$, L·min^{-1}	$\dot{V}O_{2f}$, L·min^{-1}	A_f, L·min^{-1}	TD_f, s	τ_f, s	TD_s, s	A_s', L·min^{-1}	A_s'/A_{tot}, %
MODERATE	123 ± 12	0.354 ± 0.07	1.936 ± 0.10	1.586 ± 0.08	12.5 ± 2.7*	36.7 ± 13.3*			
HEAVY	237 ± 48.7	0.344 ± 0.08	2.919 ± 0.64	2.575 ± 0.64	14.0 ± 5.6*	30.4 ± 11.9*	153.9 ± 53.4*	0.775 ± 0.33	23.3 ± 9.0*
SEVERE	268 ± 49.9	0.351 ± 0.07	3.101 ± 0.59	2.750 ± 0.55	13.3 ± 4.9*	27.7 ± 12.6*	103.4 ± 35.6*	0.636 ± 0.34	18.7 ± 8.8*
Intensity Domain	**Work Rate, W**	**HR_{BAS}, bpm**	**HR_f, bpm**	**A_f, bpm**	**TD_f, s**	**τ_f, s**	**TDs, s**	**A_s', bpm**	**A_s'/A_{tot}, %**
MODERATE	123 ± 12	77 ± 8	123 ± 10	46 ± 7	1.7 ± 5.1	24.4 ± 13.6	162.9 ± 89.6	16 ± 8	24.8 ± 11.0
HEAVY	237 ± 48.7	77 ± 10	152 ± 13	75 ± 18	3.9 ± 6.7	26.2 ± 13.7	102.8 ± 53.9	33 ± 10	31.6 ± 11.2
SEVERE	268 ± 49.9	80 ± 11	155 ± 12	74 ± 12	6.4 ± 4.6	21.0 ± 12.3	63.3 ± 16.7	27 ± 9	26.9 ± 8.0

Values are presented as mean ± SD.
*$P < 0.05$, significantly different from HR values.
HR_{BAS}, heart rate baseline; HR_f, heart rate baseline + fundamental component; $\dot{V}O_{2BAS}$, oxygen uptake baseline; $\dot{V}O_{2f}$, oxygen uptake baseline + amplitude of the fundamental component; A_f, amplitude of the fundamental component; TD_f, time delay fundamental; τ_f, time constant; TD_s, time delay slow component; A_s', actual amplitude of the slow component; A_s'/A_{tot}, total amplitude of the response.

For MODERATE, in all subjects Equation 1 represented the best fit of the data. For HEAVY and SEVERE, in all subjects Equation 2 or Equation 3, respectively, represented the best fit of the data. As expected, at all investigated work rates the fundamental component of the HR kinetics was faster than that of the $\dot{V}O_2$ kinetics (see both the TD_f and t_f values). During both HEAVY and SEVERE the onset of the slow component occurred earlier for HR than for $\dot{V}O_2$ (see TD_s values).

During both HEAVY and SEVERE the amplitude of the slow component, relative to the entire responses (A_s'/A_{tot}), was greater for the HR kinetics than for the $\dot{V}O_2$ kinetics (P=0.008 and P=0.011 respectively). This observation is confirmed by **Figure 3**, in which the percentage increases in $\dot{V}O_2$ and HR, with respect to the value obtained at the end of the fundamental component of the kinetics, arbitrarily set equal to 100%, are shown. When no slow component was detected, the 100% value was set at the value corresponding to that determined at four times the fundamental τ values. During MODERATE, only HR showed an increase. During both HEAVY and SEVERE the HR increase was more pronounced than the $\dot{V}O_2$ increase.

SV and CO mean values obtained during MODERATE, HEAVY and SEVERE are plotted as a function of time in **Figure 2**, lower panels. For SV, during both MODERATE and HEAVY the variable, after reaching a steady state at about 5-6 minutes, remained substantially constant until exhaustion. For CO the pattern was very similar to that described for HR in **Figure 2**: a slight but significant progressive increase from the 3rd to the 20th minute in MODERATE, whereas in both HEAVY and SEVERE the variable sharply increased until it reached the exhaustion values, which were not different from the peak values determined during the incremental exercise.

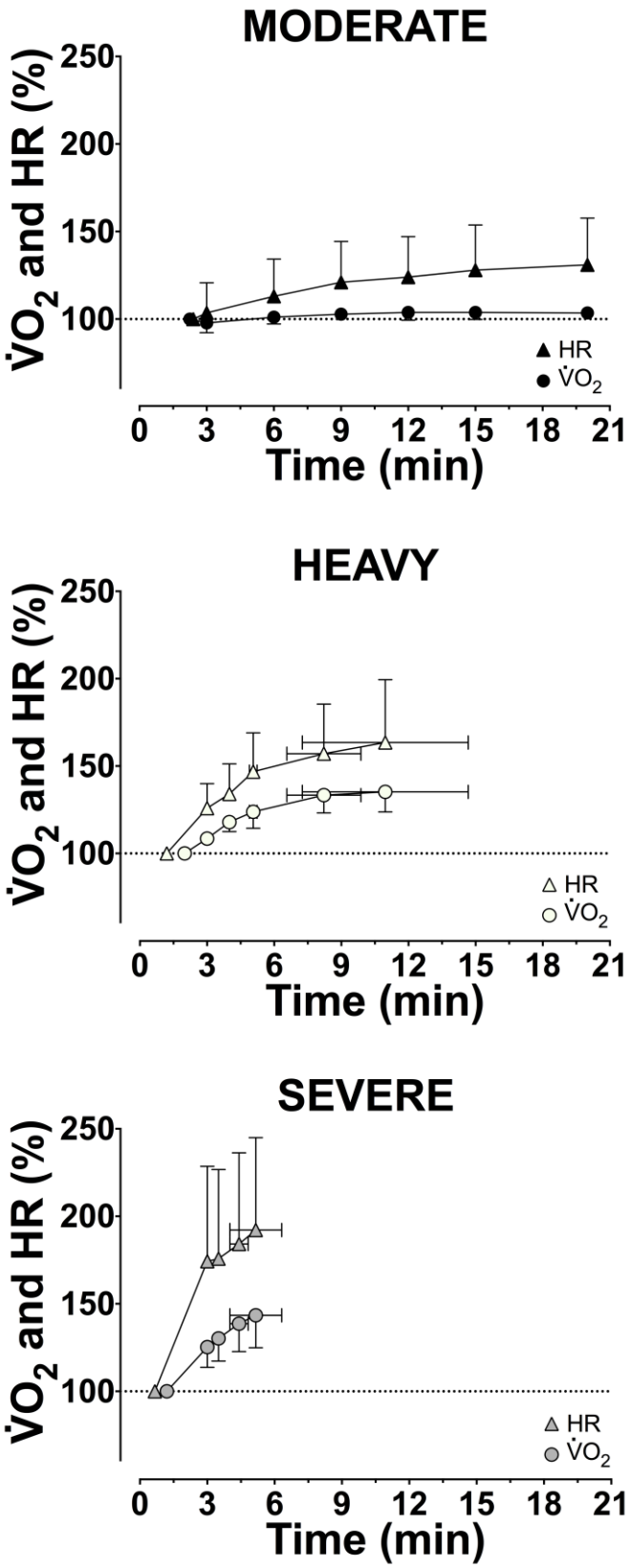

FIGURE 3. Percent change (%) from the values corresponding to the end of the fundamental component of the heart rate (HR) kinetics, and pulmonary O_2 uptake ($\dot{V}O_2$) during constant work rate (CWR) exercise at three investigated intensity domains MODERATE, HEAVY and SEVERE.

HR, work rate and $\dot{V}O_2$ values obtained in a typical subject during the $HR_{CLAMPED}$ exercise are shown in the left panels of **Figure 4**. A work rate corresponding to GET +10% was imposed for the first 3 minutes, and then the work rate was adjusted in order to maintain HR constant. In the right panels of **Figure 4** mean values of the variables are presented. The HR target value was reached on average at minute 6 (range from 3 to 9 minutes) and it remained substantially constant throughout the test, indicating that the task was successfully completed. As hypothesized, work rate had to decrease (by about 14%) in order to maintain the target HR. Interestingly, the reduced work rate was associated with a decreased $\dot{V}O_2$ (from 3.09 to 2.77 $l \cdot min^{-1}$). This observation indirectly confirms that the slow component of the HR kinetics was more pronounced than the slow component of the $\dot{V}O_2$ kinetics.

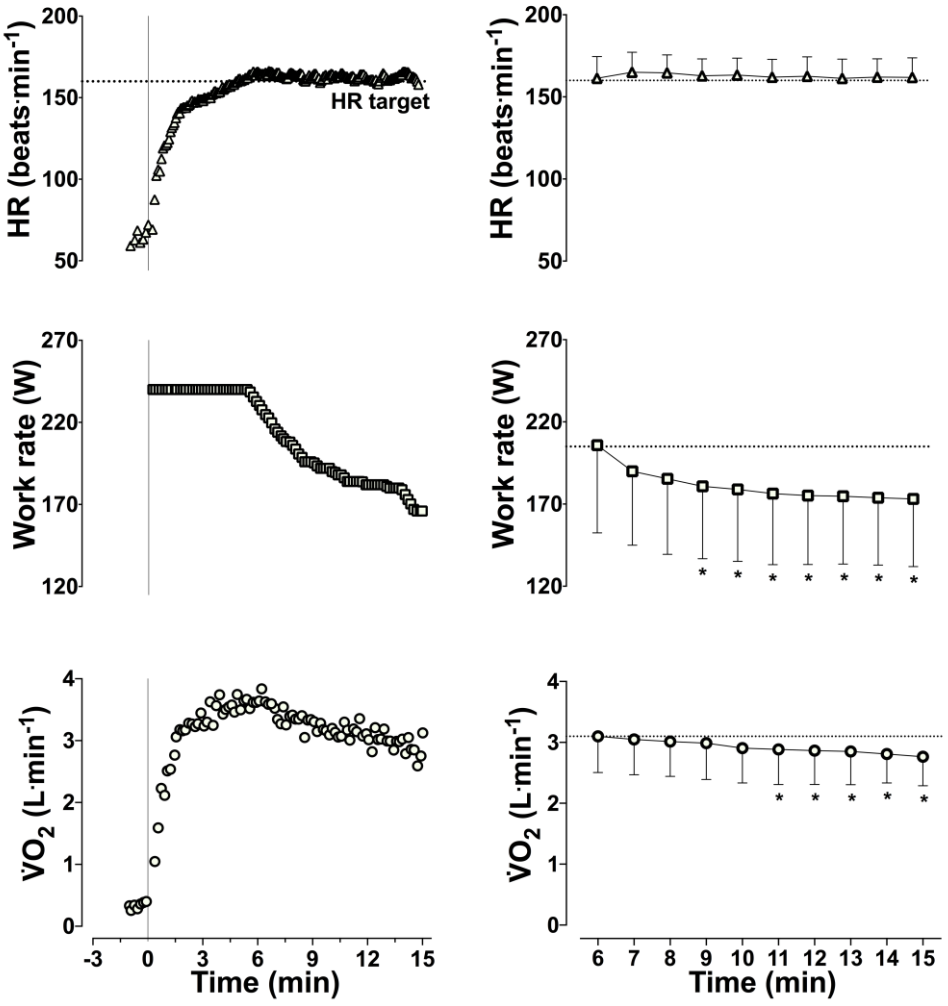

FIGURE 4. In the left panels, heart rate (HR), work rate (W) and pulmonary O_2 uptake ($\dot{V}O_2$), for a representative subject during heart rate controlled ($HR_{CLAMPED}$) exercise. In the right panels, mean (± SD) heart rate (HR), work rate (W) and pulmonary O_2 uptake ($\dot{V}O_2$) for the HR controlled exercise. The horizontal dashed lines indicate mean target values. *Statistically different than respective 6-min value ($P<0.05$).

DISCUSSION

The main results of the present study can be summarized as follows: (i) differently from what observed for the $\dot{V}O_2$ kinetics, a slow component of the HR kinetics occurred also during constant work rate (CWR) exercise in the moderate-intensity domain (<GET); (ii) during CWR exercise above GET the relative amplitude of the HR slow component was greater than the relative amplitude of the slow component of the $\dot{V}O_2$ kinetics; (iii) during CWR exercise slightly above GET, in order to keep HR constant both work rate and $\dot{V}O_2$ had to decrease, further confirming that the relative amplitude of the HR slow component is more pronounced than the relative amplitude of the $\dot{V}O_2$ slow component.

Overall, these data suggest the absence of a linear relationship between $\dot{V}O_2$ and HR during CWR exercises in different intensity domains. As a consequence, caution is needed in prescribing exercise training programs based on HR values. Whereas for $\dot{V}O_2$ the concept of a value corresponding to a specific CWR holds true only for moderate-intensity exercise (below GET), for HR the concept may not hold true also for moderate-intensity CWR exercise, in which HR may keep increasing as a function of time. This increase is more pronounced during heavy-intensity CWR. Thus, exercise prescriptions at specific HR values, when carried out for periods longer than a few minutes, could lead to premature fatigue and exercise termination.

In the present study the subject performed three repetitions of 20-min exercise of moderate intensity, and the values were ensemble-averaged into a single response profile. $\dot{V}O_2$ kinetics were mathematically represented by a typical mono-exponential increase (after excluding the "cardiodynamic phase") and no slow component was evident in any subject. In terms of the HR kinetics, on the other hand, during moderate-intensity exercise a slow component with an amplitude corresponding to about 25% of the total response was observed. Previous studies had indeed anectdotically reported an increase in HR after the first minutes of exercise for intensities higher than 30% of $\dot{V}O_2$max (Wassweman et al., 1967; Orizio et al., 1988). For work rates up to ~120 W, anecdotal reports of an increase of HR after the first minutes of exercise have been published (Wassweman et al., 1967; Orizio et al., 1988; Hebestreit et al., 1998). These studies, however, did not analyze the HR kinetics with respect to GET or CP. An exception is represented by a few subjects among those studied by Engelen et al. (1996), in whom a slow component of HR kinetics was observed during heavy-intensity exercise, but not during moderate-intensity exercise. This last observation is not in agreement with the results of the present study.

In our study, during heavy-intensity exercise subjects cycled up to voluntary exhaustion (which was reached in about 11 minutes) and a slow component was observed for both $\dot{V}O_2$ and HR. However, the relative amplitude of the HR slow component (estimated as a fraction of the overall response of

the variable, that is as A_s'/A_{tot} [see Methods]) was significantly greater than the relative amplitude of the $\dot{V}O_2$ slow component. This feature was further confirmed by the analysis presented in Figure 3, as well as by the results of the "$HR_{CLAMPED}$" trial, in which the subjects, during a prolonged exercise slightly above GET, in order to keep HR constant had to decrease the work rate, but to an extent that led to a decrease also in $\dot{V}O_2$. This observation represents a sort of a "mirror image" of the notion that the slow component of the HR kinetics is more pronounced than the slow component of the $\dot{V}O_2$ kinetics.

Previous studies investigated the dissociation among the patterns of responses for various physiological variables determined in conditions of constant work rate exercise, such as HR, $\dot{V}E$, $\dot{V}O_2$ and RPE (Martin et al., 1979; Ribeiro et al., 1986; Steed et al., 1994; Stoudemire et al., 1996; Herman et al., 2003; Lander et al., 2009; Cochrane et al., 2015). For example, Herman et al. (2003) reported that 15-min of exercise "clamped" at a constant HR, corresponding to 75% of $\dot{V}O_2$max, resulted in decreases in power output and in the amplitude of the $\dot{V}O_2$ slow component. A progressive decrease in power output was also described when $\dot{V}O_2$ was maintained constant at a work rate corresponding to the "anaerobic threshold" (Ribeiro et al., 1986). Similarly, Stoudemire et al. (1996) reported a decreased velocity during a 30-min treadmill run in which the subjects kept a constant RPE value. Interestingly, a fall in force output (electrically stimulated isometric tetanic contractions) in association with a constant $\dot{V}O_2$ was also observed in the isolated dog gastrocnemius in situ model (Zoladz et al., 2008).

The mechanism(s) responsible for the different behaviors of the slow components of HR and $\dot{V}O_2$ are somewhat difficult to hypothesize. The slow components of the two variables likely recognize different mechanistic determinants. The mechanisms responsible for the slow component of the $\dot{V}O_2$ kinetics have been discussed in detail in several recent reviews (Rossiter, 2011; Poole & Jones 2012; Grassi et al., 2015; Poole et al., 2016). Several factors have been identified which can lead to a decreased efficiency of muscle contractions (with "slow components" of $\dot{V}O_2$, blood lactate accumulation, PCr splitting [see Grassi et al., 2015]) and fatigue during exercise carried out above GET, and particularly above CP. As for HR, during dynamic exercise the HR adjustments are regulated by the autonomic nervous system on the basis of signals arising in a central area of the brain ("central command") and originating in the contracting skeletal muscles (exercise pressor reflex, baro- and chemo-reflexes), eventually modulated by afferent fibers (group III and IV) responsive to mechanical and chemical changes in the working muscles (Mitchell, 2013; Nobrega et al., 2014). These regulatory mechanisms are directly related to exercise intensity and could in theory be responsible for the slow component of the HR kinetics.

At the onset of CWR exercise the initial rise in HR has been attributed to parasympathetic withdrawal (Rowell & O'Leary, 1990; Fisher et al., 2015). Further increases have been attributed to an increased sympathetic nervous activity (Fisher et al., 2015), and follow a bi-exponential function with a fast and a much slower component (Linnarsson, 1974; Orizio et al., 1988; Engelen et al., 1996). In our study, during moderate-intensity exercise HR kinetics were well described by a bi-exponential process with a fast (τ≅25 s) and a slow (τ≅500 s) component. A fast (τ≅26 s) and a slow (τ≅288 s) component were also observed during heavy-intensity exercise.

The mechanisms responsible for the slow component of the HR kinetics in the present study should be different from those associated with the progressive increase of HR and the parallel decrease in SV (cardiac output being substantially constant) occurring after ~10 min of moderate-intensity exercise (Coyle & Gonzalez-Alonso 2000). This phenomenon is usually associated with hyperthermia and dehydration (Gonzalez-Alonso et al., 1997). In the present study, we did not measure core body temperature or indices of dehydration. Our data, however, revealed an increase in HR but not a decrease in SV during moderate-intensity exercise (see **Figure 2**), thereby negating the presence of the phenomenon mentioned above. Moreover, an increased body temperature is usually associated with an increased $\dot{V}E$ (Powers et al., 1982), which was not observed in the present study after the first few minutes of exercise (data not shown). Finally, in the present study the HR slow component occurred well before the 10^{th} minute of exercise, differently from the hyperthermia and dehydration phenomena mentioned above.

Whereas it is well accepted that the slow component of the $\dot{V}O_2$ kinetics is directly associated with reduced efficiency and fatigue (Grassi et al., 2015), at present it is not clear if the slow component of HR kinetics, appearing during moderate-intensity exercise, is also somehow related to fatigue, possibly of delayed onset. Textbook physiology says that for the same work rate a higher HR indicates a reduced exercise tolerance. The potential role on fatigue by the progressive increase in HR, observed in the present study, should be investigated in future studies. It could be hypothesized that the effects on fatigue could manifest only during really prolonged exercise. If present, the association between the HR slow component and fatigue could have a significant impact also on exercise tolerance, and not only on exercise prescription.

Exercise prescription is usually done with respect to indices such as the gas exchange (or lactate) threshold or the critical power. The data of the present study suggest that the "translation" of the work rates, or of the percentages of $\dot{V}O_2$ peak, associated with these variables into HR values is not straightforward. Prescription of an exercise intensity corresponding to a specific $\dot{V}O_2$ could translate, for exercises in the moderate- and heavy-intensity exercise domains, into a disproportionate increase in HR. As an example, **Figure 4** shows that, during exercise carried out just above GET, in order to

maintain HR constant work rate must decrease substantially. Further studies are needed in order to clarify this critical issue.

In the present study no supramaximal validation test could be performed to strengthen the confidence in the $\dot{V}O_2$peak measurements (as estimates of $\dot{V}O_2$max) obtained during the incremental test, as recently suggested by Poole & Jones (2017). Supramaximal tests were not comprehended in the ethical approval. However, although no "supramaximal validation" was performed, it should be noted that peak $\dot{V}O_2$ values were not different at the end of the incremental test compared to the values obtained at the end of the severe-intensity CWR, although in the presence of significantly different work rates. This observation represents strong evidence that the observed $\dot{V}O_2$peak values were indeed maximal.

In conclusion, the present study indicates that a “slow component” of the HR kinetics occurs at a lower work rate than the slow component of the $\dot{V}O_2$ kinetics and that, at the same absolute work rate, the relative amplitude of the slow component of the HR kinetics is greater than the relative amplitude of the slow component of the $\dot{V}O_2$ kinetics. The present findings may have profound implications on exercise prescription. The data suggest indeed that the “translation” of work rates, or of percentages of $\dot{V}O_2$peak associated with variables such as the gas exchange threshold or critical power, into HR values (attractive, in practical terms, considering the facility of measurement and recording of this variable) is not straightforward. Exercise prescriptions at specific HR values, when carried out for periods longer than a few minutes, could lead to premature fatigue and to exercise termination. Further studies are needed in order to clarify this critical issue and better understand the mechanistic bases of these phenomena.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors have no conflict of interest, and the results of the present study do not constitute endorsement by the American College of Sport Medicine. The results of this study are presented clearly, honestly, and without fabrication, or inappropriate data manipulation.

3.2 OBESE PATIENTS DECREASE WORK RATE IN ORDER TO KEEP A CONSTANT TARGET HEART RATE – *STUDY 2*

This article has been accepted for publication in "*Medicine and Science in Sport and Exercise*" ahead of print (2020) as "Obese patients decrease work rate in order to keep a constant target heart rate" by Lucrezia Zuccarelli, Alessandro Sartorio, Roberta De Micheli, Gabriella Tringali, Bruno Grassi.

ABSTRACT

Purpose: "Slow components" of heart rate (HR) kinetics, occurring also during moderate-intensity constant work rate (CWR) exercise, represent a problem for exercise prescription at fixed HR values. This problem, described in young healthy subjects, could be more pronounced in obese patients. **Methods:** Sixteen male obese patients (age: 22±7 years; body mass: 127±19 kg; body mass index: 41.6±3.9 $kg \cdot m^{-2}$) were tested before (PRE) and after (POST) 3-wk multidisciplinary body mass reduction program, entailing moderate-intensity exercise. They performed on a cycle ergometer an incremental exercise to voluntary exhaustion (to determine $\dot{V}O_{2peak}$ and gas exchange threshold [GET]) and CWR exercises: moderate-intensity (MODERATE) (80% of GET determined in PRE); heavy-intensity (HEAVY) (120% of GET determined in PRE); "$HR_{CLAMPED}$" exercise, in which work rate was continuously adjusted to maintain a constant HR corresponding to that at 120% of GET. Breath-by-breath $\dot{V}O_2$ and HR were determined. **Results:** $\dot{V}O_{2peak}$ and GET (expressed as a % of $\dot{V}O_{2peak}$) were not significantly different in PRE vs. POST. In POST, vs. PRE, the HR slow component disappeared (MODERATE) or was reduced (HEAVY). In PRE work rate had to decrease by ~20% over a 15-min task in order to keep HR constant; this decrease was significantly smaller (~5%) in POST. **Conclusion:** In obese patients a 3-wk multidisciplinary body mass reduction intervention: i) increased exercise tolerance by eliminating (during MODERATE) or by reducing (during HEAVY) the slow component of HR kinetics; ii) facilitated exercise prescription by allowing to translate a fixed submaximal HR value into a work rate slightly above GET.

INTRODUCTION

Exercise intolerance is both a cause and a consequence of obesity (Han et al., 1998; Ortega et al., 2018), within a vicious circle characterized by obesity → early fatigue → reduced exercise tolerance → reduced physical activity → obesity. Thus, exercise training, in association with nutritional and psychological interventions, aimed at reducing body mass and exercise intolerance, is considered a cornerstone in obesity treatment (Donnelly et al., 2009; Swift et al., 2018). The positive aspects of exercise training in obese patients may go well beyond those related to a reduced body mass. According to Gaesser & Blair (2019), for example, a higher level of cardiorespiratory fitness may attenuate or eliminate the mortality risk associated with an elevated body mass index.

Although health benefits can be presumably gained by any regular exercise and physical activity, a targeted exercise prescription is needed to elicit specific physiological responses and/or adaptations (Black et al., 2017). It has been recently shown that exercise prescription based on fixed percentage of maximum values (i.e., $\dot{V}O_2$, heart rate [HR], work rate) does not take into account the specific exercise-intensity domain metabolic responses (Iannetta et al., 2020). Exercise prescription, both in healthy and diseased populations, is indeed carried out with respect to variables such as the gas exchange threshold (GET) (Iannetta et al., 2020; Lansley et al., 2011) or critical power (CP) (Poole et al., 2016). By doing so, exercise prescription takes into account the non-linearity (higher slope) of the pulmonary O_2 uptake ($\dot{V}O_2$) *vs.* work rate relationship for constant work rate exercises above GET, and particularly above CP, as a consequence of the appearance of the $\dot{V}O_2$ "slow component" (Jones et al., 2011). GET and CP are however difficult to determine outside an exercise physiology laboratory, and therefore the identification of the training intensity is most often done by choosing a work rate corresponding to a fixed percentage of peak HR (Franklin et al., 2000; Powers et al., 2004). Also the HR *vs.* work rate relationship, however, shows a non-linear behavior (higher slope) during constant work rate exercises above GET and above CP. The issue is further complicated by the recent observation by our group (Zuccarelli et al., 2018) of a slow component of the HR kinetics also for work rates below GET. In that study, moreover, the relative amplitude of the HR slow component was more pronounced that the relative amplitude of the $\dot{V}O_2$ slow component (Zuccarelli et al., 2018). Some mechanisms potentially responsible for the HR slow component are mentioned below in the Discussion. In any case, as a consequence of the HR slow component, in order to keep HR constant at a value slightly above that corresponding to GET the work rate had to be decreased by ~14% during a 15-min exercise task (Zuccarelli et al., 2018). In other words, the appearance of HR slow component makes exercise prescription based on some percentage of HR peak an inaccurate approach.

The study by Zuccarelli et al. (2018) was carried out in young healthy physically active subjects. We hypothesize that the phenomena discussed above could be more pronounced in diseased populations,

such as obese patients. Obese patients are indeed characterized (*vs.* healthy subjects) by lower exercise tolerance, by higher $\dot{V}O_2$ and HR values for the same submaximal work rate, and by a more pronounced slow component of the $\dot{V}O_2$ kinetics (Salvadego et al., 2010). Thus, the mentioned differences in the metabolic responses to exercise in the obese subjects *vs.* healthy individuals, lead us to hypothesize that in obese patients a more pronounced slow component of HR kinetics as well. This would make exercise prescription based on a fixed submaximal HR even more questionable in these patients.

The aim of the present study was to test this hypothesis. More specifically, we hypothesized that in obese patients, as a consequence of a more pronounced slow component of the HR kinetics, in order to keep HR constant during a work rate slightly above GET the decrease in work rate would be more pronounced than that observed in young healthy physically active subjects (Zuccarelli et al., 2018). We also hypothesize that in the obese patients a standard 3-wk multidisciplinary body mass reduction program, consisting of moderate-intensity exercise, caloric restriction and psychological counseling would attenuate the work rate decrease aimed at keeping a constant HR, thereby improving exercise tolerance and facilitating exercise prescription, which is often done at work rates slightly above GET.

MATERIALS AND METHODS

Subjects

Sixteen male obese subjects, 7 young adults and 9 adolescents (age: 22 ± 7 years [mean ± SD]; height: 174 ± 7 cm; body mass: 127 ± 19 kg; body mass index: 41.6 ± 3.9 $kg \cdot m^{-2}$) were hospitalized (Division of Metabolic Diseases for young adults and Division of Auxology for adolescents, Istituto Auxologico Italiano, IRCCS, Piancavallo, Italy) and tested before and after a 3-wk multidisciplinary body mass reduction program. The program included: constant and monitored physical activity (5 days *per* week training, including 1 h dynamic aerobic standing and floor exercise with arms and legs, at moderate intensity and under the guide of a therapist, and either 20-30 min cycle ergometer exercise at 60 W or 3–4 km out-door walking on flat terrain, according to individual capabilities and clinical status); psychological counseling; nutritional education and moderate energy restriction. The adult patients and both parents of the adolescents provided signed consent statements, after being fully advised about the purposes and testing procedures of the investigation, which were approved by the ethics committee of the Italian Institute for Auxology, Milan, Italy (reference code: 01C827; acronym: COLEESEROB-RC18). All procedures were in accordance with the recommendations set forth in the Helsinki Declaration (2001).

Inclusion criteria were: *1*) body mass index (BMI) standard deviation score (SDS) > 2 for age and sex (adolescents), using the Italian growth charts (Cacciari et al., 2006) and BMI > 30 for young

adults; *2*) no involvement in structured physical activity programs (regular activity >120 $min \cdot wk^{-1}$) during the 8 months preceding the study; *3*) absence of overt uncompensated diabetes; *4*) absence of signs or symptoms referable to any major cardiovascular, respiratory, or orthopedic disease contraindicating or significantly interfering with the tests; *5*) absence of any kind of disease related to gastrointestinal tract (i.e., obstruction of the digestive tract, motility disorders, previously surgical procedures, swallowing disorders).

BMI was calculated as body mass (BM) divided by $height^2$, expressed in ($kg \cdot m^{-2}$). Body composition was determined by bioelectrical impedance (Human-IM Scan, DS-Medigroup, Milan, Italy). Whole body resistance to an applied current (50 kHz, 0.8 mA) was measured with a tetrapolar device, with electrodes placed on the right wrist and ankle of the supine subjects lying comfortably in bed with limbs abducted from the body. Fat free mass (FFM) was calculated with equations derived with a two-compartment model (Gray et al., 1989). Fat mass (FM) was calculated as the difference between total BM and FFM; both the variables were expressed as kg and as a percentage of body mass (see **Table 1**). The same investigators performed all examinations before and after the 3-wk intervention period (see below).

Exercise protocols

Before (PRE) and after (POST) the 3-wk multidisciplinary body mass reduction program exercise tests were conducted in three separate occasions over a four-day period. Exercise tests were carried out in a well-ventilated laboratory under continuous medical supervision.

During the first visit the subjects completed an incremental exercise (INCR) up to voluntary exhaustion on an electronically braked cycle ergometer (Corival cpet, Lode, The Netherlands), to determine $\dot{V}O_2peak$ and GET. The test started with 20 W for two minutes and then 20 W increases of work rate were imposed every minute until voluntary exhaustion. Pedaling frequency was digitally displayed to the subjects, who were asked to keep a constant cadence throughout the tests at their preferred value (between 60 and 80 rpm). Voluntary exhaustion was defined as the incapacity to maintain the imposed load and pedaling frequency despite vigorous encouragement by the researchers. After the first visit the subjects performed in two different days one repetition of 10-min constant work rate (CWR) submaximal exercise corresponding to 80% of GET determined in PRE (moderate-intensity, MODERATE), followed by one repetition of 15-min (or until exhaustion) at 120% of GET determined in PRE (heavy-intensity, HEAVY), and one repetition of 15-min "HR-controlled" exercise ($HR_{CLAMPED}$). The HEAVY CWR exercise was performed when subjects reached again baseline values of the investigated variables (*i.e.* after ~30 minutes of recovery). Participants performed the CWR exercises (MODERATE + HEAVY) and the $HR_{CLAMPED}$ exercise in a randomized order.

During $HR_{CLAMPED}$ a target HR corresponding to 120% of GET determined during the incremental exercise in PRE was identified. The work rate was kept constant for the first 2 minutes or until HR reached its target value, and then it was adjusted by the operator every 5 s to maintain a constant HR at the target value (for more details see [Zuccarelli et al., 2018]). Before the trial the subjects were familiarized with the protocol. CWR exercises were carried out at the same absolute work rate in PRE and POST; the same was true for the initial work rate during $HR_{CLAMPED}$.

Measurements

Pulmonary ventilation ($\dot{V}E$), $\dot{V}O_2$ and CO_2 output ($\dot{V}CO_2$) were determined breath-by-breath by a metabolic cart (Ergostick, Geratherm Respiratory, Bad Kissingen, Germany). Expiratory flow measurements were performed by a turbine flow meter, calibrated before each experiment by a 3 L syringe at different flow rates. $\dot{V}O_2$ and $\dot{V}CO_2$ were determined by continuously monitoring PO_2 and PCO_2 at the mouth throughout the respiratory cycle and from established mass balance equations. Calibration of O_2 and CO_2 analyzers was performed before each experiment by utilizing gas mixtures of known composition. Peak values of the main variables were taken as the highest 20-s mean values attained prior to the subject's voluntary exhaustion. Gas exchange ratio (R) was calculated as $\dot{V}CO_2/\dot{V}O_2$. GET was determined by standard methods (Beaver et al., 1986). In order to identify the work rate corresponding to $\dot{V}O_2$ at GET, the effect of the delayed $\dot{V}O_2$ adjustment (Boone & Bourgois, 2012; Iannetta et al., 2019) to the increased work rate during the incremental test was corrected by shifting the linear $\dot{V}O_2$ *vs.* time (and work rate) relationship to the left, by an amount corresponding to the mean response time of the $\dot{V}O_2$ kinetics (Whipp et al., 1981) previously determined by our group in an obese population (30 s) (Salvadego et al., 2010).

HR was determined continuously by a chest band (Polar Electro, Oulu, Finland); mean values were calculated every 5 s. Considering that only one repetition of each CWR exercise was carried out, a formal $\dot{V}O_2$ and HR kinetics analysis was not performed (Lamarra et al., 2003). The presence or absence of a steady state in $\dot{V}O_2$ and HR after the first minutes of CWR exercise was evaluated by fitting linear regressions on the data obtained from the third to the last minute of exercise (Zuccarelli et al., 2018).

Core body temperature was continuously monitored using ingestible telemetric temperature capsules (e-Celsius, BodyCap, Caen, France) during each CWR exercise in PRE. The validity and reliability of this device have been recently confirmed against a temperature-controlled water bath (Bongers et al., 2018). Briefly, the ingestible core body temperature sensor (17.7 mm length, 8.9 mm diameter and 1.7 g) wirelessly transmits signals by a radio-frequency of 433 MHz - 434 MHz trough the body to the data recorder (e-Viewer, BodyCap, Caen, France), worn on the outside of the body. Each capsule was activated and then swelled by the patients 3 hours before the CWR exercises. Sampling

rate was set at 5 s and mean capsule temperature values were calculated during the last 30 s of every minute of CWR exercise.

Ratings of perceived exertion (RPE) were obtained every minute during exercise using the Borg's 6-20 scale (Borg, 1982).

Statistical analysis

Results are expressed as mean ± SD values. Statistical significance of differences in PRE and POST for the main respiratory, cardiovascular, and metabolic variables during INCR, MODERATE and HEAVY were checked using a two-tailed Student's t-test for paired data. Linear regression line and correlation analysis were carried out by the last-squared residual method. A two-way analysis of variance (ANOVA) with repeated measures (intervention X time) was used to assess changes in $\dot{V}O_2$, HR and work rate during CWR and $HR_{CLAMPED}$ exercises. When significant differences were found, a Bonferroni post-hoc test was used to determine the exact location of the difference. The level of significance was set at $P<0.05$. Statistical analyses were carried out by a commercially available software package (Prism 6.0; GraphPad). Power analysis was conducted *a priori* taking the work rate decrement seen during $HR_{CLAMPED}$ exercise in our previous paper (Zuccarelli et al., 2018) as the main variable. In order to identify significant differences, with an α error of 0.05 and a statistical power (1-β) of 0.90, an n value of 11 subjects resulted to be necessary (G*Power 3.1).

RESULTS

All patients carried out the entire protocol except one, who did not complete the INCR exercise in POST for medical reasons (this subject was not taken into account for analyses related to the INCR exercise). The main anthropometric characteristics of the patients are reported in **Table 1**.

Table 1. Anthropometric characteristics and age of participants before (PRE) and after (POST) a 3-wk multidisciplinary body mass reduction program.

	PRE	POST	P value
Age (years)	22±7	22±7	---
Height (m)	1.74±0.09	1.74±0.09	---
BM (kg)	127±19.5	121 ±19.3*	<0.0001
BMI ($kg \cdot m^{-2}$)	41.6±3.9	39.8±3.6*	<0.0001
FFM (% BM)	58.6±8.27	61.8±7.78*	0.0006
FFM (kg)	74.7±20.32	75.1±19.72	0.67
FM (% BM)	43.6±3.88	40.7±3.96*	0.003
FM (kg)	55.6±11.85	49.5±10.74*	<0.0001

Values are mean ± SD. BM, body mass; BMI, body mass index; FFM, fat free mass; FM, fat mas.
*P<0.05 different from PRE.

BM (by ~6 kg, corresponding to ~5% of the initial body mass), BMI and FM were significantly lower in POST *vs.* PRE.

Peak values of the main respiratory, cardiovascular, and metabolic variables determined during INCR are shown in **Table 2**. Patients attained peak HR values corresponding to ~89% and ~86% of the age-predicted maximum (calculated as 208 - 0.7 X age [Tanaka & Monahan, 2001]), respectively, in PRE and POST. Taking into account also R peak and RPE peak values, it can be assumed that exhaustion was indeed reached, although no validation test for determination of maximal $\dot{V}O_2$ (Poole & Jones 2017) was carried out in the recovery phase. $\dot{V}O_2$ peak values were typical for obese subjects (Salvadego et al., 2017; Rasica et al., 2018) and they were not significantly different (P=0.44) in PRE (19.4 ± 3.0 $ml \cdot kg^{-1} \cdot min^{-1}$) *vs.* POST (20.1 ± 4.3 $ml \cdot kg^{-1} \cdot min^{-1}$). In PRE GET occurred at 59% of $\dot{V}O_2$ peak, corresponding to 1.395 $L \cdot min^{-1}$, not significantly different (P=0.14) from the value in POST (61% of $\dot{V}O_2$ peak, corresponding to 1.487 $L \cdot min^{-1}$). Respiratory compensation point (RCP) was higher in POST *vs.* PRE (1.942 ± 0.262 and 1.805 ± 0.300 $L \cdot min^{-1}$, respectively; P=0.048).

Main respiratory, cardiovascular and metabolic end-exercise or steady-state values, determined in PRE and POST during MODERATE and HEAVY CWR exercises are also shown in **Table 2**.

Table 2. Main respiratory, cardiovascular, and metabolic end-exercise or steady state values, determined during incremental exercise (INCR), constant work rate exercises (MODERATE and HEAVY) and $HR_{CLAMPED}$ exercise, before (PRE) and after (POST) a 3-wk multidisciplinary body mass reduction program.

	INCR			**MODERATE**			**HEAVY**			**$HR_{CLAMPED}$**		
	PRE	POST	P value	PRE	POST	P value	PRE	POST	P value	PRE	POST	P value
Work rate, W	196 ± 28	205 ± 28*	0.03	58 ±13	58 ±13	---	118 ± 18	118 ± 18	---	95 ± 23	112 ± 21*	0.0002
$\dot{V}O_2$, $l \cdot min^{-1}$	2.400 ± 0.327	2.456 ± 0.380	0.52	1.146±0.257	1.077±0.264	0.14	1.854 ± 0.295	1.846 ± 0.310	0.88	1.566 ± 0.202*	1.679 ± 0.307*	0.04
$\dot{V}O_2$, $ml \cdot kg^{-1} \cdot min^{-1}$	19.4 ± 3.0	20.1 ± 4.3	0.43	9.2 ± 2.1	8.7 ± 1.9	0.17	15.0 ± 3.0	15.1 ± 3.1	0.84	12.6 ± 3.9*	14.0 ± 3.5*	0.04
$\dot{V}CO_2$, $l \cdot min^{-1}$	3.018 ± 0.397	2.990 ± 0.417	0.93	1.028 ± 0.228	0.940 ± 0.295	0.15	1.866 ± 0.317	1.837 ± 0.333	0.66	1.573 ± 0.217	1.641 ± 0.314	0.35
R	1.26 ± 0.06	1.25 ± 0.10	0.87	0.90 ± 0.04	0.88 ± 0.17	0.63	1.01 ± 0.07	1.00 ± 0.05	0.27	1.00 ± 0.09	0.99 ± 0.05	0.71
$\dot{V}E$, $l \cdot min^{-1}$	101.2 ± 19.4	102.8 ± 20.1	0.57	31.2 ± 7.0	29.5 ± 8.5	0.32	63.7 ± 15.3	60.6 ±14.1	0.23	47.6 ± 14.6	54.7 ± 12.1	0.06
fR, $breaths \cdot min^{-1}$	42 ± 6	44 ± 8	0.30	24 ± 4	24 ± 8	0.81	36 ± 8	34 ± 8	0.10	29 ± 9	34 ± 7*	0.02
HR, $beats \cdot min^{-1}$	176 ± 11	171 ± 14	0.06	118 ± 9	111 ± 10*	0.0001	157 ± 15	147 ± 17*	0.0001	148 ± 17	146 ± 15	0.17
RPE, 6-20	20 ± 1	20 ± 1	0.87	8 ± 3	8 ± 3	0.36	18 ± 3	16 ± 4*	0.048	13 ± 3	13 ± 4	0.78
Time exercise, min	10 .7± 1.4	11.3± 1.4*	0.03	10 .0± 0	10.0 ± 0	---	12.4 ± 4.3	13.5 ± 2.5*	0.0043	14.8 ± 0.8	14.9 ± 0.5	0.33

Mean values ± SD. $\dot{V}O_2$, pulmonary oxygen uptake; $\dot{V}CO_2$, CO_2 output; R, gas exchange ratio; $\dot{V}E$, pulmonary ventilation; fR, breathing frequency; HR, heart rate; RPE, rate of perceived exertion. *$P<0.05$ different from PRE

Work rates for MODERATE and HEAVY were set to be identical in the two conditions, and they were 29 ± 5% and 62 ± 9% of peak work rate, respectively. For MODERATE all patients completed the imposed 10-min of exercise. No significant differences were found in $\dot{V}O_2$ end-exercise values in PRE *vs.* POST, whereas HR values determined during the last 20-s were significantly lower in POST *vs.* PRE (-6 %; P=0.005). As for HEAVY, seven patients in PRE and eleven patients in POST completed the imposed 15-min exercise. Time to exhaustion was significantly higher in POST *vs.* PRE (+8 %; P=0.004). In **Figure 1** HR (upper panel) and pulmonary $\dot{V}O_2$ (lower panel) mean values obtained during MODERATE and HEAVY in PRE and POST are plotted as a function of the time of exercise. To obtain this figure, individual values were grouped for discrete work rate intervals, which were determined in order to have, in each interval, each subject represented by one data point. When the subject had more than one "original" data point in the interval, mean individual values were calculated, both for the x and the y variables. Data were fitted by linear regression from the 3rd minute to the end of exercise. During MODERATE, the slopes of the linear regressions of HR *vs.* time were significantly different from zero in PRE (0.408 ± 0.105 b·min^{-2}; P=0.008) but not in POST (0.166 ± 0.168 b·min^{-2}; P=0.36). Thus, HR was not in steady-state during the 10-min MODERATE exercise in PRE, suggesting the presence of a slow component (Zuccarelli et al., 2018), whereas a steady-state was present in POST. HR values were lower in POST *vs.* PRE (P=0.008; F=9.058) starting from the 3rd minute of exercise. As for $\dot{V}O_2$, the slopes of the linear regressions were not significantly different from zero in both conditions (PRE and POST). In other words, in both conditions $\dot{V}O_2$ values were in steady-state. At all time points $\dot{V}O_2$ values were not different in POST *vs.* PRE. In the lower panel of **Figure 1**, horizontal lines indicating $\dot{V}O_2$peak in PRE (no differences before *vs.* after training were observed for this variable, as well as for GET [see above]), RCP in PRE and POST, and GET in PRE are shown. Steady-state $\dot{V}O_2$ values during HEAVY corresponded to RCP in PRE, and were slightly below RCP in POST. In both cases, however, $\dot{V}O_2$ values appeared in steady-state between the 10th minute and the end of exercise. During HEAVY, the slopes of the $\dot{V}O_2$ *vs.* time regression lines were significantly different from zero both in PRE and in POST (P=0.04 and P=0.0008, respectively), and they were not significantly different in POST (0.012 ± 0.001 L·min^{-2}) *vs.* PRE (0.015 ± 0.004 L·min^{-2}). The slopes of the HR *vs.* time linear regressions were significantly different from zero both in PRE and in POST (P=0.03 and P=0.004, respectively), and they were lower (P=0.001) in POST (1.103 ± 0.184 b·min^{-2}) vs. PRE (2.348 ± 0.567 b·min^{-2}). HR values were lower in POST *vs.* PRE (P=0.0012; F=15.73) starting from the 5th minute of exercise. The rate of HR and $\dot{V}O_2$ increases, calculated as the percentage increases with respect to the value obtained at the 3rd minute of exercise, arbitrarily set at 100%, were greater for HR than for $\dot{V}O_2$, both in PRE (119% and 109% for HR and $\dot{V}O_2$, respectively; P=0.001) and in POST (111% and 106% for HR and $\dot{V}O_2$, respectively; P=0.047). The

% increases in $\dot{V}O_2$ were significantly correlated with the % increases in HR ($r=0.57$; $r^2=0.32$; $P=0.0019$).

Core body temperature at the 3rd and at the end of the exercise was 37.5 ± 0.3 °C and 37.6 ± 0.3 °C ($P<0.0001$), respectively, during MODERATE, and 37.5 ± 0.2 °C and 37.8 ± 0.2 °C ($P<0.0001$) during HEAVY. A positive and significant correlation ($r^2=0.41$; $P=0.0001$) was found between the individual increases in HR and the corresponding increases in core body temperature, both calculated as end-exercise values minus values calculated at the 3rd minute of exercise. As mentioned above, core body temperature measurements were obtained only in PRE.

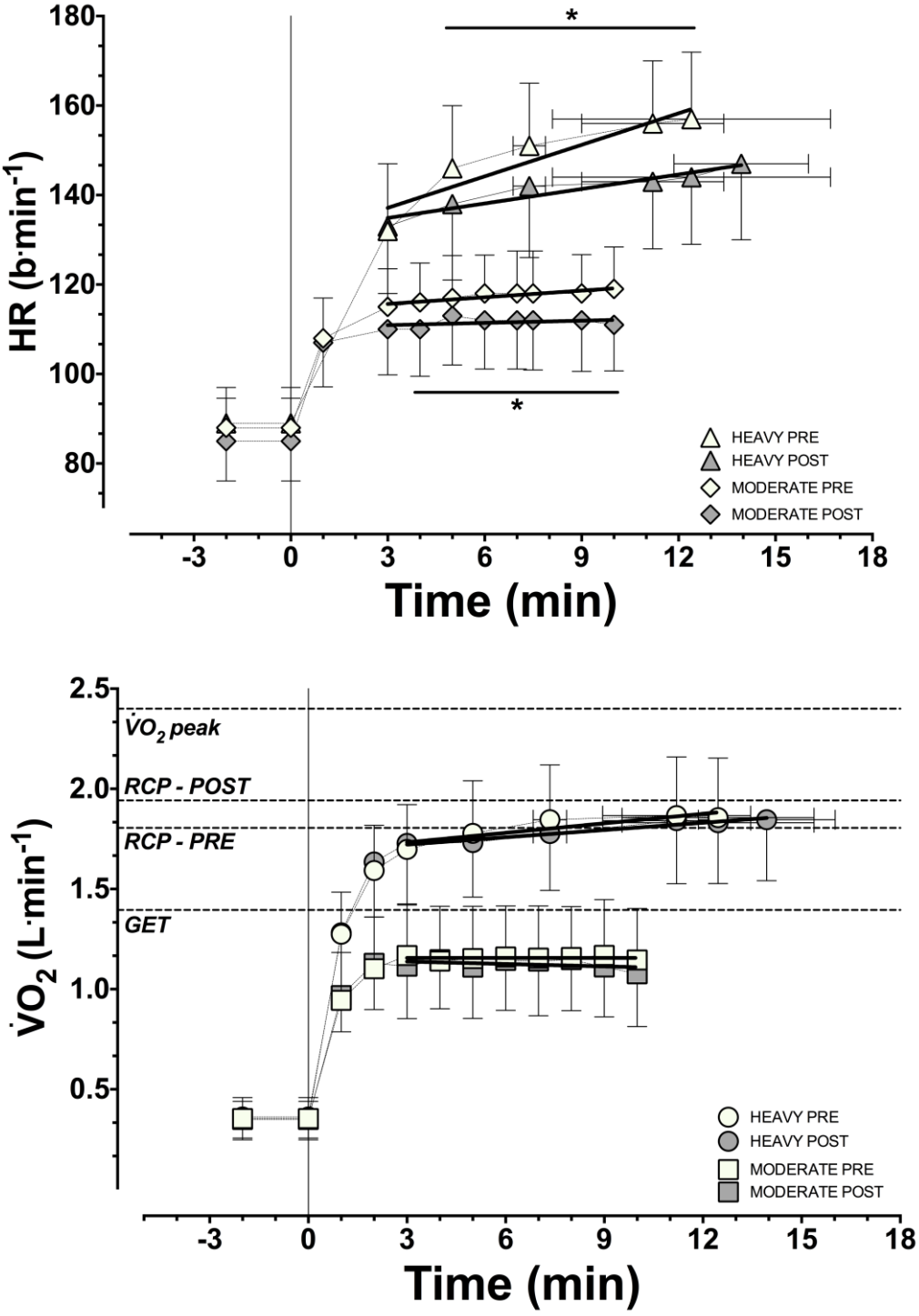

Figure 1. Mean values (± SD) of heart rate (HR) (upper panel) and oxygen consumption ($\dot{V}O_2$) (lower panel), grouped for discrete time intervals, during constant work rate (CWR) exercise at two investigated intensity domains, MODERATE and HEAVY, before and after a 3-wk multidisciplinary body mass reduction intervention. Vertical lines indicate that exercise started at *time 0*. Horizontal lines indicate $\dot{V}O_2$peak in PRE, RCP in PRE and POST, and GET in PRE. The fitted linear regression lines are also shown. *Significantly different ($P<0.05$) from PRE. See text for further details.

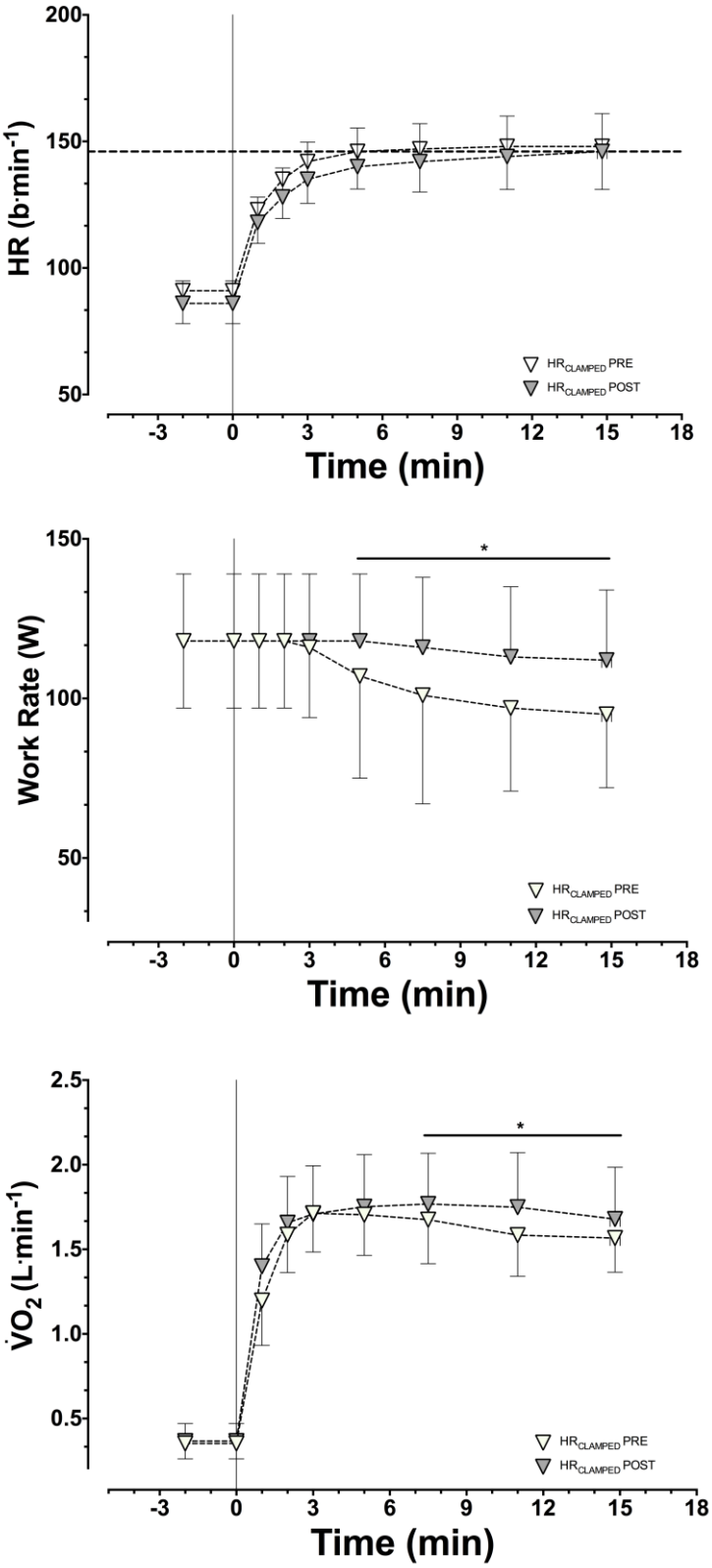

Figure 2. Shows mean (± SD) HR, work rate, and $\dot{V}O_2$ values obtained during the $HR_{CLAMPED}$ exercise in PRE and POST. In the two conditions the work rate imposed at the start of the exercise was the same, corresponding to HEAVY (GET +20%) determined in PRE; this work rate was imposed for the first 2 minutes, and then the work rate was adjusted to maintain HR constant (see METHODS).

Main respiratory, cardiovascular and metabolic end-exercise values, determined in PRE and POST during $HR_{CLAMPED}$ exercise are shown in **Table 2.** All patients but one (who terminated the exercise for voluntary exhaustion at the 12th minute in PRE and at the 13th minute in POST) completed the 15-min $HR_{CLAMPED}$ exercise.

The HR target value was reached on average after 2 minutes in PRE and after 5 minutes in POST, and afterwards it remained substantially constant throughout the test (upper panel of the Figure), indicating that the HR "clamp" was successful. In PRE, in order to maintain the target HR, work rate had to significantly decrease (middle panel of the Figure) from the 5th minute until the end of exercise; at the end of the exercise (15 minutes) the percentage decrease in work rate, *vs.* the value obtained at the 3rd minute, was approximately 19%. On the other hand, in POST the decrease in work rate was significantly (P=0.0002) less pronounced (~5%), although it was still statistically significant (P=0.005). From the 5th minute until the end of exercise work rate was higher in POST *vs.* PRE (P=0.0023; F=13.36). As shown in the lower panel of the Figure, the reduced work rate in PRE and POST during $HR_{CLAMPED}$ was associated with a decreased $\dot{V}O_2$ (by ~10% and ~5%, respectively).

DISCUSSION

The main findings of the present study can be summarized as follows: (i) Differently from $\dot{V}O_2$, during-moderate-intensity (<GET) constant work rate exercise HR increased significantly from the 3rd to the 10th minute of exercise, suggesting the presence of a slow component for this variable also in this exercise domain. (ii) During heavy-intensity exercise (>GET), from the 3rd to the 10th minute of exercise the percentage increase in HR was greater than the percentage increase in $\dot{V}O_2$, thus, the HR slow component was more pronounced than the $\dot{V}O_2$ slow component. (iii) In order to keep a constant target HR value, slightly above that corresponding to GET, both work rate (by ~20% over a 15-min task) and $\dot{V}O_2$ had to decrease. Thus, in untrained obese patients exercise prescription at a fixed submaximal HR translates into a work rate which must be progressively decreased during the training session; this obviously makes exercise prescription quite problematic. The work rate decrease was similar to that (~15%) observed in young healthy physically active subjects (Zuccarelli et al., 2018). (iv) A 3-wk multidisciplinary body mass reduction program, including moderate-intensity exercise, eliminated the HR slow component during exercise <GET and reduced the amplitude of the HR slow component during exercise >GET, thereby increasing exercise tolerance (lower HR for the same work rate). (v) After the body mass reduction program, the decrease in work rate in order to keep HR constant at a value slightly above GET was substantially eliminated, confirming the increased exercise tolerance (Clausen, 1977) and facilitating exercise prescription, by allowing to translate a fixed submaximal HR into a work rate slightly above GET.

To the best of our knowledge this is the first study analyzing the relationship between HR and $\dot{V}O_2$ responses during CWR exercise carried out in different intensity domains in obese patients. In the present study no formal analyses of the $\dot{V}O_2$ and HR kinetics and their different components (Jones et al., 2010) were carried out, since only one repetition of each exercise was performed. The presence *vs.* absence of a steady-state (in other words, the absence *vs.* the presence of a slow component) of the investigated variables was evaluated by a simplified approach, as proposed in previous studies by our group (see e.g. [Alemayehu et al., 2018]). We fitted a linear function to the data from the 3rd to the 10th minute of exercise: a significant positive slope suggests the absence of a steady-state, and therefore the presence of a slow component, whereas a slope not significantly different from zero suggests a steady-state and negates the presence of a slow component.

Whereas the slow component of the $\dot{V}O_2$ kinetics reflects a loss of efficiency of oxidative metabolism and is associated with fatigue (Grassi et al., 2015), also in obese patients (Salvadego et al., 2010), the functional significance of the HR slow component, particularly during moderate-intensity exercise, is less clear (Zuccarelli et al., 2018). The results of the present study are, in this respect, of interest: the body mass reduction program, which included exercise training, decreased (heavy-intensity exercise) or abolished (moderate-intensity exercise) the amplitude of the HR slow component, and increased exercise tolerance. This may indirectly suggest that also the HR slow component is associated with fatigue and a decreased exercise tolerance, as it occurs for the $\dot{V}O_2$ slow component. Although HR responses during CWR exercises in relation to the physiological thresholds have been poorly studied so far, a slow component is known to occur also for the HR kinetics (Zuccarelli et al., 2018; Orizio et al., 1988; Engelen et al., 1996). Increases in core body temperature could have had a role in determining the HR increases during constant work rate exercise. In previous studies (Gonzalez-Alonso et al., 1997; Coyle & Gonzalez-Alonso 2001), 10 minutes of exercises at ~60% of $\dot{V}O_2$peak were associated with ~0.3 °C increases in core body temperature. In the present study we have similar data: the increases in core body temperature from the 3rd to the end of exercise in PRE were indeed ~0.1 °C during 10 minutes of moderate- and ~0.2 °C during 15 minutes of heavy-intensity exercise. Core temperature increases were significantly correlated with the HR increases. Although a correlation does not imply a cause-effect relationship, it is legitimate to hypothesize that the slow component of the HR kinetics could be attributable, at least in part, to the increases in core temperature. Unfortunately, the measurements of core temperature could not be repeated in POST for logistic reasons, and thus we miss information about the possible effects on this variable by the body mass reduction program.

A causative role in determining the HR slow components could be attributed to blood catecholamine levels. Increments in catecholamine concentration were correlated with increments in HR (slow

component) during short dynamic exercise at ~45% of $\dot{V}O_{2peak}$ (Orizio et al., 1988). On the contrary, no increase in norepinephrine occurred during very low intensity exercise (~23% of $\dot{V}O_{2peak}$), in which no HR slow component was identified (Orizio et al., 1988). Obese patients, however, show lower plasma catecholamine concentrations compared with normal weight individuals (Zouhal et al., 2010); this occurs at rest, during dynamic exercise and after training interventions (Vettor et al. 1997; Del Rio, 2000; Zouhal et al., 2010; Salvadori et al., 2015). A reduced epinephrine secretion has been described in obese patients (Del Rio, 2000), possibly due to higher plasma levels of leptin, insulin and cortisol, and higher catecholamine elimination rate (Zouhal et al., 2010).

The different behavior of $\dot{V}O_2$ and HR during submaximal exercises was further confirmed during the $HR_{CLAMPED}$ exercise. In order to keep HR constant at the target value, the obese patients had to decrease the work rate to an extent at which $\dot{V}O_2$ not only did not present a slow component, but actually decreased, confirming what previously observed by Zuccarelli et al. (2018) in healthy physically active young subjects. This indirectly confirms that the slow component of the HR kinetics was more pronounced that the slow component of the $\dot{V}O_2$ kinetics.

Not confirming our hypothesis, the work rate decrease (-20%) during $HR_{CLAMPED}$ exercise observed in the present study was only slightly greater than that described by Zuccarelli et al. (2018) in healthy physically active young subjects (-15%). Thus, the uncertainty in exercise prescription and the consequences on exercise tolerance deriving from the HR slow components are not more severe in obese patients compared to healthy controls. Further studies should be conducted on different patient populations. An interesting group would be represented by patients treated with β-blockers.

In the present study the evidence of increased exercise tolerance after the body mass reduction intervention, discussed above, did not translate into an increased $\dot{V}O_{2peak}$. This appears in agreement with previous studies carried out in obese patients by our group, in which interventions which improved exercise tolerance during submaximal exercise (such as normoxic helium breathing [Salvadego et al., 2015] or respiratory muscles endurance training [Salvadego et al., 2017; Alemayehu et al., 2018]) did not affect $\dot{V}O_{2peak}$. This variable, therefore, in obese patients may be more resistant to changes compared to other submaximal variables. GET was not significantly different in POST *vs.* PRE, whereas RCP was higher in POST *vs.* PRE; it cannot be excluded that the lack of a significant difference for GET could be related to lack of statistical power. Moreover, $\dot{V}O_2$ values during HEAVY corresponded to RCP in PRE, and were slightly below RCP in POST. In both cases, however, $\dot{V}O_2$ values appeared in steady-state during the last minutes of exercise, and they did not show the continuous increase that would characterize the severe exercise domain. Both in PRE and in POST, therefore, exercise was at upper boundary of the heavy exercise domain. In any case, it should be stressed that even if changes in exercise domain had occurred following training, they

would not have detracted from the message of the study: exercise training would improve exercise tolerance, as manifested (for the same absolute work rate) by a change in exercise domain, by less pronounced slow components of HR and $\dot{V}O_2$ kinetics, and by a less pronounced decrease in work rate for the same fixed HR.

To conclude, in untrained obese patients exercise prescription at a fixed submaximal HR, slightly above that corresponding to GET (as it is often done for endurance exercise prescription), translates into a work rate which must be progressively decreased during the training session. A 3-wk multidisciplinary body mass reduction intervention, including moderate-intensity exercise, increased exercise tolerance by eliminating (during moderate-intensity exercise) or by reducing (during heavy-intensity exercise) the slow component of HR kinetics. As a consequence, the decrease in work rate, occurring in order to maintain a constant HR slightly above that corresponding to GET, substantially disappeared after the body mass reduction intervention, thereby facilitating exercise prescription, universally recognized as a cornerstone in the treatment of obesity.

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Conflicts of interest

The authors have no conflict of interest, and the results of the present study do not constitute endorsement by the American College of Sport Medicine. The results of this study are presented clearly, honestly, and without fabrication, or inappropriate data manipulation.

3.3 DECREASE IN WORK RATE IN ORDER TO KEEP A CONSTANT HEART RATE: EFFECTS OF A 10-DAY BED REST. PRELIMINARY RESULTS – *STUDY 3*

ABSTRACT

Purpose: Aerobic exercise prescription is usually set at specific heart rate (HR) values. However, it has been recently demonstrated that during exercise carried out at a HR slightly above that corresponding to the gas exchange threshold (GET), the work rate has to decrease in order to maintain HR constant. It is well documented that simulated microgravity (i.e., bed rest, BR) determines a significant impairment of skeletal muscle oxidative metabolism and exercise tolerance. This led us to hypothesize that the decrease in work rate needed to keep HR constant could be significantly aggravated by BR. Testing this hypothesis would significantly affect exercise evaluation and prescription in microgravity. **Methods**: Ten young and healthy men (age, 23 ± 5 yr) were tested before (PRE) and after (POST) a 10-day horizontal BR and, performed on a cycle ergometer: a) an incremental exercise up to voluntary exhaustion (INCR); b) a 15-min (''$HR_{CLAMPED}$'') exercise, in which work rate was continuously adjusted in order to maintain HR constant, slightly higher than that determined at the "gas exchange threshold" (GET). Pulmonary O_2 uptake ($\dot{V}O_2$) was assessed breath-by-breath, cardiac output (CO) and stroke volume (SV) were estimated by impedance cardiography. Fractional O_2 extraction (Δ[deoxy(Hb+Mb)]) was evaluated in muscles of the anterior and posterior compartments of the thigh by near-infrared spectroscopy (NIRS). **Results:** During INCR, Work rate (230±41 w *vs.* 251±50, P=0.02) and $\dot{V}O_{2peak}$ (40.3±6.1 $ml \cdot kg^{-1} \cdot min^{-1}$ *vs.* 44.4±7.2, P<0.001) were significantly lower in POST *vs.* PRE, whereas HR_{peak} remained unchanged (189±6 $b \cdot min^{-1}$ *vs.* 187±8). $\Delta[deoxy(Hb+Mb)]_{peak}$ significantly decreased (p<0.05) in POST compared to PRE. Work rate at GET was not different in POST (113±33 W) *vs.* PRE (118 ±33). During $HR_{CLAMPED}$ (set at 145±11 $b \cdot min^{-1}$), the decrease in work rate needed to maintain a constant HR was more pronounced in POST *vs.* PRE (39±10% *vs*. 29±14%) and it was associated with decreases (both in POST and in PRE) of $\dot{V}O_2$ (14% and 13%), Δ[deoxy(Hb+Mb)] (41% and 18%), whereas SV and CO did not change. **Conclusion**: During 15-min cycling initially set at a w corresponding to a HR slightly above GET (as frequently done for exercise prescription), in order to keep HR constant, work rate had to decrease by ~29% and ~39%, respectively, before and after a 10-day bed rest. The work rate decrease, whose cause(s) still need to be determined, is a sign of exercise intolerance, and was more pronounced compared to that needed to prevent "slow components" of $\dot{V}O_2$ and muscle fractional O_2 extraction kinetics. Exercise evaluation and prescription at fixed submaximal HR are problematic, also in microgravity.

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INTRODUCTION

The recent observations made by our group (Zuccarelli et al., 2018; Zuccarelli et al., 2019) regarding a progressive increase in heart rate (HR) during constant work rate exercise (CWR) for intensities below the gas exchange threshold (GET), complected the exercise prescription. Since HR can be easily measured and recorded, aerobic exercise is commonly prescribed at specific heart rate values mainly because of the practicality of the approach. From a less practical point of view, this common practice is based on the linear relationship occurring between HR and $\dot{V}O_2$ (Astrand et al., 1986). However, confirming anecdotical observations (Engelen et al., 1996; Orizio et al., 1988), HR and $\dot{V}O_2$ show different behaviours during CWR. We have recently demonstrated both in healthy and diseased populations (i.e., obese patients) that a "slow component" of HR kinetics occurs also during moderate-intensity CWR (below GET), so that HR does not reach a steady-state value but keeps increasing as a function of the time of exercise (Zuccarelli et al., 2018; Zuccarelli et al., *In press*). This issue is further complicated by the observations that the relative amplitude (or slope) of the HR slow component is more pronounced than the relative amplitude of the well-known slow component of $\dot{V}O_2$. This translates into the fact that when healthy people and obese subjects are asked to perform an exercise of 15-20 minutes keeping constant an HR target value slightly higher than that corresponding to GET, they are forced to decrease the exercise intensity to an extent of which also $\dot{V}O_2$ decreases. Interestingly, when measurements on obese patients were done before and after a 3-week body mass reduction program, the decrease in work rate needed to keep HR constant following the intervention was less pronounced. Thus, the decreases in work rate were interpreted as a "new" marker of exercise (in)tolerance. Having the opportunity to be part of a10-day horizontal bed rest campaign in Summer 2019, allowed us to test the above-mentioned interpretation also in simulated microgravity (i.e., bed rest), a condition which significantly effects oxidative metabolism and exercise tolerance. The aim of the present study was therefore to identify, in the needed decrease in work rate in order to keep HR constant, a systemic biomarker of impairment of oxidative metabolism, possibly more sensitive than traditional ones, such as $\dot{V}O_{2peak}$ or GET. Moreover, if confirmed also in microgravity, the phenomenon mentioned above would stress the need to reconsider the whole approach to exercise prescription and exercise evaluation in microgravity.

METHODS

This study was part of the 10-day horizontal bed rest campaign financed within the "MARS-PRE Bed Rest SBI 2019" project by the Agenzia Spaziale Italiana (ASI) whose aim was to monitor adaptations to simulated microgravity in different organs and systems of living organisms, in order to identify early biological and functional biomarkers of altered state of health. Detailed data related to participants and exercise protocols are reported in paragraph 3.5.

Briefly, ten healthy recreationally active men participated in this study, and their main physical characteristics at baseline were as follows: age, 23 ± 5 yr (mean ± SD); height, 1.81 ± 0.04 m; body mass, 77.5 ± 10.0 kg; body mass index, 23.6 ± 2.5 $kg \cdot m^{-2}$. Subjects were tested before (PRE) and after (POST) a 10-day horizontal bed rest without countermeasures, carried out at the General Hospital of Izola, Slovenia. Measurements included in this study were performed over the last 2 days before subjects were put to bed, and over the first 2 days after they arose from bed. Participants completed during the first day, an incremental exercise on an electronically braked cycle ergometer (Monark 818E; Stockholm, Sweden) to determine $\dot{V}O_{2peak}$ and GET. During the test cycling clip-in pedals and shoes were used, thereby allowing to utilize hamstring muscles to a greater extent in the pedal upstroke, and thus generating power through the entire rotation of the crank. During the second day the subjects performed, after an initial 2-min of unloaded pedaling, a 15-min ‘‘HR-controlled’’ exercise ($HR_{CLAMPED}$), in which work rate was continuously adjusted to maintain a constant HR, slightly higher than that determined at GET (GET + 10 %) in PRE (see Zuccarelli et al. 2018). During the exercise the work rate was kept constant for the first 2-3 minutes, or until HR reached its target value, and then it was adjusted by the operator every 5 s in order to maintain HR constant throughout the exercise.

Gas exchange parameters were assessed breath-by-breath by a metabolic cart (Quark PFTergo, Cosmed, Rome, Italy).

At the end of the incremental exercise and at specific time intervals (5, 10, 15 min) during the $HR_{CLAMPED}$ exercise the rate of perceived exertion (RPE) was determined using the Borg 6-20 scale (Borg, 1973). Both at rest and at specific time intervals (1, 3, 5 min) during the recovery period following the incremental exercise, or during the $HR_{CLAMPED}$ exercise (5, 10 and 15 min), 20 μL of capillary blood was collected from a pre-heated earlobe for the determination of blood lactate concentration ($[La]_b$) by means of an automated electro-enzymatic analyzer (Biosen C-line, EKF, Germany). At the same time points during the $HR_{CLAMPED}$ exercise, systolic (SBP) and diastolic blood pressure (DPB) were measured using an automatic sphygmomanometer placed over the brachial artery of the right arm (Omron M6; Omron Healthcare Ltd., Milton Keynes, United Kingdom).

Oxygenation changes in 4 different sites of both the anterior and posterior compartments of the right thigh were evaluated by near-infrared spectroscopy (NIRS) (Grassi & Quaresima, 2016; Barstow, 2019;), thereby allowing an evaluation of the distribution of the variables in different portions of the same muscles or in different muscles. A portable near-infrared continuous-wave instrument (OctaMon M; Artinis Medical Systems, The Netherlands) was used in this study, which consists of 8 light transmitters/channels (2 wavelengths at 760 and 850 nm), separated by 35 mm from the receiving optode (1 receiver every 4 transmitters). One probe was firmly attached to the skin

overlying the quadriceps femoris muscle, more precisely the vastus lateralis and rectus femoris muscles, whereas the other one was situated on the hamstring muscles, more specifically over the biceps femoris and sartorious muscles. Adipose tissue thicknesses (ATT) at the sites of application of the NIR probes were estimated by a caliper (Gima, Milan, Italy). During $HR_{CLAMPED}$ exercise, changes of measured variables were calculated as the difference between the highest mean value (over about 30 seconds) obtained during the first minutes of exercise and the mean value calculated at the end of exercise. To confirm the absence of an increase/decrease in HR as a function of time, a linear regression from the second minute to the end of exercise was calculated. The absence of a slope significantly different from zero would confirm that the variable remained constant throughout exercise, as planned by the experimental protocol.

Statistical analysis

Results are expressed as mean ± SD values. Statistical significance of differences between the two conditions (PRE vs. POST) was checked, for variables of the incremental tests, by two-tailed Student's paired t-tests. Dependent variables measured over several time periods during $HR_{CLAMPED}$ exercises were analyzed using a two-way (condition–time) repeated measures ANOVA. Significant interaction effects were followed up by Bonferroni-corrected paired t-tests. Regression analysis was performed by the least-squared residuals method. The level of significance was set at 0.05. Statistical analyses were carried out with a commercially available software package (Prism 8.0; GraphPad).

RESULTS

Peak values of the main respiratory, cardiovascular and metabolic variables obtained during the incremental exercises are shown in **Table 1**. Work $rate_{peak}$, $\dot{V}O_{2peak}$, CO_{peak} and SV_{peak} were significantly lower in POST vs. PRE. Mean values of GET expressed as absolute $\dot{V}O_2$ values, as a percentage of $\dot{V}O_{2peak,}$ as HR and $\dot{w}$ are shown in **Figure 1**. In all cases GET was not significantly different in the two conditions. HR_{peak} [corresponding to 94 and 96 % of the age-predicted maximum values (calculated as 208 - 0.7 x age (Tanaka et al. 2001)), in PRE and POST, respectively], R_{peak}, $[La]_{b\ peak}$ and RPE_{peak} were not significantly different in POST vs. PRE. These data confirm that in both conditions the exercises were maximal from a cardiorespiratory perspective.

The adipose tissue thickness (ATT) at the sites overlying quadriceps and hamstrings muscles where NIRS probes were positioned were (PRE vs. POST) 5.4 ± 1.3 vs. 4.7 ± 0.7 mm (P = 0.03) and 4.1 ± 1.5 vs. 3.6 ± 1.2 mm (P = 0.16), respectively. $\Delta[deoxy(Hb+Mb)]_{peak}$ of muscles of the anterior (8.9 ± 3.1 vs. 7.9 ± 3.0 µM, P = 0.02) and of the posterior compartments of the thigh (6.1 ± 4.9 vs. 4.9 ± 3.4 µM, P = 0.04) significantly decreased after BR. Values were significantly higher (P < 0.001) in the anterior compartment vs. the posterior compartment.

Table 1. Peak values of the main respiratory, cardiovascular and metabolic variables determined during incremental exercises before (PRE) and after (POST) bed rest.

	PRE	**POST**
Work rate $_{peak}$ (W)	251 ± 50	230 ± 41*
$\dot{V}O_{2peak}$ ($L \cdot min^{-1}$)	3.436 ± 0.67	3.039 ± 0.46***
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	44.4 ± 7.2	40.3 ± 6.1***
$\dot{V}CO_{2peak}$ ($L \cdot min^{-1}$)	4.020 ± 0.76	3.527 ± 0.56**
R_{peak}	1.17 ± 0.07	1.16 ± 0.07
$\dot{V}E_{peak}$ ($L \cdot min^{-1}$)	150.5 ± 20.5	133.2 ± 20.3*
HR_{peak} ($b \cdot min^{-1}$)	187 ± 8	189 ± 6
SV_{peak} (mL)	134 ± 28	101 ± 17**
CO_{peak} ($L \cdot min^{-1}$)	25.2 ± 5.8	19 ± 3.2**
SaO_2 (%)	97.7 ± 1.3	98.1 ± 1.3
$[La]_{b\ peak}$ (mM)	11.9 ± 2.7	11.2 ± 3.1
RPE_{peak} (6–20)	18.6 ± 0.9	19 ± 0.9

Data are means ± SD. $\dot{\mathbf{V}}O_2$ pulmonary oxygen uptake; $\dot{\mathbf{V}}CO_2$, CO_2 output; R, gas exchange ratio; $\dot{\mathbf{V}}$E, pulmonary ventilation; HR, heart rate; SV, stroke volume; CO, cardiac output; SaO_2, arterial blood O_2 saturation; $[La]_b$, blood lactate concentration; RPE, rate of perceived exertion. Asterisks denote differences from PRE by means of Student's paired t-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

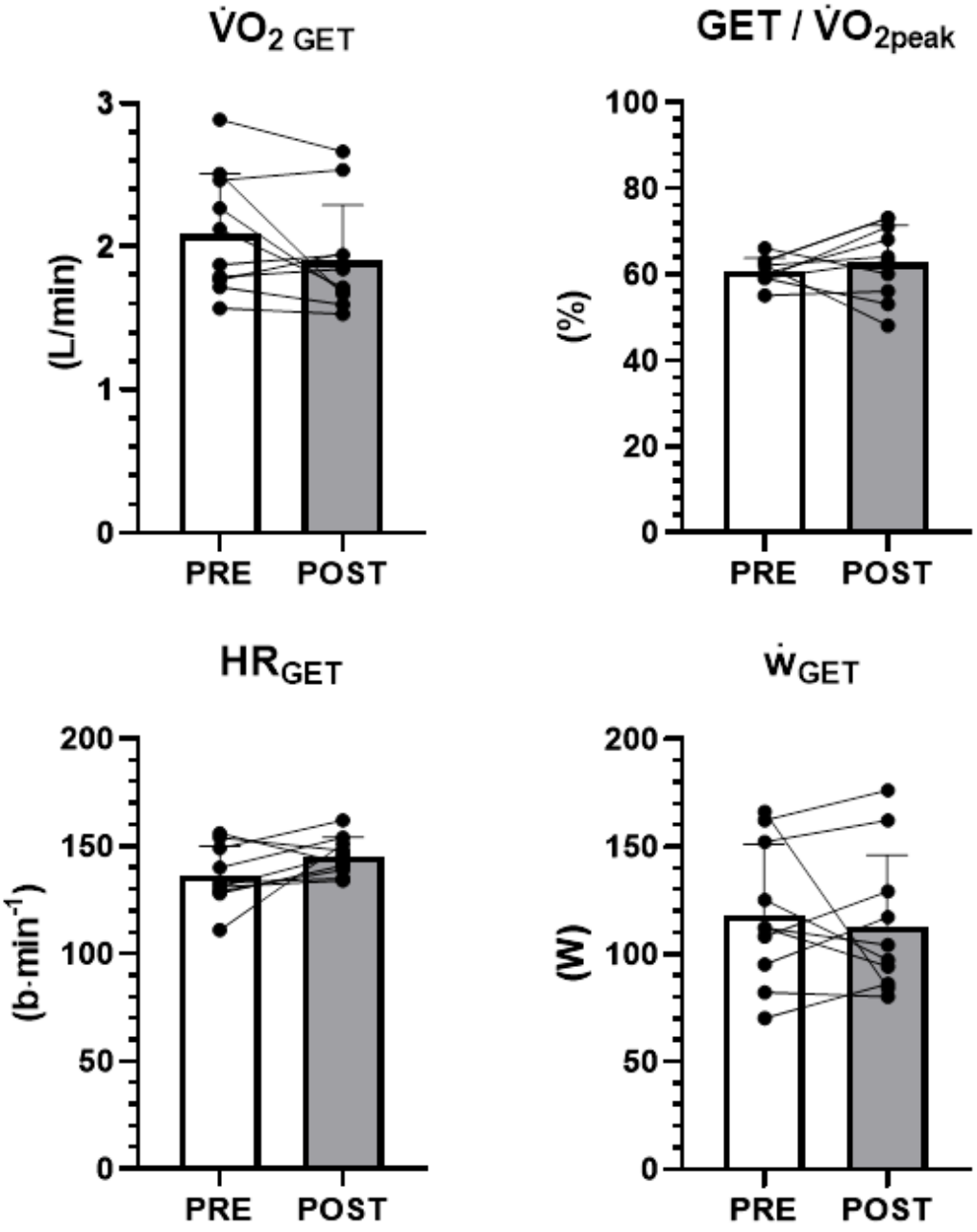

Figure 1. Individual and mean (± SD) values of gas exchange threshold (GET), expressed as absolute $\dot{V}O_2$ values, as a percentage of $\dot{V}O_{2peak}$, as HR and work rate at GET ($\dot{W}$GET), obtained before (PRE) and after (POST) bed rest.

In **Figure 2** mean values of HR, work rate and $\dot{V}O_2$ obtained during the $HR_{CLAMPED}$ exercise are shown. Both in PRE and in POST HR mean target value (set at 145 ± 11 b·min^{-1}, corresponding to GET +10% in PRE) was reached within the first 2-3 minutes of exercise and remained substantially constant throughout the test. The slopes of the linear regression lines were not significantly different from zero ($P > 0.05$). Both in PRE and in POST work rate decreased in order to maintain HR constant. The work rate decrease was more pronounced and occurred earlier during the 15-min task in POST

vs. PRE. At the end of the task the work rate decrease was 39 ± 10 % in POST vs. 29 ± 14% in PRE ($P < 0.01$), and was associated with a decreased $\dot{V}O_2$ (**Figure 2**) (-14 ± 7 % in POST vs. -13 ± 8 % in PRE, $P > 0.05$).

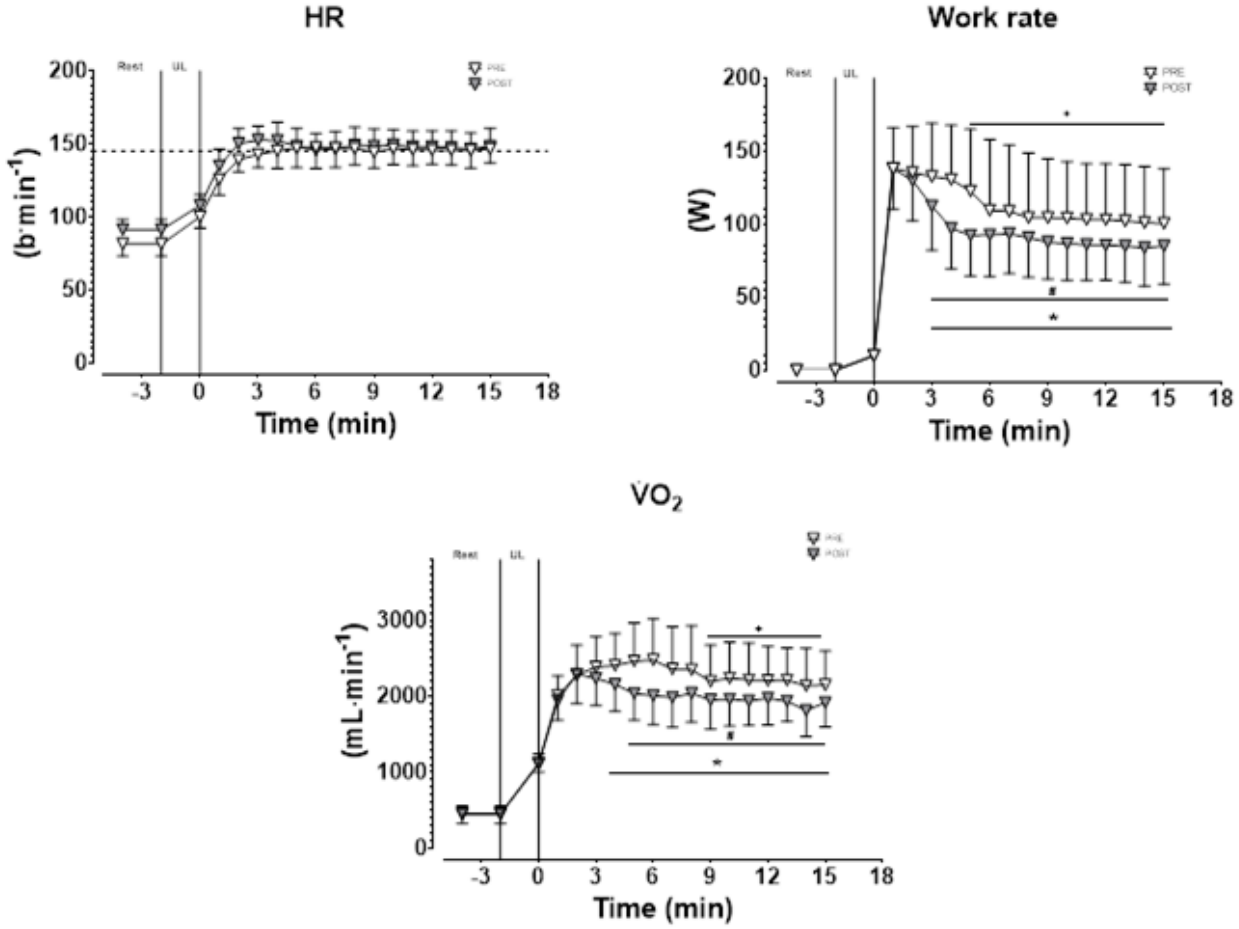

Figure 2. Mean (± SD) values of HR, work rate and $\dot{V}O_2$ during $HR_{CLAMPED}$ exercises, before (PRE) and after bed rest (POST). The horizontal dashed line indicates HR mean target value. UL=unloaded pedalling; *statistically different from PRE; $^{+,\#}$ statistically different from respective 1-min value (for work rate); $^{+}$ statistically different from respective 6-min value (for $\dot{V}O_2$); $^{\#}$ statistically different from respective 2-min value (for $\dot{V}O_2$).

Initial and end exercise values of the other respiratory, cardiovascular and metabolic variables determined during $HR_{CLAMPED}$ exercises before and after BR are given in **Table 2**. $\dot{V}E$, $\dot{V}CO_2$, R, $[La]_b$ and SBP significantly decreased during the 15-min task, both in PRE (14, 22, 13, 12 and 7%, respectively) and in POST (18, 26, 14, 23 and 10%, respectively). RPE, SV and DBP did not decrease at the end of the exercise in both conditions, whereas CO decreased significantly only in POST (-7%, $P = 0.01$) (see **Table 2**).

Table 2. Initial and final values of the main respiratory, cardiovascular and metabolic variables determined during $HR_{CLAMPED}$ exercises before (PRE) and after (POST) bed rest.

	PRE		POST	
	Initial	**Final**	**Initial**	**Final**
$\dot{V}E$ ($L \cdot min^{-1}$)	67.1 ± 13.0	57.0 ± 10.1*	60.5 ± 9.8	49.2 ± 6.6***
$\dot{V}CO_2$ ($L \cdot min^{-1}$)	2.371 ± 0.4	1.867 ± 0.4***	2.171 ± 0.3	1.604 ± 0.3***
R	0.99 ± 0.05	0.86 ± 0.04***	0.97 ± 0.05	0.83 ± 0.03***
SV (mL)	140.8 ± 24	140.7 ± 26	121.9 ± 15	118 ± 17
CO ($L \cdot min^{-1}$)	20.5 ± 4	20.7 ± 4	18.6 ± 3	17.3 ± 3*
$[La]_b$ (mM)	3.7 ± 1.4	3.2 ± 1.3*	3.3 ± 0.8	2.6 ± 0.8***
RPE (6–20)	13 ± 3	13 ± 4	11 ± 2	13 ± 1
SBP (mmHg)	165 ± 17	153 ± 20*	164 ± 15	146 ± 11*
DBP (mmHg)	94 ± 18	80 ± 13	93 ± 14	93 ± 19

Data are means ± SD. Highest values obtained during the first minutes of $HR_{CLAMPED}$ exercise (Initial). Values obtained during the last minute of exercise (Final). $\dot{V}E$, pulmonary ventilation; $\dot{V}CO_2$, CO_2 output; R, gas exchange ratio; HR, heart rate; SV, stroke volume; CO, cardiac output; $[La]_b$, blood lactate concentration; RPE, rate of perceived exertion; SBP, systolic blood pressure; DPB, diastolic blood pressure. * denote differences from respective initial value by means of Student's paired t-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 3 shows mean (± SD) values of skeletal muscle fractional O_2 extraction (Δ[deoxy(Hb+Mb)]) of quadriceps femoris (anterior compartment) and hamstring (posterior compartment) muscles during $HR_{CLAMPED}$ exercise. To obtain this figure, data of the four sites overlying the anterior and posterior compartments of the thigh were averaged. In muscles of the anterior compartment, a decrease of fractional O_2 extraction was observed both in PRE and POST (-22 ± and -43 %, respectively). Δ[deoxy(Hb+Mb)] values in muscles of the posterior compartment were significantly lower than those of the anterior compartment ($P < 0.001$), and decreased to a similar extent by the end of the exercise both in PRE and POST. During the first 2 minutes of exercise values were slightly but significantly greater in POST compared to PRE.

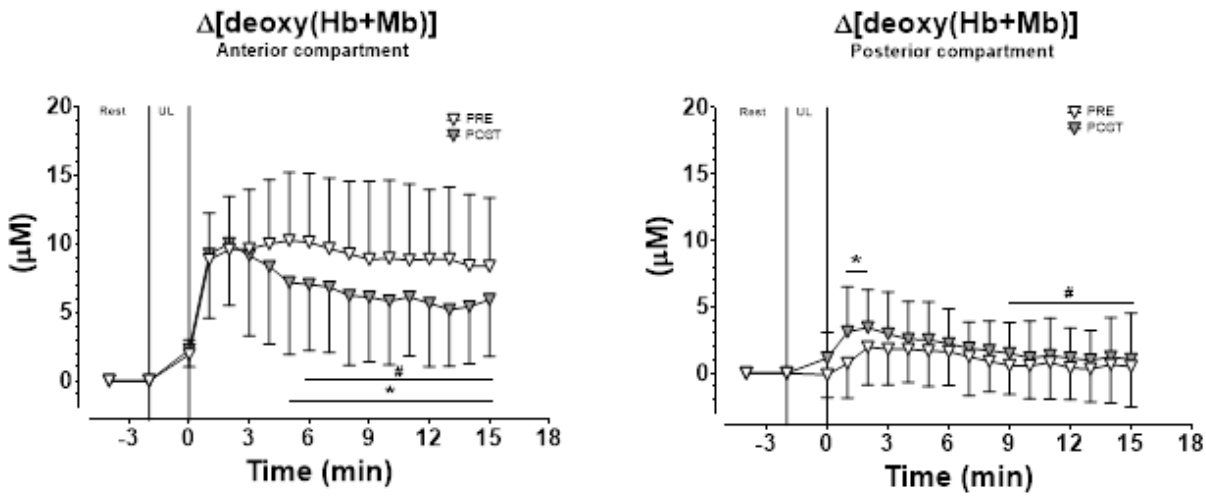

Figure 3. Mean (± SD) values of the near-infrared spectroscopy (NIRS)-obtained muscle oxygenation variable (Δ[deoxy(Hb+Mb)]), in muscles of the anterior and posterior compartments of the tight, during $HR_{CLAMPED}$ exercise PRE and POST BR. UL=unloaded pedalling; *statistically different from PRE; # statistically different from respective 2-min value.

DISCUSSION

The main finding of the present study was that the work rate decrease necessary to keep HR constant at a value slightly above GET was significantly greater (by ~40%) after vs. before (~30%) 10 days of bed rest (BR). This finding can be considered a "systemic biomarker" of impaired exercise tolerance following BR and has profound implications on exercise evaluation and exercise prescription, in microgravity conditions or after a period of immobilization. The results confirm the concept, put forward by recent studies by our group (Zuccarelli et al. 2018, Zuccarelli et al. in press), that exercise prescription at fixed submaximal HR slightly above GET (as it is frequently done when submaximal "aerobic" training is involved) is not associated with a specific work rate, and demonstrate that the problem is more significant following microgravity/immobilization exposure.

In the present study subjects performed a 15-min ''$HR_{CLAMPED}$'' trial initially set at a work rate corresponding to a HR slightly above GET, which was continuously adjusted to maintain HR constant. Confirming our hypothesis and recent studies by our group (Zuccarelli et al. 2018, Zuccarelli et al. in press), in order to keep the fixed value of HR, work rate had to decrease both in PRE and POST, but more markedly POST. This phenomenon was also associated with $\dot{V}O_2$ decreases of 14% and 13%, respectively, in POST and PRE. Overall, these data suggest the absence of a linear relationship between HR, $\dot{V}O_2$ and work rate, which makes aerobic exercise prescription at fixed submaximal HR values problematic, particularly during or immediately after exposure to microgravity. As discussed before, the behaviours of HR and $\dot{V}O_2$ observed during $HR_{CLAMPED}$ exercises were different, suggesting that the slow components of these two variables likely recognize different mechanistic determinants. Whereas the slow component of the $\dot{V}O_2$ kinetics has been widely studied (see e.g. the reviews by Rossiter et al. 2011; Poole & Jones, 2012; Jones et al. 2011, Grassi et al. 2015), and seems to be related to a decreased efficiency of oxidative metabolism and with muscle fatigue, the HR slow component has not received the same attention in literature. In previous studies (Zuccarelli et al. 2018) we demonstrated that, differently from the $\dot{V}O_2$ slow component, the HR slow component occurs also during moderate intensity exercise (below GET), and that above GET it is more pronounced than the $\dot{V}O_2$ slow component. But, is the HR slow component associated with fatigue? In the present study during the $HR_{CLAMPED}$ trial we observed *decreases* of variables whose continuous increase is generally associated with muscle fatigue (Grassi et al. 2015), namely $\dot{V}O_2$, $[La]_b$, R, skeletal muscle fractional O_2 extraction. This suggests that during $HR_{CLAMPED}$ the work rate decrease in order to keep HR constant, was greater than that needed to prevent a progressive increase of the variables associated with fatigue described above. This leaves the search of the cause(s) responsible for the work rate decrease during $HR_{CLAMPED}$ still open.

In conclusion, in the present study, after a 10-day horizontal BR we documented an important impairment of oxidative metabolism as demonstrated by a significant decrease in $\dot{V}O_{2peak}$, CO_{peak}, as well as in the peak of fractional O_2 extraction in skeletal muscles located in the anterior and posterior compartments of the thigh. Secondly, confirming our hypothesis, during 15-min cycling initially set at a work rate corresponding to a HR slightly above GET (as frequently done for exercise prescription), in order to keep HR constant, work rate had to decrease more markedly after BR compared to before. The work rate decrease, whose cause(s) still need to be determined, is a sign of exercise intolerance, which may significantly affect exercise tolerance evaluation as well as exercise prescription, especially during and after exposure to microgravity. Our data demonstrated indeed that prescribing exercise at fixed HR values, slightly higher than that corresponding to GET, would correspond to decreasing work rate by ~40%; hence, the concept of a HR value corresponding to a specific CWR does not hold true. Consequently, caution is needed in prescribing aerobic exercise training programs based on HR values, also during and after exposure to microgravity.

Funding

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3.4 SKELETAL MUSCLE $\dot{V}O_2$ KINETICS BY THE NIRS REPEATED OCCLUSIONS METHOD DURING THE RECOVERY FROM CYCLE ERGOMETER EXERCISE – *STUDY 4*

This article has been published in the "*Journal of Applied Physiology*" 123(3): 534-544 (2020) as "Skeletal muscle $\dot{V}O_2$ kinetics by the NIRS repeated occlusions method during the recovery from cycle ergometer exercise" by Lucrezia Zuccarelli, Paulo Cesar do Nascimento Salvador, Alessio Del Torto, Riccardo Fiorentino, Bruno Grassi.

ABSTRACT

Near-infrared spectroscopy (NIRS) has been utilized as a non-invasive method to evaluate skeletal muscle mitochondrial function in humans, by calculating muscle $\dot{V}O_2$ ($\dot{V}O_2m$) recovery (off-) kinetics following short light-intensity plantar flexion exercise. The aim of the present study was to determine $\dot{V}O_2m$ off- kinetics following standard cycle ergometer exercise of different intensities. Fifteen young physically active healthy males performed an incremental exercise (INCR) up to exhaustion and two repetitions of constant work-rate (CWR) exercises at 80% of gas exchange threshold (GET) (MODERATE) and at 40% of the difference between GET and peak pulmonary $\dot{V}O_2$ ($\dot{V}O_2p$) (HEAVY). $\dot{V}O_2p$ and vastus lateralis muscle fractional O_2 extraction by NIRS (Δ[deoxy(Hb+Mb)]) were recorded continuously. Transient arterial occlusions were carried out at rest and during the recovery for $\dot{V}O_2m$ calculation. All subjects tolerated the repeated occlusions protocol without problems. The quality of the monoexponential fitting for $\dot{V}O_2m$ off- kinetics analysis was excellent ($0.93 \leq r^2 \leq 0.99$). According to interclass correlation coefficient the test-retest reliability was moderate to good. $\dot{V}O_2m$ values at the onset of recovery were ~27, ~38 and ~35 times higher (in MODERATE, HEAVY and INCR, respectively) than at rest. The time constants (τ) of $\dot{V}O_2m$ off-kinetics were lower ($P<0.001$) following MODERATE (29.1±6.8 s) *vs.* HEAVY (40.8±10.9) or INCR (42.9±10.9), suggesting an exercise intensity dependency of $\dot{V}O_2m$ off-kinetics. Only following MODERATE the $\dot{V}O_2m$ off-kinetics were faster than the $\dot{V}O_2p$ off-kinetics. $\dot{V}O_2m$ off-kinetics, determined non-invasively by the NIRS repeated occlusions technique, can be utilized as a functional evaluation tool of skeletal muscle oxidative metabolism also following conventional cycle ergometer exercise.

"NEW & NOTEWORTHY"

This is the first study in which muscle $\dot{V}O_2$ recovery kinetics, determined non-invasively by near-infrared spectroscopy (NIRS) by utilizing the repeated occlusions method, was applied following standard cycle ergometer exercise of different intensities. The results demonstrate that muscle $\dot{V}O_2$ recovery kinetics, determined non-invasively by the NIRS repeated occlusions technique, can be utilized as a functional evaluation tool of skeletal muscle oxidative metabolism also following

conventional cycle ergometer exercise, overcoming significant limitations associated with the traditionally proposed protocol.

INTRODUCTION

The functional evaluation of skeletal muscle oxidative metabolism is crucially important in the assessment of exercise tolerance in normal subjects, athletes and patients affected by many chronic diseases such as heart failure and chronic obstructive pulmonary disease (Grassi et al., 2016; Poole & Jones 2012; Rossiter et al., 2011). Since the pioneering studies by Meyer (see *e.g.* Meyer, 1988), the rate of phosphocreatine (PCr) recovery following exercise, as determined by ^{31}P nuclear magnetic resonance spectroscopy (^{31}P-MRS), has been traditionally considered a valuable functional evaluation tool of skeletal muscle oxidative performance (Kent & Fitzgerald 2016). The main limitations of this approach are represented by the cost of the equipment and by the limited exercise paradigms which can be performed in the NMR magnet.

Since PCr resynthesis is accomplished solely by oxidative phosphorylation (Kent & Fitzgerald 2016), however, the kinetics of PCr recovery (off- kinetics) should be closely related to the off- kinetics of muscle O_2 uptake ($\dot{V}O_2m$) decrease during the same recovery period. The importance of the $\dot{V}O_2m$ off-kinetics following exercise as a functional evaluation tool is underlined by the fact that in single muscle fibers the velocity constant of this variable is directly related to maximal O_2 uptake ($\dot{V}O_2$max) (Wüst et al., 2013). Direct measurements of $\dot{V}O_2m$ off- kinetics, however, have been traditionally difficult to perform in humans, and implied invasive measurements (see *e.g.* Krustrup et al., 2009). A proxy of $\dot{V}O_2m$ kinetics could be pulmonary $\dot{V}O_2$ ($\dot{V}O_2p$) kinetics. Although the two variables appear reasonably similar during the on- (rest-to-exercise) transition (Grassi et al., 1996), however, different results have been seen during the off- (exercise-to-recovery) transition (Krustrup et al., 2009).

A reliable and non-invasive method to determine $\dot{V}O_2m$ off- kinetics in exercising humans would therefore be highly desirable. An answer to this need could derive from a method recently proposed by two groups (Ryan et al., 2012; Adami & Rossiter 2018), based on a concept originally developed by Hamaoka et al. (1996) and by Van Beekvelt et al. (2001): in ischemic conditions, the linear rate of increase in deoxy-(hemoglobin+myoglobin), or the linear rate of decrease of oxy-(hemoglobin+myoglobin), as determined by near-infrared spectroscopy (NIRS) (Grassi & Quaresima 2016; Barstow 2019), represent an index of $\dot{V}O_2m$. By performing a series of repeated short ischemia (blood flow occlusions induced by rapid inflation and subsequent deflation of a pneumatic cuff with suprasystolic pressure) during the recovery from exercise, $\dot{V}O_2m$ measurements have been obtained with a temporal resolution allowing to perform a reliable $\dot{V}O_2m$ off- kinetics analysis (Ryan et al., 2014; Adami & Rossiter 2018). The method has been validated against other approaches of functional

evaluation of skeletal muscle oxidative metabolism, such as [PCr] (squared brackets denote concentrations) recovery kinetics (Ryan et al., 2013) and high-resolution respirometry of permeabilized skeletal muscle fibers (Ryan et al., 2014).

In a recent review Adami and Rossiter (2018) have summarized the key methodological issues of the repeated ischemia approach to determine $\dot{V}O_2m$ off- kinetics. Some limitations, in our opinion, should be acknowledged in the proposed protocols. According to Adami and Rossiter (2018), in order for the method to effectively evaluate skeletal muscle oxidative metabolism, mitochondrial enzymes should be "maximally activated". In our opinion it is not clear if this actually happens with the proposed protocol (Ryan et al., 2012; Adami & Rossiter 2018). A short (about 15 s) cyclical plantar flexion/relaxation exercise against a "manually applied resistance" (Adami et al., 2017a) is usually performed. Fifteen seconds of contractions are not enough to reach a $\dot{V}O_2m$ steady state. No measurement of external work rate can be performed. No inferences on exercise intensity can be made. The extent of the $\dot{V}O_2m$ increase during exercise, with respect to the resting baseline, is not known. Since the involved muscle mass is relatively small, and the exercise is very short, no systemic measurements of exercise intensity (in order to identify exercise intensity domains, which characterize the physiological responses to exercise [Rossiter et al., 2011; Poole & Jones 2012; Grassi et al., 2016]) can be made. Moreover, no comparisons with $\dot{V}O_2p$ kinetics (on- and off- transients) are possible. No inferences on O_2 availability (another pre-requisite for the measurement, see Adami & Rossiter 2018), or on its consequences, can be directly made, although some cautionary rules have been proposed by the authors (Adami & Rossiter 2018).

The aim of the present study was to provide some answers to the issues raised above. More specifically, we applied the repeated ischemia approach in order to determine $\dot{V}O_2m$ off- kinetics during the recovery from standard cycle ergometer exercise, carried out for several minutes at moderate-intensity, below the gas exchange threshold (GET), at heavy-intensity above GET and during the recovery from an incremental exercise. $\dot{V}O_2p$ off- kinetics were concurrently determined, as well as muscle deoxygenation off- kinetics by NIRS (see Grassi & Quaresima 2016) in the opposite leg compared to the one in which the repeated ischemia protocol was performed. We hypothesized an exercise intensity dependency of $\dot{V}O_2m$ off- kinetics (slower kinetics as a function of exercise intensity), paralleled by an exercise intensity dependency of $\dot{V}O_2p$ off- kinetics. Faster $\dot{V}O_2m$ *vs.* $\dot{V}O_2p$ off- kinetics were expected at all exercise intensities.

The obtained results, besides clarifying some of the issues/doubts discussed above, would allow insights into basic physiological mechanisms during metabolic transitions, and would allow to put

the repeated ischemia approach to determine $\dot{V}O_2m$ kinetics in the "real life" context of cycle ergometer exercise in different intensity domains.

MATERIALS AND METHODS

Subjects

Fifteen healthy, habitually active males (age 25 ± 4 years; height 180 ± 6 cm; body mass 77 ± 8 kg; body mass index 23.8 ± 2.1 $kg \cdot m^{-2}$) were tested. All participants were moderately trained and attained the American College of Sports Medicine (ACSM) exercise recommendations for adults (at least 150 $min \cdot week^{-1}$). Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state and to avoid strenuous exercise in the 24 h preceding each testing session. Subjects abstained from drinking alcohol (24 h) and caffeine (5 h) before the exercise test, and had their last meal at least 3 h before each testing session. The procedures used in this study were approved by the local Institutional Review Board, and were conducted in accordance with the Declaration of Helsinki. The subjects were fully informed of any potential risk associated with the experiments before verbal and written consents were obtained.

Exercise protocols

Exercise tests were carried out in a well-ventilated laboratory at 19–21 °C, under continuous medical supervision and 12-lead electrocardiography (Quark C12x, Cosmed). The participants were required to report to the laboratory on three separate occasions over a two-week period. On their first visit, anthropometric measurements were performed and subjects completed a ramp incremental exercise (INCR) (30 $W \cdot min^{-1}$) up to voluntary exhaustion on an electronically braked cycle ergometer (Ergomedic 839 E, Monark), to determine $\dot{V}O_2$peak and GET. Pedaling frequency was digitally displayed to the subjects, who were asked to keep a constant cadence throughout the tests at their preferred value (between 70 and 90 rpm). Voluntary exhaustion was defined as the incapacity to maintain the imposed load and pedaling frequency despite vigorous encouragement by the researchers. Peak values of the main variables were taken as the highest 15-s mean values attained prior to the subject's voluntary exhaustion. $\dot{V}O_2p$ at GET was determined by two independent investigators by standard methods (Beaver et al., 1986). In order to identify the work rate corresponding to $\dot{V}O_2p$ at GET, the effect of the delayed $\dot{V}O_2p$ adjustment to the increased work rate during the incremental test was corrected by shifting the linear $\dot{V}O_2p$ *vs.* time (and work rate) relationship to the left, by an amount corresponding to the individual mean response time of the $\dot{V}O_2p$ kinetics (21.8 ± 8.9 s) (Whipp et al., 1981).

After the first visit the subjects performed in two different days two repetitions of 6-min constant work rate (CWR) submaximal exercise corresponding to 80% of GET (MODERATE) and 40% of

the difference between GET and $\dot{V}O_2p$ peak (HEAVY). MODERATE was always carried out before HEAVY. HEAVY exercise was performed when subjects reached again baseline values of the main investigated variables (about 30 minutes of recovery).

Measurements

Pulmonary ventilation ($\dot{V}E$), $\dot{V}O_2$ and CO_2 output ($\dot{V}CO_2$) were determined breath-by-breath by a metabolic cart (Quark PFTergo, Cosmed, Rome, Italy). Expiratory flow measurements were performed by a turbine flow meter calibrated before each experiment by a 3 L syringe at different flow rates. $\dot{V}O_2p$ and $\dot{V}CO_2$ were determined by continuously monitoring PO_2 and PCO_2 at the mouth throughout the respiratory cycle and from established mass balance equations. Calibration of O_2 and CO_2 analysers was performed before each experiment by utilizing gas mixtures of known composition. Gas exchange ratio (R) was calculated as $\dot{V}CO_2/\dot{V}O_2p$. HR was determined from the electrocardiogram signal.

Oxygenation changes in a superficial portion of *vastus lateralis* muscles of both limbs were evaluated by near-infrared spectroscopy (NIRS). The main advantages and limitations of this technology have been recently discussed in reviews (Grassi & Quaresima 2016; Barstow, 2019). Portable continuous-wave, spatially-resolved near-infrared (NIR) light photometers (PortaLite, Artinis Medical Systems, Netherlands) were utilized. The PortaLite probe consists of three light transmitters (each emitted two wavelengths of 760 nm and 850 nm) separated by 3, 3.5 and 4 cm from the receiving optode. The deepest signal (4 cm) was taken into account for the analysis. Thus, the light penetration depth can be estimated to be at least 2 cm (i.e., at least about half of the source detector distance (Hamaoka et al., 2007). The instruments provide measurements of micromolar (μM) changes in deoxygenated hemoglobin (Hb) + myoglobin (Mb) concentrations (Δ[deoxy(Hb + Mb)]) and in oxygenated (Hb + Mb) (Δ[oxy(Hb + Mb)]). The sum between the two variables (Δ[deoxy(Hb + Mb)+ oxy(Hb + Mb)]) is related to changes in the total Hb volume (blood volume in the investigated tissue). An increased Δ[deoxy(Hb + Mb)] or a decreased Δ[oxy(Hb + Mb)], would indicate an increased fractional O_2 extraction (ratio between $\dot{V}O_2m$ and O_2 delivery in the investigated tissue, see [Grassi & Quaresima 2016]) only when Δ[deoxy(Hb + Mb)+ oxy(Hb + Mb)] is constant. This is unlikely in exercising muscles. In the past the problem was circumvented, at least in part, by taking as an index of deoxygenation the Δ[deoxy(Hb + Mb)] variable, which is relatively insensitive to blood volume changes, and has been demonstrated to nicely correlate with other variables related to fractional O_2 extraction (Grassi & Quaresima 2016). In the present study the problem was solved by utilizing, for values during exercise, the method proposed by Ryan et al. (Ryan et al., 2012), which allows to correct the deoxy Δ[deoxy(Hb + Mb)] variable for changes in blood volume.

The probes were firmly attached to the skin overlying the lower third of vastus lateralis muscles (~10 cm above the knee joint) of the right and left limbs, parallel to the major axis of the thigh, by a belt secured by Velcro straps and by adhesive tape. The skin was carefully shaven prior to the experimentation. The places where the probes were attached were recorded using a skin marker and reproduced throughout the tests. Black clothes were put around the probes and the skin to prevent contamination from ambient light. The sampling frequency was set at 10 Hz. Skinfold thicknesses at the sites of application of the NIR probes were determined by a caliper (Gima, Milan, Italy) in order to estimate adipose tissue thickness (ATT). The averaged values of skin and subcutaneous tissue thickness were 3.6 ± 1.0 and 4.1 ± 1.0 mm for the right and left limb, respectively. The NIRS probe on the left leg was utilized to determine muscle deoxygenation changes during recovery, whereas the NIRS probe on the right leg was utilized to determine muscle oxygenation changes during exercise and for the repeated ischemia protocol and the determination of the $\dot{V}O_2m$ off- kinetics during the recovery (see below).

Δ[deoxy(Hb + Mb)] values with respect to an initial value arbitrarily set equal to zero, were calculated and expressed in arbitrary units. Before the exercise period, an ischemic/hyperemia calibration of the right limb (i.e., physiologic normalization) was utilized to normalize Δ[deoxy(Hb + Mb)] values (McCully et al., 1994) by inflating a pressure cuff (~300 mmHg) positioned at the inguinal crease of the thigh (subjects in the sitting position on the cycloergometer) for a few minutes (from 2 to 4) until a signal plateau (indicating maximal deoxygenation) was reached. Δ[deoxy(Hb + Mb)] values obtained during exercise were then expressed as a percentage of the values obtained during the ischemic calibration. All subjects were seated on the cycle ergometer during the recovery period. They were instructed to place the leg on which the occlusions were performed on a wooden platform (height 10 cm), with the foot fixed to the pedal, and to keep the other leg relaxed, with the foot fixed to the pedal.

$\dot{V}O_2p$ and HR kinetics

$\dot{V}O_2p$ kinetics were mathematically evaluated during transitions from rest to low (MODERATE), and high (HEAVY) intensity CWR exercises (on- kinetics) and during the recovery from MODERATE, HEAVY and INCR exercises (off- kinetics). Breath-by-breath $\dot{V}O_2p$ values were initially examined to exclude outlier values caused by sighs, swallowing, and coughs, time aligned and then superimposed for each subject (Lamarra et al., 1987). Average $\dot{V}O_2p$ values every 10 s were calculated. Data obtained during the first 20 s of the on- transition (cardiodynamic phase [Whipp et al., 2002]) were excluded from analysis. Thus, on- $\dot{V}O_2p$ kinetics analysis dealt mainly with the phase 2 (or fundamental component) of the response. To evaluate mathematically the $\dot{V}O_2p$ kinetics, data were first fitted by the function:

$$y(t) = y_{BAS} + A_f [1 - e^{(t - TD)_f / \tau_f}] \quad (1)$$

and parameter values (TD_f, τ_f) were determined that yielded the lowest sum of squared residuals. In *equation 1*, t is the time, y_{BAS} indicates the baseline, A_f is the amplitude between the y_{BAS} and the steady state during the fundamental component, TD_f is the time delay, and τ_f is the time constant of the function for the fundamental component. To check the presence of a slow component (Whipp et al., 2002) of the kinetics, data were also fitted by other two functions. For details, please see Zuccarelli et al. (2018).

Average HR values every 5s were calculated. HR kinetics were analyzed by applying the same equations described above for $\dot{V}O_2p$, as suggested by previous authors (see *e.g.* Engelen et al., 1996; Zuccarelli et al., 2018).

For the $\dot{V}O_2p$ off- kinetics, a mono-exponential function based on previous literature (29) was utilized:

$$y(t) = y_{END} - A [1 - e^{(t - TD) / \tau}] \quad (2)$$

where y(t) represents the $\dot{V}O_2p$ value at a given time (t), y_{END} is the average value over the last 60 s of exercise, A is the amplitude of the exponential term describing changes in $\dot{V}O_2p$ from exercise to its asymptote during the recovery, τ is the time constant and TD is the time delay of the function.
Equation 2 was also used for the analysis of skeletal muscle reoxygenation off- kinetics (Δ[deoxy(Hb + Mb)]) in the leg without occlusions.

$\dot{V}O_2m$ off- kinetics

Following the method proposed by Ryan et al. (2012) and Adami and Rossiter (2018), $\dot{V}O_2m$ was estimated by calculating the slope of the initial linear increase (~3 s) in NIRS-measured Δ[deoxy(Hb + Mb)] during short (5-10 seconds) bouts of ischemia induced by rapid (less than 1 s) inflation and deflation (DN 200/10/5, Stanley) of a pneumatic cuff during the recovery from MODERATE, HEAVY and INCR exercises. A repeated arterial occlusion method (see Ryan et al., 2012; Adami & Rossiter 2018) was carried out at the end of each exercise protocol (i.e., INCR, MODERATE and HEAVY). When muscle reached a desaturation target of 50% of the physiological normalization (Adami et al., 2017a) (see below), several intermittent arterial occlusions were performed: 6

occlusions lasting 5 s each, separated by 10 s, and subsequently 6 occlusions lasting 10 s separated by 30-60 s. When the target of 50% was not reached at the end of the exercise protocol the first arterial occlusion was performed after 10 s.

$\dot{V}O_2m$ values were then fit by a monoexponential function according to equation 3 (Ryan et al., 2014):

$$y(t) = y_{END} - A \times e^{-kt} \quad (3)$$

where y(t) represents the value of $\dot{V}O_2m$ at a given time (t), y_{END} the $\dot{V}O_2m$ immediately after the cessation of the exercise, A is the amplitude of the response, *k* is the exponential recovery rate constant ($k = [1/\tau]$; expressed in min^{-1}) and *t* is time. Resting $\dot{V}O_2m$ values were estimated by the same approach, described above, on the data obtained during an arterial occlusion carried out at rest before the physiologic normalization procedure (see above).

Statistical analysis

Results are expressed as mean ± SD. Data fitting by exponential functions was performed by the least-squared residuals method. Statistical significance of differences between HR and $\dot{V}O_2p$ slow component amplitudes was checked by two-tailed Student's *t*-test for paired data. A one-way ANOVA with repeated measures was used to analyze the differences of $\dot{V}O_2m$, $\dot{V}O_2p$ and muscle deoxygenation parameters at the different exercise intensities. Assumptions of sphericity were assessed using the Mauchly test, and any violations were corrected using the Geisser-Greenhouse correction factor. When significant effects were observed a Tukey's post hoc test was used to determine the exact location of the difference. The level of significance was set at $P < 0.05$. Statistical analyses were carried out with a commercially available software package (Prism 7.0; GraphPad). Coefficient of variation (CV) and intraclass correlation coefficient (ICC) were utilized to analyze test-retest reliability. ICC estimates were calculated using SPSS statistical package version 23 (SPSS Inc, Chicago, IL) based on a mean-rating (K=2), absolute-agreement, 2-way mixed-effects model. Bland-Altman test for repeated measurements was used to assess the agreement between the two evaluations of the $\dot{V}O_2m$ off- kinetics.

RESULTS

All subjects completed the entire protocol, with no adverse events. Adipose tissue thickness was not significantly different in the right when compared to the left limb (P=0.30). Main respiratory, cardiovascular, and metabolic end-exercise or steady-state values, determined during INCR (peak values) and MODERATE and HEAVY CWR exercises are shown in **Table 1**.

Table 1. Main respiratory, cardiovascular, and metabolic end-exercise values or steady state values, determined during incremental exercise (INCR) and constant work rate exercises (MODERATE and HEAVY).

	INCR	**MODERATE**	**HEAVY**
Work rate, W	315 ± 45*#	118 ± 28§#	201 ± 36§*
$\dot{V}O_2p$, l·min^{-1}	3.596 ± 0.442*#	2.067 ± 0.297§#	3.183 ± 0.356§*
$\dot{V}O_2$p, ml·kg^{-1}·min^{-1}	47.5 ± 6.7*#	28.0 ± 5.4§#	41.0 ± 6.0§*
$\dot{V}CO_2$, l·min^{-1}	4.351 ± 0.428*#	1.994 ± 0.243§#	3.145 ± 0.313§*
R	1.21 ± 0.06*#	0.93 ± 0.06§#	1.00 ± 0.04§*
$\dot{V}$E, l·min^{-1}	143.6 ± 28.5*#	52.8 ± 7.5§#	88.7 ± 11.1§*
V_T, l	2.95 ± 0.33*#	2.04 ± 0.28§#	2.61 ± 0.29§*
fR, breaths·min^{-1}	49.4 ± 11.3*#	26.4 ± 4.5§#	34.2 ±5.8§*
$PETO_2$, mmHg	114.5 ± 4.8*#	100.1 ± 4.2§#	104.7 ± 4.2§*
$PETCO_2$, mmHg	36.6 ± 5.2*#	43.8 ± 2.3§#	41.80 ± 3.3§*
HR, beats·min^{-1}	181 ± 10*#	124 ± 17§#	162 ± 14§*
RPE, 6-20	19 ±1.06*#	8±2.03§#	16±1.60§*
Δ[deoxy(Hb+Mb)], %ischemia	68.3 ± 31.9*	20.7 ± 19.6§#	71.2 ± 19.9*

Mean values ± SD. $\dot{V}O_2p$, pulmonary oxygen uptake; $\dot{V}CO_2$, CO_2 output; R, gas exchange ratio; $\dot{V}$E, pulmonary ventilation; V_T, tidal volume; fR, breathing frequency; $PETO_2$, end-tidal O_2 partial pressure; $PETCO_2$, end-tidal CO_2 partial pressure; HR, heart rate; RPE, rate of perceived exertion; Δ [deoxy(Hb+Mb)], changes in deoxygenated Hb and myoglobin Mb concentrations, muscle oxygenation index obtained by near-infrared spectroscopy. *P<0.05 vs MOD; #P<0.05 vs HEAVY; §P<0.05 vs INC

$\dot{V}O_2p$ peak values were typical for young physically active subjects. R peak, HR peak (corresponding to 96% of the age-predicted maximum [calculated as 208 - 0.7 X age] [Tanaka et al., 2001]), and RPE peak values indirectly confirm that the INCR exercise was maximal. GET occurred at 58% of $\dot{V}O_2p$ peak. Work rates for MODERATE and HEAVY were 37 ± 6% and 64 ± 5% of peak work rate, respectively. Mean values of $\dot{V}O_2p$ and HR determined during the last 30 s of MODERATE were 58 ± 6% of $\dot{V}O_2p$ peak and 69 ± 9% of HR peak, respectively, whereas during HEAVY values were 89 ± 6% of $\dot{V}O_2p$ peak and 90 ± 6% of HR peak. Skeletal muscle fractional O_2 extraction (as indicated

by Δ[deoxy(Hb + Mb)]) during the last 10 s of MODERATE, HEAVY and INCR was about 21%, 71% and 68% of the ischemic/hyperemia calibration, respectively. Δ[deoxy(Hb + Mb)] values were significantly lower in MODERATE compared to INCR and HEAVY ($F=39.80$; $P<0.01$). No significant differences were observed between HEAVY and INCR.

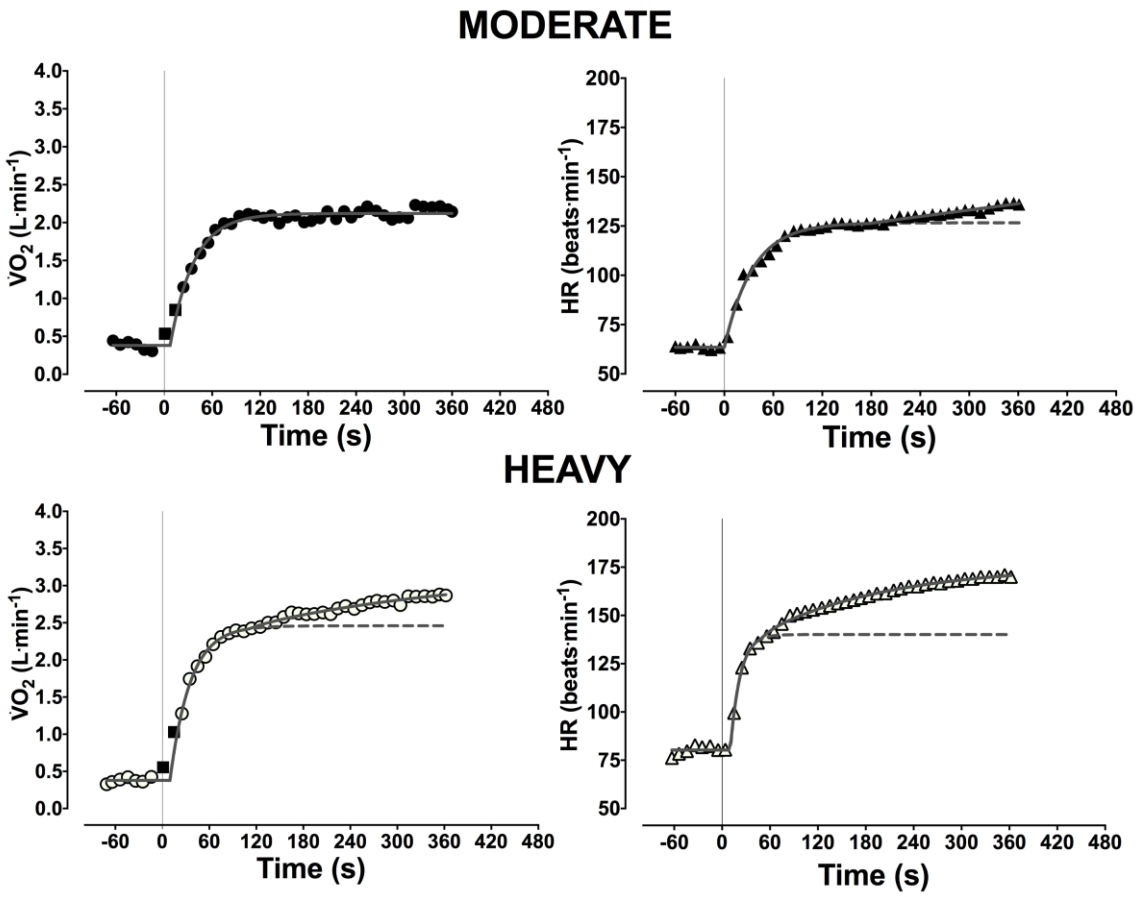

Figure 1. Pulmonary O_2 uptake ($\dot{V}O_2p$) (left panels) and heart rate (HR) (right panels) on- kinetics for a representative subject during constant work rate (CWR) exercise at two investigated intensity domains, MODERATE and HEAVY. The fitted functions are also shown. The dashed lines indicate the continuation of the monoexponential fitting; vertical distances between experimental data and the dashed lines indicate the amplitude of the slow components of the responses. The first two $\dot{V}O_2p$ data points (cardiodynamic phase) were excluded from the fitting. The vertical lines indicate the transitions from rest to the imposed work rate. See text for further details.

In HEAVY slow components were detected both for $\dot{V}O_2p$ and HR. In **Table 2**, parameters deriving from the fitting of $\dot{V}O_2p$ and HR on- kinetics are presented. For MODERATE, equation 1 represented the best fit for the $\dot{V}O_2p$ data in all subjects with the exception of one, who did show a slow component with an amplitude relative to the entire responses (A'_s/A_{tot}) equal to 7.8%. For HR, confirming the data obtained in a recent study by our group (Zuccarelli et al., 2018) a slow component with a relative amplitude of 21.4 ± 13.0% of A'_s/A_{tot} was detected in 12 subjects out of 15. For HEAVY a slow component was observed in all subjects, both for $\dot{V}O_2p$ and HR. The relative amplitude of the HR

slow component was greater than the relative amplitude of the $\dot{V}O_2p$ slow component (23.0 ± 11.0 and 13.3 ± 6.4%, respectively); also these data confirm those obtained in a previous study by our group.

Table 2. *Pulmonary O_2 uptake ($\dot{V}o_2p$) and heart rate (HR) kinetics parameters determined during constant work rate (CWR) exercises*

Intensity Domain	Work Rate, W	$\dot{V}o_2p_{bas}$, L/min	$\dot{V}o_2p$, L/min	A_f, L/min	TD_f, s	T_f, s	TD_s, s	A_s', L/min	A_s'/A_{tot}, %
MODERATE	118 ± 28	0.420 ± 0.069	2.067 ± 0.297	1.647 ± 0.301	10.3 ± 6.2	19.4 ± 4.9			
HEAVY	201 ± 36	0.470 ± 0.096	3.183 ± 0.356	2.361 ± 0.403	10.2 ± 3.4	23.5 ± 7.1	109.8 ± 38.3	0.489 ± 0.208	13.3 ± 6.4
Intensity Domain	Work Rate, W	HR_{bas}, beats/min	HR, beats/min	A_f, beats/min	TD_f, s	τ_f, s	TD_s, s	A_s', beats/min	A_s'/A_{tot}, %
MODERATE	118 ± 28	72 ± 11	124 ± 17	44 ± 12	3.1 ± 4.5*	15.5 ± 11.1*	105.1 ± 51.5	12 ± 9	21.4 ± 13.0
HEAVY	201 ± 36	78 ± 12	162 ± 14	65 ± 12	2.8 ± 3.4*	23.8 ± 11.0	81.4 ± 44.9*	20 ± 10	23.0 ± 11.0*

Values are means ± SD. MODERATE, moderate CWR exercise; HEAVY, heavy CWR exercise; $\dot{V}o_{2bas}$, oxygen uptake baseline; $\dot{V}o_2$, end-exercise oxygen uptake; HR_{bas}, heart rate baseline; HR, end-exercise heart rate; A_f, amplitude of the fundamental component; TD_f, time delay fundamental; τ_f, time constant fundamental; TD_s, time delay slow component; A_s', actual amplitude of the slow component; A_s'/A_{tot}, total amplitude of the response. *$P < 0.05$, significantly different from HR values.

Representative $\dot{V}O_2p$ and $\dot{V}O_2m$ off- kinetics curves for a typical subject following MODERATE, HEAVY and INCR are shown in **Figure 2**. A monoexponential decrease was observed in all conditions for both variables. For $\dot{V}O_2m$, individual values of the coefficient of determination (r^2) ranged between 0.93 and 0.99. For $\dot{V}O_2p$, the r^2 range was 0.96-0.99. In the panel with the $\dot{V}O_2m$ off-data, values obtained at rest before the exercise are also shown (dashed horizontal line). Δ[deoxy(Hb + Mb)] values during the first occlusion following MODERATE, HEAVY and INCR were about 11%, 49% and 48%, respectively, of the ischemic/hyperemia calibration. $\dot{V}O_2m$ values at the onset of recovery (extrapolated to time = 0 s according to the fitted monoexponential curve) were about 27, 38 and 35 times higher than those determined at rest (dashed horizontal line) for MODERATE, HEAVY and INCR, respectively.

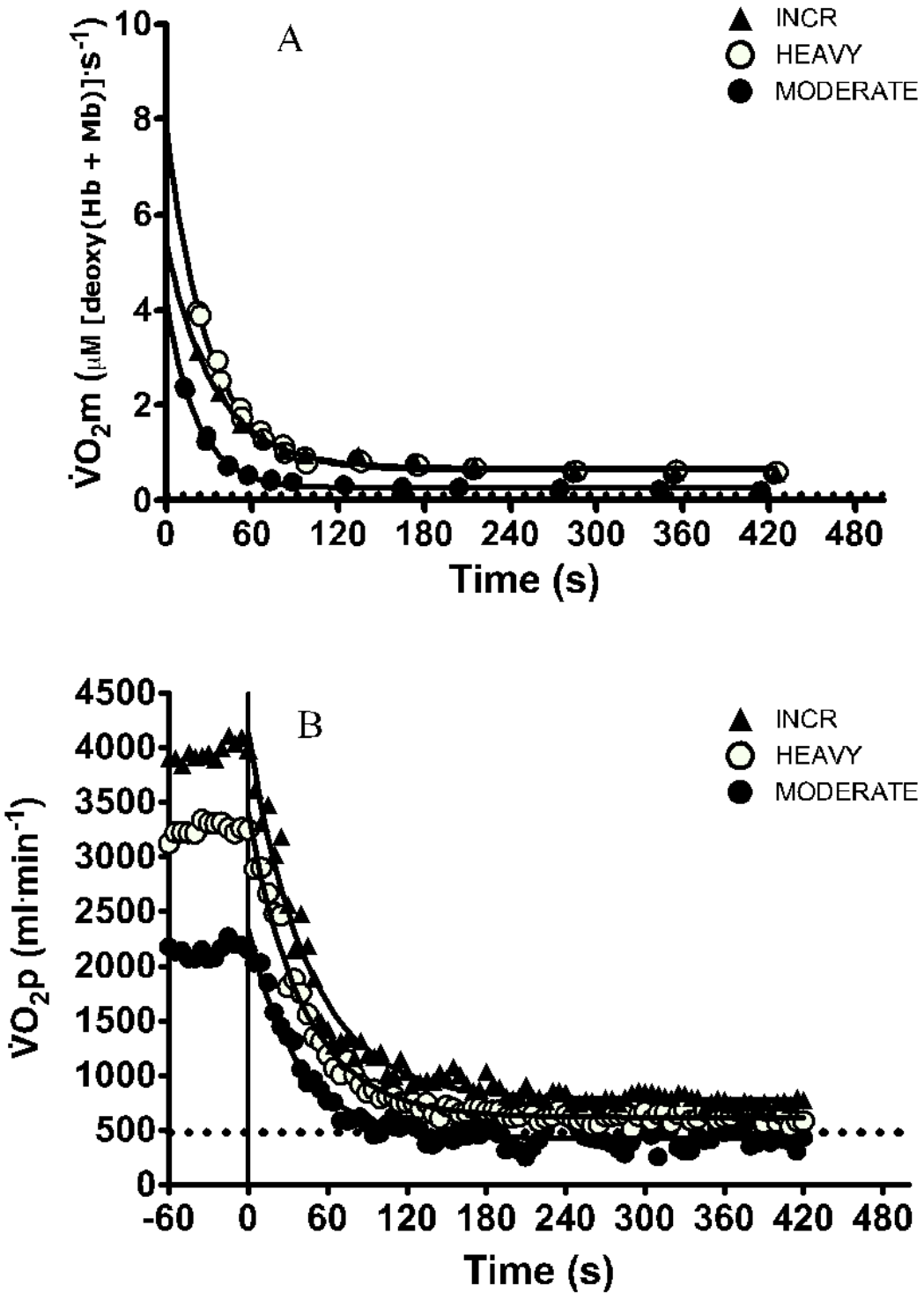

Figure 2. In panel A, muscle $\dot{V}O_2$ ($\dot{V}O_2m$) recovery kinetics for a representative subject following INCR, MODERATE and HEAVY exercises are shown. Panel B shows pulmonary $\dot{V}O_2$ ($\dot{V}O_2p$) recovery kinetics following INCR, MODERATE and HEAVY exercises for a representative subject. In both panels experimental data and fitted functions are shown. The dotted horizontal lines indicate the resting baseline values. See text for further details.

As mentioned above, following MODERATE and HEAVY each subject performed two repetitions of the protocol for $\dot{V}O_2m$ off- kinetics determination: individual test-retest reproducibility was moderate and good for MODERATE and HEAVY, respectively (interclass correlation coefficient [ICC] = 0.65; coefficient of variation [CV] = 43.5% for MODERATE and ICC = 0.76, CV = 29.9% for HEAVY). Corresponding Bland-Altman plot revealed mean bias of -2.76 s and a 95% confidence interval of -24.91, 19.38 s. Parameters of the $\dot{V}O_2p$ and $\dot{V}O_2m$ off- kinetics are reported in **Table 3**.

Table 3. Muscle ($\dot{V}O_2m$) and pulmonary O_2 uptake ($\dot{V}O_2p$) kinetics parameters determined in the recovery from incremental exercise (INCR) and constant work rate (CWR) exercises.

	MODERATE (95%CI)		**HEAVY (95%CI)**		**INCR (95%CI)**	
	$\dot{V}O_2$m (95%CI)	$\dot{V}O_2$p (95%CI)	$\dot{V}O_2$m (95%CI)	$\dot{V}O_2$p (95%CI)	$\dot{V}O_2$m (95%CI)	$\dot{V}O_2$p (95%CI)
TD, s	--	1.08±1.35 (0.33-1.84)	--	1.6±2.0 (0.47-2.58)	--	1.9±1.7 (0.93-2.84)
τ, s	29.1±6.8*# (25.3-32.9)	37.5±6.2# (34.0-40.9)	40.8±10.9 (34.8-46.8)	42.1±6.0 (38.8-45.2)	42.2±10.9 (36.2-48.2)	44.7±5.1 (41.8-47.5)
MRT, s	--	38.6±7.1# (34.7-42.5)	--	43.5±6.9 (39.7-47.4)	--	46.5±5.0 (43.8-49.3)
k, min^{-1}	2.16±0.45* (1.91-2.41)	1.64±0.25# (1.50-1.77)	1.58±0.44 (1.33-1.82)	1.45±0.22 (1.34-1.57)	1.51±0.38 (1.30-1.72)	1.36±0.15 (1.28-1.44)

Mean values ± SD. TD, Time delay; τ, time constant; MRT, mean response time; k, recovery rate constant. CI, confidence interval. *Significantly different (P<0.05) from corresponding value for $\dot{V}O_2$p; #P<0.05 vs HEAVY and INCR.

For both $\dot{V}O_2p$ off- and $\dot{V}O_2m$- off, τ and mean response time (MRT = τ + time delay) values were significantly lower in MODERATE (see statistical details in Table 3) *vs.* HEAVY and INCR, whereas no significant differences were observed between HEAVY and INCR. τ of $\dot{V}O_2m$ off- and $\dot{V}O_2p$ off- values are also presented in **Figure 3**. τ $\dot{V}O_2m$ off- was significantly lower (faster kinetics) than the τ $\dot{V}O_2p$ off- following MODERATE, whereas no significant differences were observed following HEAVY or INCR.

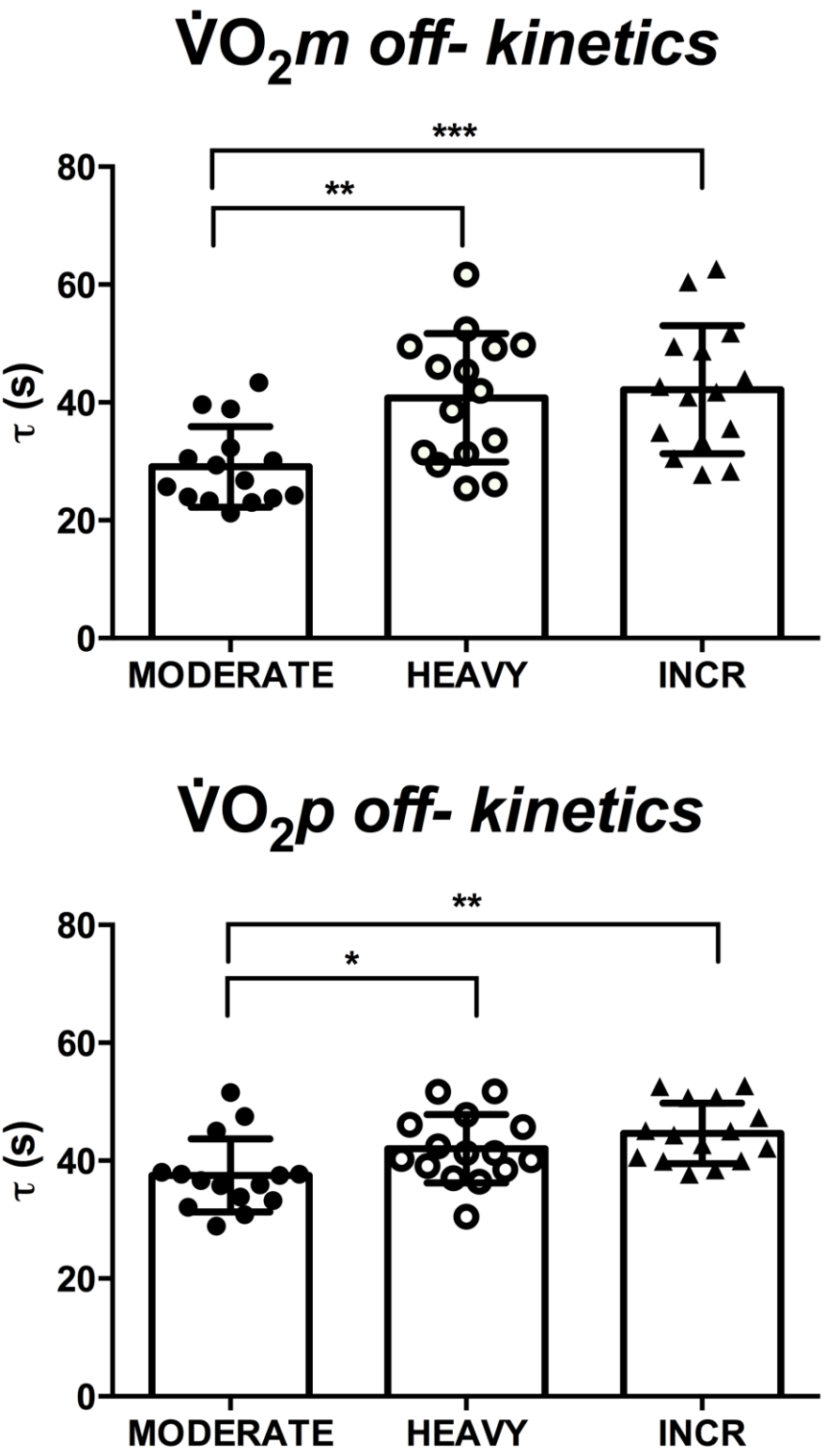

Figure 3. Time constant (τ) values (individual values and mean ± SD values) of muscle $\dot{V}O_2$ ($\dot{V}O_2m$) and pulmonary $\dot{V}O_2$ ($\dot{V}O_2p$) recovery kinetics following MODERATE, HEAVY and INCR. *Significant difference ($P<0.05$). See text for further details.

The same conclusions applied to k ($k = [1/\tau]$). A significant correlation between individual values of the τ $\dot{V}O_2p$ off- and the τ of $\dot{V}O_2m$ off- was observed following MODERATE, but not following HEAVY or INCR (**Figure 4**). In **Figure 4** the identity lines (y = x) are also shown. Following MODERATE, all experimental points (with the exception of one) lied above the identity line, confirming that the τ $\dot{V}O_2p$ off- overestimated the τ $\dot{V}O_2m$ off-. A significant correlation ($r = 0.43$, $p = 0.003$) between individual values of k of $\dot{V}O_2m$ off- and $\dot{V}O_2p$ peak values during INCR, HEAVY and MODERATE was found (**Figure 5**).

For $\dot{V}O_2p$ off- the asymptotic values of the monoexponential functions were higher in INCR (0.673 ± 0.076 L·min^{-1}) *vs.* HEAVY (0.516 ± 0.053 L·min^{-1}) and MODERATE (0.411 ± 0.061 L·min^{-1}), and were higher in HEAVY *vs.* MODERATE. The same trend was also observed for $\dot{V}O_2m$ off- values.

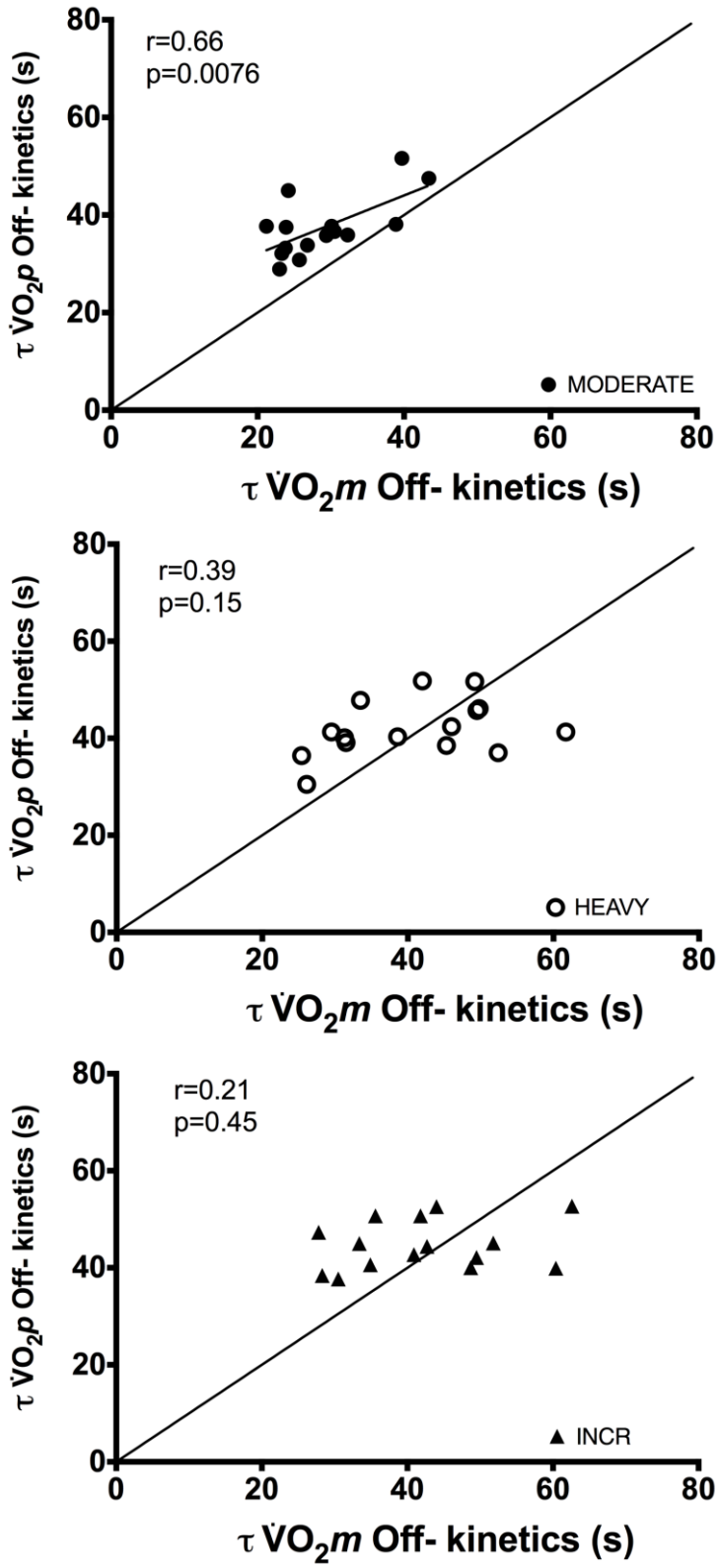

Figure 4. Individual values of the time constant (τ) of the pulmonary $\dot{V}O_2$ ($\dot{V}O_2p$) recovery kinetics as a function of the τ of the muscle $\dot{V}O_2$ ($\dot{V}O_2m$) recovery kinetics, following MODERATE, HEAVY and INCR. The identity lines (y = x) are also shown. Only following MODERATE a significant linear correlation (shown in the Figure) was observed between the two variables. See text for further details.

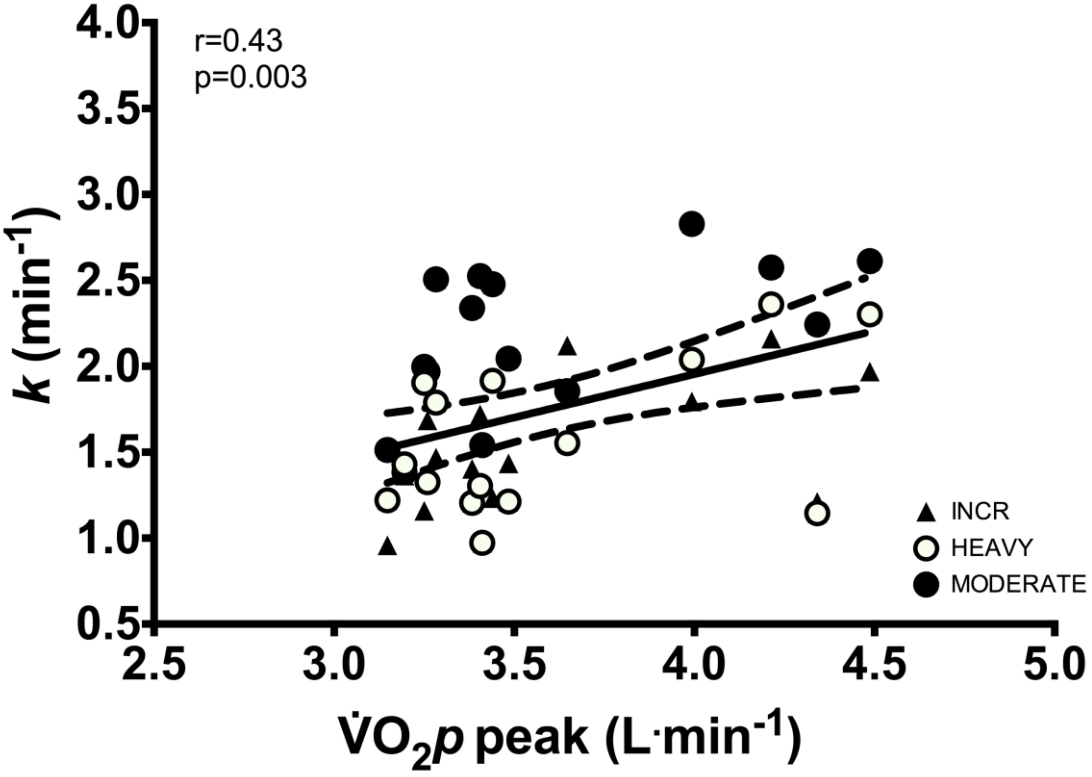

Figure 5. Individual values of the recovery rate constant (k) of muscle $\dot{V}O_2$ ($\dot{V}O_2m$) recovery kinetics following MODERATE, HEAVY and INCR as a function of pulmonary $\dot{V}O_2$ peak. Dashed lines indicate the 95% confidence intervals.

The kinetics of *vastus lateralis* deoxygenation (Δ[deoxy(Hb+Mb)]) off- kinetics in the leg which did not undergo the repeated occlusions protocol, fitted by a monoexponential function, were significantly faster following MODERATE (TD = 11.3 ± 4.9 s; τ = 22.3 ± 14.0 s; MRT = 33.6 ± 17.7 s) *vs.* following HEAVY (TD = 19.8 ± 8.1 s; τ = 48.5 ± 24.9 s; MRT = 68.3 ± 28.2 s) and INCR (TD = 17.2 ± 11.5 s; τ = 59.4 ± 25.8 s; MRT = 76.4 ± 30.8 s). No significant differences were observed between HEAVY and INCR. The MRT of the Δ[deoxy(Hb+Mb)] off- kinetics was not different from the τ of $\dot{V}O_2m$ off- following MODERATE, whereas is was substantially slower following HEAVY and INCR.

In **Figure 6** typical examples of the off- kinetics of Δ[deoxy(Hb+Mb)], Δ[oxy(Hb+Mb)] and Δ[oxy+deoxy(Hb+Mb)] obtained on the *vastus lateralis* muscles of the leg undergoing (upper panels) and not undergoing (lower panels) the repeated occlusions protocol are shown. As expected, in the leg undergoing the repeated occlusions, during each occlusion (vertical lines) an increase in Δ[deoxy(Hb + Mb)] and a decrease in the Δ[oxy(Hb + Mb)], with no significant change in Δ[oxy+deoxy(Hb+Mb)], were observed. The pattern of the three variables is typical for an increased fractional O_2 extraction, induced by the occlusion of blood flow. Interestingly, in the contralateral

leg, not undergoing the repeated occlusion protocol, in the period corresponding to the occlusions occurring in the contralateral leg Δ[deoxy(Hb+Mb)] decreased, whereas Δ[oxy(Hb+Mb)] and Δ[oxy+deoxy(Hb+Mb)] increased, following a pattern typical for vasodilation. In other words, the occlusions in one leg induced a reflex vasodilation in the contralateral leg.

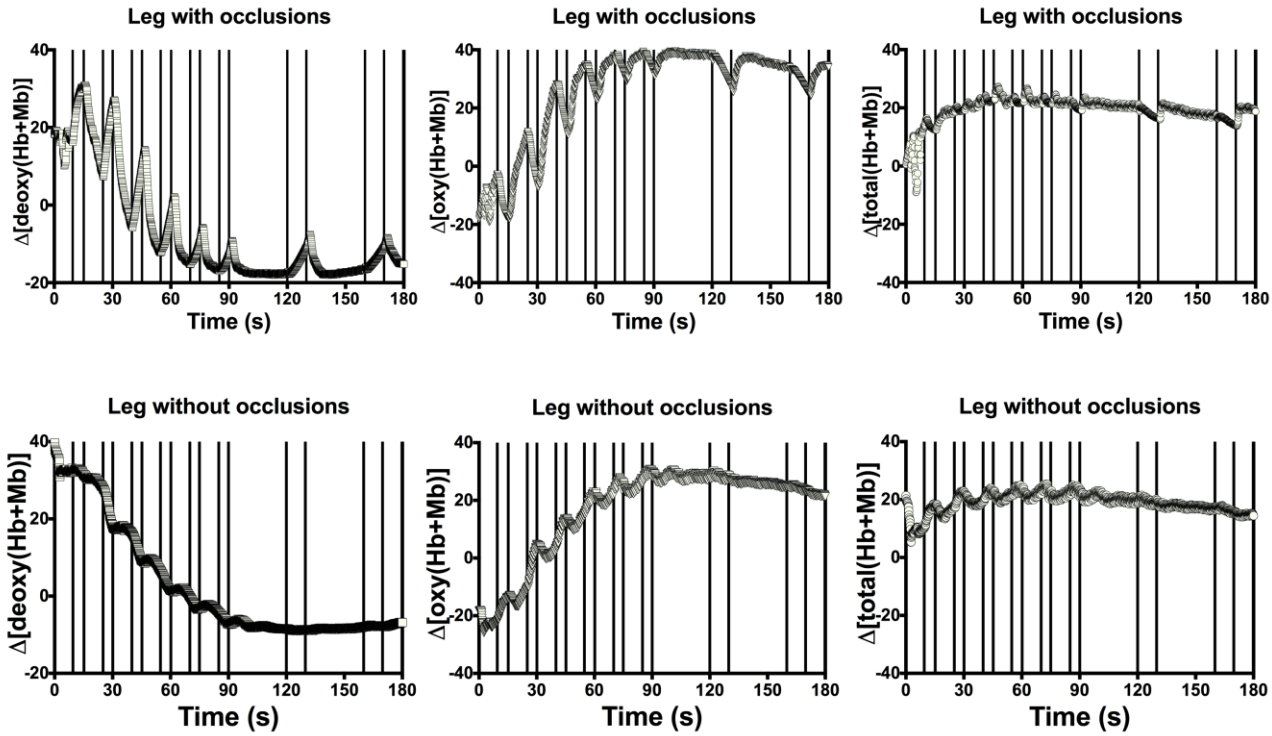

Figure 6. Typical individual examples of Δ [deoxy(Hb+Mb)], Δ [oxy(Hb+Mb)] and Δ [oxy+deoxy(Hb+Mb)] NIRS derived signals during the recovery from HEAVY exercise in the leg in which the ischemia protocol was performed (leg with occlusions) and in the leg in which the occlusions were not performed. The vertical lines indicate the beginning and the cessation of each period of ischemia. See text for further details.

DISCUSSION

To the best of our knowledge, this is the first study to analyze $\dot{V}O_2m$ off- kinetics by NIRS, by applying the repeated occlusions approach, during the recovery of constant work rate (CWR) cycle ergometer exercise carried out at moderate-intensity (MODERATE), at heavy-intensity (HEAVY), and during the recovery from an incremental exercise (INCR). The study demonstrates the feasibility of proposed approach and negates the need to perform a specific protocol of plantar flexion exercise, as proposed by Ryan et al. (2012) and by Adami and Rossiter (2018). In other words, $\dot{V}O_2m$ off-kinetics can be effectively determined following standard cycle ergometer exercises carried out for other purposes ($\dot{V}O_2p$ peak, GET, $\dot{V}O_2p$ on-kinetics evaluation). As discussed in the Introduction,

$\dot{V}O_2m$ off- kinetics represents a valuable functional evaluation tool of skeletal muscle oxidative metabolism, which can be utilized in normal subjects, athletes and in patient populations.

More specifically, the quality of the monoexponential fitting utilized for $\dot{V}O_2m$ off- kinetics analysis was excellent (r^2 values between 0.93 and 0.99). All subjects tolerated the repeated occlusions protocol without significant problems, even following the intense or exhaustive exercise, and the study had no drop-outs. Based on the ICC results, the test-retest reliability was moderate to good. The values of the time constant (τ) of the $\dot{V}O_2m$ off- kinetics were significantly lower (indicating a faster kinetics) following MODERATE compared to HEAVY and INCR. No significant differences were observed between τ values in HEAVY and INCR. The mechanisms responsible for the slower $\dot{V}O_2m$ off- kinetics at higher work rates could be similar to those responsible for the slow component of $\dot{V}O_2p$ on- kinetics: the recruitment of intrinsically slower fibers, in terms of oxidative metabolism; the presence of acidosis; reduced efficiency/fatigue; a relative lack of O_2 (Jones et al., 2012; Grassi et al., 2015). In the present study, however, no significant correlation was observed between the relative amplitude of the slow component of $\dot{V}O_2p$ on- kinetics and the difference in τ of the $\dot{V}O_2m$ off- kinetics determined following MODERATE and HEAVY. The individual values of skeletal muscle rate constant (*k*) correlated with $\dot{V}O_2p$ peak, confirming the data obtained by Wüst et al. (2013) in an animal model, as mentioned in the Introduction. The observation confirms the role of $\dot{V}O_2m$ off- kinetics as a functional evaluation tool of oxidative metabolism.

In terms of O_2 availability during the recovery phase, our study allows to make some indirect inferences. According to Adami and Rossiter (2018), adequate O_2 availability is a pre-requisite for a reliable functional evaluation of oxidative metabolism by the determination of the $\dot{V}O_2m$ off- kinetics. Following the recommendations by Adami and Rossiter (2018), in order to assure adequate O_2 availability the repeated occlusions protocol was initiated at specific percentages of the physiologic calibration (the range between Δ[deoxy(Hb + Mb)] at the end of the sustained arterial occlusion and the peak value reached during the reactive hyperemia, see Methods). Whereas the occlusions following MODERATE exercise were conducted under conditions of almost maximum O_2 availability (first occlusion occurring at approximately 10% of the distance between maximum oxygenation and maximum deoxygenation; the following occlusions at even lower percentages), for HEAVY and INCR the availability of O_2 was relatively minor (first occlusion at about 50% of the physiologic calibration range). According to Adami and Rossiter (2018), this percentage should correspond to an adequate O_2 availability, but no experimental data have been provided to support this concept. Thus, in strict terms it cannot be excluded that the slower $\dot{V}O_2m$ off- kinetics observed

following HEAVY and INCR, with respect to MODERATE could be attributable, at least in part, to a reduced availability of O_2. In any case, the exercise-intensity dependency of the τ of $\dot{V}O_2m$ off- observed in the present study underscores the need to quantify the absolute and relative (*i.e.* with respect to GET, critical power, $\dot{V}O_2p$ peak) intensity of the exercise preceding the recovery phase during which the $\dot{V}O_2m$ off- kinetics is determined. This quantification was impossible following the experimental approach proposed by Adami and Rossiter (2018) and by Ryan et al. (2012), whereas it was feasible by following the approach utilized in the present study.

Another methodological aspect which remained unresolved with the approach proposed by Ryan et al. (2018) and by Adami and Rossiter (2012) is the following: how much, in quantitative terms, was $\dot{V}O_2m$ increased (*vs.* rest) during the exercise preceding the recovery phase? The issue is critical, since, as suggested by Adami and Rossiter (2018), only in the presence of a significant activation of oxidative metabolism the determination of the $\dot{V}O_2m$ off- kinetics would represent a valid functional evaluation tool. The data of the present study allow to give an indirect answer to this question, based on two assumptions: (i) The back-extrapolation to time 0 of the monoexponential function describing $\dot{V}O_2m$ off- kinetics represents a reliable estimate of the $\dot{V}O_2m$ at the end of exercise. Considering the very precise fitting of $\dot{V}O_2m$ data, and since the adopted monoexponential functions substantially yield no time delay, the mentioned assumptions appear legitimate. (ii) $\dot{V}O_2m$ values obtained during the ischemia carried out with the subject in resting conditions (see Materials and Methods) represent a reliable estimate of the resting oxidative metabolism of the muscle. When it was calculated for MODERATE, HEAVY and INCR the ratio between the $\dot{V}O_2m$ extrapolated to the end of exercise and the resting $\dot{V}O_2m$ (horizontal dashed lines in **Figure 2a**), values equal to ~27, 35 and 38 were obtained. In other words, at the end of the exercise $\dot{V}O_2m$ values were ~25-40 times higher than at rest. These values appear to be compatible with literature data (Andersen & Saltin 1985; Bangsbo et al., 2000; Krustrup et al., 2009), and confirm the significant increase in oxidative energy expenditure during the exercise preceding the off- kinetics. The resting $\dot{V}O_2m$ values indicated by the dashed line in **Figure 2** allow us to make a further observation. At the end of the recovery phase considered in the present study (~7 minutes), the $\dot{V}O_2m$ values (see the asymptote of the function describing $\dot{V}O_2m$ off- kinetics) were still significantly higher than the $\dot{V}O_2m$ values at rest. In other words, a very slow component of the $\dot{V}O_2m$ -off kinetics was presumably present, and it could not be considered by our analysis (Margaria et al., 1933).

By utilizing the velocity constant *k* as the parameter to evaluate the $\dot{V}O_2m$ off- kinetics, we were able to compare the data obtained in the present study, following the three investigated work rates (2.16

min^{-1} following MODERATE, 1.58 min^{-1} following HEAVY, 1.51 min^{-1} following INCR), with literature data obtained in different populations by utilizing the plantar flexion exercise protocol (see Introduction), and summarized by Adami and Rossiter (2018). Since $k = 1/\tau$, a faster kinetics is indicated by a lower value of τ (time constant) or by a higher value of k (velocity constant). The k data of the present study (see above) substantially correspond to the higher (MODERATE) and lower (HEAVY and INCR) ends of the spectrum for "normal" subjects reported by Adami and Rossiter (2018). As expected, the $\dot{V}O_2m$ off- kinetics of the present study were significantly slower than those observed in endurance athletes (Brizendine et al., 2013), and significantly faster than those observed in patient populations (chronic heart failure [Southern et al., 2015; Zamani et al., 2015], spinal cord injury [Erickson et al., 2013; Erickson et al., 2017], chronic obstructive pulmonary disease [Adami et al., 2017a; Adami et al., 2017b]). As mentioned above, our data underscore the exercise intensity dependency of the $\dot{V}O_2m$ off- kinetics. On the other hand, an exercise intensity dependency could not be identified by the plantar flexion protocol proposed by Ryan et al. (2012) and by Adami and Rossiter (2018), in which exercise intensity cannot be quantified.

A slightly slower $\dot{V}O_2m$ off- kinetics following sprint *vs.* moderate running was described by Buchheit et al. (2011) by utilizing an experimental approach similar to that of the present study. On the other hand, no difference in $\dot{V}O_2m$ off- kinetics, determined by a different method (invasive measurements and Fick equation to calculate $\dot{V}O_2m$ across the exercising muscles), was described by Krustrup et al. (2009) following moderate- *vs.* heavy-intensity knee extension exercise. Rossiter et al. (2002) observed no difference in τ for the PCr off- kinetics (considered a close proxy of $\dot{V}O_2m$ - off, see Introduction) following moderate- *vs.* heavy-intensity knee-extension exercise. After considering that following heavy-intensity exercise a slow component of PCr off- was observed in the study by Rossiter et al. (2002), the results of the present study (slower $\dot{V}O_2m$ off- following heavy-intensity exercise) appear in substantial agreement with those obtained by Rossiter et al. (2002). Ryan et al. (2013) observed no differences in $\dot{V}O_2m$ off- kinetics (NIRS + repeated occlusions, as in the present study) following plantar-flexion exercises carried out with increasing contraction frequencies; the difference with the results of the present study could relate, at least in part, with the very short duration (15 s) of the exercise employed by Ryan et al. (2013), which obviously precluded the reaching of a steady-state for $\dot{V}O_2m$.

Regarding the $\dot{V}O_2p$ off- kinetics, they followed a pattern similar to that described above for $\dot{V}O_2m$ off-: the kinetics were faster following MODERATE *vs.* following HEAVY or INCR, with no significant difference between these last two conditions. As far as the comparison between the $\dot{V}O_2m$ off- and the $\dot{V}O_2p$ off- kinetics, no significant differences were described following HEAVY and

INCR, whereas following MODERATE the $\dot{V}O_2m$ off- kinetics was faster. On the other hand, only following MODERATE a significant correlation between the τ of $\dot{V}O_2p$ off- and the τ of $\dot{V}O_2m$ off- was observed. In other words, following MODERATE the τ of $\dot{V}O_2p$ off- was correlated with, but overestimated, the τ of $\dot{V}O_2m$ off-. Following HEAVY and INCR no correlations between the two variables were observed. Thus, following all exercise intensities the $\dot{V}O_2p$ off- kinetics cannot be utilized as a proxy for the $\dot{V}O_2m$ off- kinetics. This confirms the conclusions by the study of Krustrup et al. (2009), in which $\dot{V}O_2m$ off- kinetics were determined, by a different method, following knee-extension exercise. The mechanisms responsible for the discrepancies between the two kinetics are likely attributable to the influence of cardio-circulatory adjustments (cardiac output is exponentially decreasing in the period taken into consideration by the off- kinetics analysis) and/or of changes in O_2 stores between skeletal muscles and the subject's mouth. The faster of $\dot{V}O_2m$ off- compared to the $\dot{V}O_2p$ off- kinetics described in the present study following MODERATE confirms the observations by Krustrup et al. (2009) following knee-extension exercise.

As illustrated in **Figure 6**, during the recovery following exercise ischemia applied to one leg induced a reflex vasodilation in the contralateral leg. To the best of our knowledge, this represents a novel observation. It has been described before that voluntary contraction in another limb (Ishii et al., 2012), mental stress (Blair et al., 1959), immobile alerting and fighting behavior, imagery of voluntary exercise (Ishii et al., 2012) induce vasodilation and increase blood flow in another limb. The mechanism(s) responsible for the metaboreflex observed in the present study are not clear. Increased shear stress and increased nitric oxide (NO) and prostacyclin release by endothelial cells (Shoemaker et al., 2015) must be excluded, since the metabolic signal(s) arose from an ischemic maneuver. β_2-mediated vasodilatory effects, as a result of adrenaline release by the adrenal medulla (Shoemaker et al., 2015), must be excluded as well, since the vasodilation occurred almost immediately during the ischemia in the other limb. It can be hypothesized that metabolic signals induced by the ischemia in one limb, superimposed on metabolic signals associated with the recovery phase following exercise, reached the cardiovascular control center through group III and IV afferent fibers. The efferent arm of the metaboreflex, responsible for the neurogenic vasodilation, is less clear. Conflicting evidence is present in the literature in favor or against the occurrence of a withdrawal of sympathetic vasoconstriction (see the discussion in Ishii et al., 2012). The idea of a sympathetic cholinergic vasodilation (presumably involving nitric oxide) has been around for decades (see Joyner & Dietz 2003; Shoemaker et al., 2015), and would be supported by the observation that the initial vasodilation in the non-exercising limb is blocked by atropin but not by propanolol (Sanders et al., 1989). A strong argument against this possibility, however, lies in the lack of anatomical or histochemical evidence

for any cholinergic neural pathway in human skeletal muscles (Joyner & Dietz 2003; Shoemaker et al., 2015). In any case, the metaboreflex was present only transiently, since it disappeared after about 90 s into the recovery (see **Fig. 6**).

In conclusion, $\dot{V}O_2m$ off- kinetics determination by the NIRS repeated occlusions approach, carried out following standard cycle ergometer exercise at different intensities, is a feasible and useful functional evaluation tool for skeletal muscle oxidative metabolism. With respect to the original approach (plantar flexion exercise) proposed by Ryan et al. (2012) and by Adami and Rossiter (2018), the approach proposed in the present study can be applied during the recovery following standard cycle ergometer exercises conducted for the evaluation of other relevant variables of oxidative metabolism ($\dot{V}O_2p$ peak, gas exchange threshold, critical power, $\dot{V}O_2p$ kinetics), without the need of performing an additional protocol.

Acknowledgements

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3.5 PERIPHERAL IMPAIRMENTS OF OXIDATIVE METABOLISM AFTER A 10 - DAY BED REST ARE UPSTREAM OF MITOCHONDRIAL RESPIRATION. PRELIMINARY RESULTS – *STUDY 5*

ABSTRACT

Exposure to simulated microgravity by bed rest (BR), leads to an impairment of oxidative metabolism. The sites of this impairment are still debated. Aim of this study was to identify markers of impaired oxidative metabolism along the O_2 pathway, from ambient air to skeletal muscle mitochondria, following 10 days of BR. Methods: Before (PRE) and after (POST) 10 days of horizontal BR, ten recreationally active young males (age 23 ± 5 years [mean±SD]) performed on a cycle ergometer an incremental exercise (INCR) up to exhaustion (to determine peak pulmonary $\dot{V}O_2$ [$\dot{V}O_2p$] and the gas exchange threshold [GET]) and two repetitions of constant work-rate (CWR) exercises at 80% of GET (MOD). $\dot{V}O_2p$ and *vastus lateralis* muscle fractional O_2 extraction by near-infrared spectroscopy (NIRS) (Δ[deoxy(Hb±Mb)]) were recorded continuously. Peripheral vascular and endothelial functions were evaluated by the blood flow response (Doppler ultrasound) in the femoral artery during 1-min passive leg movements (PLM). Mitochondrial respiration was evaluated: (i) *ex vivo* by high-resolution respirometry on permeabilized *vastus lateralis* fibers obtained by biopsy and (ii) non-invasively *in vivo* by NIRS by calculating skeletal muscle $\dot{V}O_2$ ($\dot{V}O_2m$) recovery kinetics (repeated transient occlusions method) following MOD. The time constants (τ) of the monoexponential $\dot{V}O_2m$ kinetics during the first 7 minutes of recovery were determined. Results: Peak $\dot{V}O_2p$ was lower (P=0.001) in POST (41.5 ± 6.5 ml.kg-1.min-1) *vs.* PRE (44.5 ± 7.4). The area under the blood flow *vs.* time curve during PLM was lower (P=0.038) in POST (274 ± 233 mL) *vs.* PRE (427 ± 291). Skeletal muscle citrate synthase activity, an estimate of mitochondrial mass, was not different (P=0.115) in POST (131.2 ± 15.9 $mU.mg^{-1}$ protein) *vs.* PRE (137.9 ± 18.8). Maximal ADP stimulated mitochondrial respiration (66.4 ± 17.5 $pmol{\cdot}s^{-1}{\cdot}mg^{-1}$ wet weight [POST] *vs.* 72.3 ± 14.0 [PRE], P=0.127) and oxidative phosphorylation coupling efficiency (respiratory control ratio, 4.10 ± 1.19 [PRE] *vs.* 3.59 ± 1.11 [POST], P=0.443) were not affected by BR. τ of $\dot{V}O_2m$ recovery was not different (P=0.079) in POST (22.2 ± 5.9 s) *vs.* PRE (21.7 ± 5.7). Conclusions: These preliminary data suggest that the whole-body impairment of oxidative metabolism during exercise, following 10 days of horizontal BR, is associated with an impairment of peripheral vascular and endothelial functions whereas mitochondrial volume and maximal respiratory function are unaffected. Funding: ASI, MARS-PRE Project, n. DC-VUM-2017-006.

INTRODUCTION

It is well established that prolonged inactivity affects almost all the physiological systems (Teasel & Dittmer, 1993). Bed rest studies offer a unique opportunity to evaluate the effects of prolonged muscle disuse and unloading, conditions regularly experienced in patients with injuries, chronic disease or in astronauts during spaceflight missions in microgravity. Exposure to microgravity or prolonged physical inactivity lead to impairment of oxidative metabolism. However, the sites of this impairment are still debated. Whereas cardiovascular impairments associated with (or responsible for) the decreased peak pulmonary oxygen uptake ($\dot{V}O_2peak$) usually detected following bed rest or spaceflights have been well described (Ade et al., 2015; Capelli et al., 2009), more peripheral impairments have been relatively less observed. In this regard, Capelli et al., (2016) and Ferretti al., (2009) utilizing the multifactorial model of $\dot{V}O_2peak$ limitation, originally developed by Di Prampero and Ferretti (1990), arrived at the conclusion that the fractional limitation imposed by peripheral factors was about 30% after 42 days of bed rest and about 40% after 90 days. More recent investigations reinforced the peripheral factors as contributors to decreased $\dot{V}O_2peak$ (Ade at al., 2015).

Aim of the present study was to identify early functional biomarkers that indicate changes in oxidative metabolism during exercise following a 10-day exposure to microgravity, with a particular interest to the peripheral level.

METHODS

Ethical approval

The study was part of the Italian Space Agency (ASI) project "MARS-PRE Bed Rest SBI 2019". It was approved by the ethical committee and was performed in accordance with the standard set by the Helsinki declaration. All participants were informed about the aims, procedures and potential risks of the investigations before written consent was obtained.

Subjects

Ten young healthy, recreationally active males (age, 23±5 yr; height, 1.81±0.04 m; weight, 78±10 kg; body mass index, 23.5±2.5 $kg \cdot m^{-2}$) participated in this study. Participants' characteristics at baseline are given in **Table 1**. Subjects underwent a medical screening before being considered for this study.

Experimental protocol

Each subject was evaluated before (PRE) and after (POST) 10-day of strict horizontal bed rest (BD) without countermeasures. The experiments were carried out at Izola General Hospital, Slovenia. Participants arrived at the hospital 3 days before BD and immediately after the pre-measurements

were finished, they entered the BD. Measurements after BD were carried out during the first 2 days after subjects arose from bed. During BD intervention no deviation from lying position, muscle stretching, or static contraction were allowed. Adherence to the assigned protocol was ensured using continuous closed-circuit television surveillance and constant supervision by researchers and medical staff. Subjects consumed an individually tailored, standardized diet and were allowed to drink water ad libitum. All tests were conducted under close medical supervision and following standard safety procedures. Exercise tests were carried out in a well-ventilated laboratory at 19–21 °C, under continuous medical supervision and 12-lead electrocardiography (ECG; Custo Med GmbH). Each subject completed the entire experimental protocol.

Before data collection, subjects familiarized with the investigators, experimental arrangement, and with the exercise protocols by means of short preliminary practice runs. For the purposes of the present study, subjects performed during day 1 a *passive leg movement* (PLM) and an *incremental exercise* (INCR) up to voluntary exhaustion on an electronically braked cycle ergometer (ErgoMedic 839 E; Monark). For PLM, measurements were made according to the recently provided guidelines (Gifford & Richardson 2017; Limberg et al., 2020). The subjects remained seated with their legs extended and supported for 15 min before the data collection. Resting doppler data were recorded for 2 min followed by 60 s of cyclical passive knee extension and flexion. The movements were performed across 90° range of motion (180°-90°-180°) at 1 Hz. The same trained researcher manually moved the subjects' leg. The protocol was repeated after 20 minutes of recovery.

Pedaling frequency was digitally displayed to the subjects, who were asked to keep a constant cadence throughout the tests between 70 and 90 rpm. Voluntary exhaustion was defined as the incapacity to maintain the imposed load and pedaling frequency despite vigorous encouragement by the researchers. The protocol began after an initial 1 minute of unloading pedaling (0-40 W) with a power output increasing by 20-40 W every minute. The peak values of the main cardiovascular, respiratory and metabolic parameters were taken as the highest 20-s mean values attained prior the subject's voluntary exhaustion. The $\dot{V}O_2$ at the gas exchange threshold (GET) was determined by one or two independent investigators by utilizing the "V-slope" methods and the "secondary criteria" (Beaver et al., 1986). In order to identify the work rate corresponding to the $\dot{V}O_2$ at GET, the effect of the delayed $\dot{V}O_2$ adjustment to the increased work rate during the incremental test was corrected by shifting the linear $\dot{V}O_2$ *vs.* time (and work rate) relationship to the left, by an amount corresponding to the individual mean response time of the $\dot{V}O_2$ kinetics determined in each subject (Whipp et al., 1981). At day 2, two repetition of 6 minutes *constant work rate (CWR) exercise* (MODERATE) of moderate intensity were performed at 80% of GET previously determined during INCR.

Each repetition was separated by 10 to 15 minutes recovery period. CWR exercises were carried out at the same absolute work rate in PRE and POST. Both before and after BR, experiments were conducted on 2 consecutive days: INCR during day 1 and MODERATE during day 2. At day 3, at the beginning of bed rest, muscle biopsies were taken from vastus lateralis.

MEASUREMENTS

Anthropometry

Body mass (BM), regional and whole body composition were assessed before and immediately after the campaign with dual-energy X-ray absorptiometry (DEXA) using a fan-beam densitometer (Discovery W – QDR series; Hologic, Marlborough, MA, USA). Skinfold measurements were made by a caliper at the site of placement of the NIRS probe on the vastus lateralis muscle, aiming to estimate skin and subcutaneous adipose tissue thickness.

Cardiopulmonary and metabolic variables

Pulmonary ventilation ($\dot{V}E$), $\dot{V}O_2$ and CO_2 output ($\dot{V}CO_2$) were determined breath-by-breath by a metabolic cart (Quark PFTergo, Cosmed, Rome, Italy). Expiratory flow measurements were performed by a turbine flow meter calibrated before each experiment by a 3 L syringe at different flow rates. $\dot{V}O_2p$ and $\dot{V}CO_2$ were determined by continuously monitoring PO_2 and PCO_2 at the mouth throughout the respiratory cycle and from established mass balance equations. Calibration of O_2 and CO_2 analyzers was performed before each experiment by utilizing gas mixtures of known composition. Gas exchange ratio (R) was calculated as $\dot{V}CO_2/\dot{V}O_2p$. HR was determined from the electrocardiogram signal and using a heart rate monitor (RS 400; Polar, Kempele, Finland).

Stroke volume (SV) was estimated beat-by-beat by means of transthoracic impedance cardiography (Physio Flow; Manatec Biomedical) and averaged every 10 beats during all exercise tests. The accuracy of this device has been previously evaluated during incremental exercise in healthy subjects against the direct Fick method (Richard et al., 2001). Heart rate was obtained from R-R interval determined on the ECG first derivative. Cardiac output (CO) was then calculated by multiplying stroke volume with heart rate. Blood pressure (BP) was measured using a standard cuff sphygmomanometer. At 1, 3, and 5 minutes of recovery, 20 µL of capillary blood were obtained from a preheated earlobe for the determination of blood lactate concentration ($[La]_b$) by an enzymatic method (Biosen C-line; EKF). Ratings of perceived exertion (RPE) were obtained every minute during exercise using the Borg's 6-20 scale (Borg, 1982).

Skeletal muscle oxygenation

Oxygenation changes in a superficial portion of vastus lateralis muscle of the dominant limb were evaluated by near-infrared spectroscopy (NIRS) (Grassi & Quaresima, 2016; Barstow, 2019).

A portable near-infrared continuous-wave instrument (OctaMon M; Artinis Medical Systems) was used in this study. One light transmitters/channels (which emitted 2 wavelengths of 760 and 850 nm), separated by 35 mm from the respective receiving optode. The instrument measures non-invasively micromolar (μM) changes in oxygenated hemoglobin (Hb) + myoglobin (Mb) concentrations (Δ[oxy(Hb+Mb)]), and in deoxygenated [Hb+Mb] (Δ[deoxy(Hb+Mb)]) with respect to an initial value arbitrarily set equal to zero and obtained during the resting condition preceding the test. The sum of the two variables (Δ[total(Hb+Mb)]) reveals changes in the total Hb+Mb volume in the muscle region of interest. An increased Δ[deoxy(Hb+Mb)] or a decreased Δ[oxy(Hb+Mb)], would indicate an increased fractional O_2 extraction in the in the tissue under consideration (Grassi & Quaresima, 2016) only if (Δ[total(Hb+Mb)]) is constant, which is not always the case in exercising skeletal muscles. In previous studies (see e.g. Refs. Ferreira et al. 2007, Porcelli at al. 2010, Salvadego et al. 2016; Salvadego et al. 2018) the problem was partially overcome, by considering changes in [deoxy(Hb+Mb)], which unlike (Δ[oxy(Hb+Mb)]) are relatively less influenced by changes in blood volume, and which have been considered an estimate of fractional O_2 extraction (Grassi & Quaresima, 2016). However, in the present study, the problem was solved by correcting NIRS signals for changes in blood volume, as proposed by Ryan et al. (2012). Before the exercise period a 5-min ischemic calibration (physiological normalization) was performed by inflating a pressure cuff (~300 mmHg) positioned at the inguinal crease of the thigh. The probe was firmly attached to the skin overlying the lower third of vastus lateralis muscle of the dominant limb. The skin overlying the investigated muscle regions was carefully shaven before the experimentation, and the places where the probes were attached were recorded using a skin marker, thereby positioning the probe in a similar position during all the tests. Adipose tissue thicknesses (ATT) at the site of application of the NIR probe were estimated by a caliper (Gima, Milan, Italy). Furthermore, black clothes were put around the probes and the skin to prevent contamination from ambient light. The sampling frequency was set at 10 Hz.

Femoral artery blood flow

Blood flow in the common femoral artery was estimated by measurements of blood flow velocity and vessel diameter distal to the inguinal ligament, 2.0-2.5 cm proximal to the bifurcation of the superficial and deep femoral artery using an ultrasound system (Vivid IQ, General Electric Medical Systems, Milwaukee, WI, USA) with a linear array transducer operating at the imaging frequency of 9 MHz. Two-dimensional measurements of the arterial lumen were made from B-mode image in longitudinal view. Measurements of the vessel diameter were taken at the same time point in the cardiac cycle (peak of the R wave derived from the integrated ECG system). Blood flow velocities were collected with the sample volume covering more than 75% of the arterial lumen, and with the insonation angle always kept <60°. Arterial blood flow was automatically calculated second by

second by commercially available software multiplying arterial cross-sectional area mean blood flow velocity.

Mitochondrial respiration *in vivo*.

$\dot{V}O_2m$ was estimated by calculating the slope of the initial linear increase (3 s) in NIRS-measured Δ[deoxy(Hb + Mb)] during short (5 s) bouts of ischemia induced by rapid (less than 1 s) inflation and deflation of a pneumatic cuff (Hokanson E20 cuff inflator, Bellevue, WA, USA) during the recovery from constant work rate exercises. A repeated arterial occlusion method (see Ryan et al., 2014, Adami & Rossiter 2018, Zuccarelli et al., 2020) was carried out at the end of each MODERATE exercise. When muscle reached a desaturation target of 50% of the physiological normalization (Adami et al., 2017), several intermittent arterial occlusions were performed: the first 5 occlusions lasting 5 s each were separated by 5 s, other 5 occlusions lasting 5 s each were separated by 10 s and finally the last 5 occlusions lasting 5 s were separated by 20 s. When the target 50% was not reached at the end of the exercise protocol the first arterial occlusion was performed after 5-10 s.

$\dot{V}O_2m$ values were then fit by a monoexponential function according to equation 1 (Ryan et al., 2014):

$$y(t) = y_{END} - \text{Delta} \times e^{-1/\tau} \qquad (1)$$

where y(t) represents the value of $\dot{V}O_2m$ at a given time (t), y_{END} the $\dot{V}O_2m$ immediately after the cessation of the exercise, Delta is the change in $\dot{V}O_2m$ from rest to end exercise and τ is the fitting rate constant ($k = [1/\tau]$ expressed in min^{-1}). Resting $\dot{V}O_2m$ values were estimated by the same approach, described above, on the data obtained during the first 60 s of the physiologic normalization procedure (see above).

Mitochondrial respiration *ex vivo*.

Skeletal muscle biopsies were obtained from the vastus lateralis muscle under local anaesthetic (2% lidocaine). The biopsy was taken immediately before bed rest intervention and after the bed rest period. Following the application of the anaesthetic, a 1.0-1.5 cm incision was made to the skin, subcutaneous tissue and muscle fascia, and the tissue sample was harvested with a Rongeur-Conchotome (GmbH&Co, Zepf Instruments, Dürbheim - Germany). The collected muscle tissue was dissected free of fat and connective tissue and rapidly divided in several portions. One portion (15-20 mg wet weight) was immediately frozen in liquid nitrogen and stored at -80°C until determination of citrate synthase (CS) activity (see below). Another small portion (2.0-6.5 mg ww) was then used immediately to evaluate mitochondrial respiration *ex vivo* (Pesta and Gnaiger, 2012). Measurements were performed in duplicate. The small portion of tissue was immediately placed in an ice-cold

preservation solution (BIOPS; Oroboros Instruments, Innsbruck, Austria) (4°C) containing: EGTA-calcium buffer (10 mM) (free Ca2+ concentration 100 nmol L^{-1}), imidazole (20 mM), taurine (20 mM), K+/4 morpholinoethanesulphonic acid (50 mM), dithiothreitol (0.5 mM), MgCl2 (6.56 mM), ATP (5.77 mM) and phosphocreatine (15 mM) (pH 7.1). Fiber bundles were trimmed from the connective and fatty tissue excess (if present) and separated with sharp-ended needles under magnification (70 x) (Stereomicroscope CRYSTAL-PRO, Konus-optical & sports systems, Italy). After this, fibers bundles were incubated into 2 mL of BIOPS containing 20 $\mu g \cdot ml^{-1}$ saponin for 30 min at 4°C (Kuznetsov et al., 2003) with continuous gentle stirring to ensure complete permeabilization. Samples were washed with the respiration medium (MIR05; Oroboros Instruments, Innsbruck, Austria) containing 0.5 mM EGTA, 60 mM potassium lactobionate, 3 mM MgCl2 6H2O, 20 mM taurine, 10 mM KH2PO4, 20 mM Hepes, 110 mM sucrose and 1 g L^{-1} BSA, pH 7.1, weighed in a balance-controlled scale (Shimatzdu) Therefore permeabilized fibers were measured for wet weight and immediately transferred into the respirometer (Oxygraph-2k Oroboros Instruments) chambers for O_2 consumption analysis. Mitochondrial respiratory function was evaluated by measuring O_2 consumption polarographically by high resolution respirometry (Pesta & Gnaiger, 2012). Data were digitally recorded using DatLab4 software (Oroboros Instruments). The instrumentation allows for O_2 consumption measurements with small amounts of sample in closed respiration chambers containing 2 mL of air-saturated respiration medium (MIR06; MIR05 + catalase 280 $IU \cdot mL^{-1}$) at 37 °C. Standardized instrumental and chemical calibrations were performed to correct for back-diffusion of O_2 into the chamber from the various components (e.g. leak from the exterior, O_2 consumption by the chemical medium and by the sensor O_2) (Pesta & Gnaiger, 2012). The O_2 concentration in the chamber was maintained between 300 and 400 µM (average O_2 partial pressure 250 mmHg) to avoid O_2 limitation of respiration. Intermittent reoxygenation steps were performed during the experiments by injections of 1-3 µl of 0.3 mM H_2O_2, which was instantaneously dismutated by catalase, already present in the medium, to O_2 and H_2O. Experiments were performed in the presence of the myosin II-ATPase inhibitor (Blebbistatin, 25 µM, dissolved in DMSO 5mM stock) (Perry et al. 2011) in order to prevent spontaneous contraction in the respiration medium.

A substrate-uncoupler-inhibitor-titration protocol, with a substrate combination that matches physiological intracellular conditions, was applied (Salvadego et al. 2013; Pesta & Gnaiger, 2012; Salvadego et al. 2016). Non-phosphorylating resting mitochondrial respiration was measured in the presence of malate (4 mM) and glutamate (10 mM) and in the absence of adenylates, so that O_2 consumption was mainly driven by the back leakage of protons through the inner mitochondrial membrane (“leak” respiration).

Succinate (10 mM) was added to support convergent electron flow into the Q-junction through complexes I and II. This was followed by submaximal titration of ADP (12.5, 25, 175, 250, 500, 1000, 2000, 4000, 6000, 8000, 10000 µM) to assess complex I+II-linked ADP sensitivity and maximal oxidative phosphorylation (OXPHOS) capacity.

Cytochrome C (10 µM) was added to test mitochondrial outer membrane integrity. The addition of cytochrome C had no significant additive effects on respiration, with minor increases of <5%, thereby confirming the integrity of the outer mitochondrial membrane. Maximal electron transport system capacity was measured by stepwise additions of chemical uncoupler protonophore carbonylcyanide-p trifluoromethoxyphenylhydrazone (FCCP). Rotenone (1 µM) and anti-mycin A (2.5 µM) were added to inhibit complexes I and III, providing a measure of residual O_2 consumption, indicative of non-mitochondrial O_2 consumption. Mitochondrial respiration indices were then corrected for O_2 flux resulting from residual O_2 consumption. The degree of coupling of oxidative phosphorylation for a specific substrate supply (glutamate and malate and succinate) was determined by calculating the ratio between state 3 respiration minus leak respiration and state 3 respiration [(OXPHOS-leak) / OXPHOS] (Pesta & Gnaiger 2012). The obtained values were also normalized by citrate synthase activity (see above), taken as an estimate index of mitochondrial mass (Jacobs et al., 2013).

Citrate synthase activity

In order to carry out citrate synthase activity, muscle samples were thawed and underwent a motor driven homogenization in a pre-cooled 1 ml glass-glass potter (Wheaton, USA). The muscle specimen was suspended 1:50 w/v in a homogenization buffer containing sucrose (250 mM), Tris (20 mM), KCl (40 mM) and EGTA (2mM) with 1:50 v/v protease (P8340-Sigma) inhibitors. The specimen was homogenised in an ice-bath with 20 strokes at 500 rpm, but before the last hit, Triton X-100 (0.1% v/v) was added to the solution. After this, the sample was left in ice for 30 minutes. The homogenate was centrifuged at 13000 rpm for 10 minutes in order to discard cellular debris. The supernatant was used to evaluate protein concentration according to method of Lowry (Lowry et al., 1951). 5-10-15 µg of protein extracts were added to each well of a 96-well-microplate along with 100 µl of 200 mM Tris, 20 µl of 1 mM 5, 5'-dithiobis-2-nitrobenzoate (DTNB) freshly prepared, 6µl of 10 mM acetyl-coenzyme A (Acetyl-Co-A) and mQ water to a final volume of 190 µl. A background ΔAbs, to detect any endogenous activity by acetylase enzymes, was recorded for 90 seconds with 10 seconds interval at 412 nm at 25°C by an EnSpire 2300 Multilabel Reader (PerkinElmer). The ΔAbs was subtracted from the one given after the addition of 10 µl of 10 mM oxalacetic acid that started the reaction. All assays were performed at 25 °C in triplicate on homogenates. Activity was expressed as mU

(nanomoles/min) per mg of protein. This protocol was modified from (Srere, 1969; Spinazzi et al., 2012).

Statistical analysis

All data are presented as mean ± SD. Statistical significance of differences between POST and PRE was checked by two-tailed Student's *t*-test for paired data. Apparent Km (Michaelis constant) values were determined using a double exponential model and then the [ADP] at 50% of the Complex I-II linked respiration was extrapolated. The level of significance was set at $P < 0.05$. Statistical analyses were carried out with a commercially available software package (Prism 6.0; GraphPad).

RESULTS

Anthropometric and body composition characteristics are reported in **Table 1**. BM and BMI decreased by ~2% after bed rest. Skin and adipose tissue thickness measured at the site of NIRS probe ranged between 8.0 and 16.8 mm and it was reduced after bed rest (P=0.03).

Table 1. Anthropometric characteristics and age of participants before (PRE) and after (POST) a 10-day horizontal bed rest.

	PRE	**POST**	**P value**
Age (years)	23±5	23±5	---
Height (m)	1.81±0.04	1.82±0.04	0.07
BM (kg)	77.5±10.0	76.0±19.4*	0.02
BMI ($kg \cdot m^{-2}$)	23.6±2.5	23.0±2.4*	0.009
Lean body mass (%)	80.9±5.6	80.8±6.0	0.74
Fat body mass (%)	19.1±5.6	19.2±6.0	0.74

Values are mean ± SD. BM, body mass; BMI, body mass index. *P<0.05 different from PRE.

Main cardiovascular, ventilatory, and metabolic variables determined in PRE and POST during INCR (peak values) and MODERATE are shown in **Table 2**. HR_{peak} corresponded to ~95% of the age-predicted maximum both in PRE and POST (calculated as 208-0.7 X age (Tanaka et al., 2001) and was unaffected after the intervention. On the contrary, SV and CO were reduced in POST (101 ± 17 ml and 19 ± 3.2 $l \cdot min^{-1}$, respectively) *vs.* PRE (134 ± 28 ml and 25.2 ± 5.8 $l \cdot min^{-1}$, respectively) of about 25%. Both work $rate_{peak}$ and $\dot{V}O_{2peak}$ decreased about 9% in POST *vs.* PRE (P<0.001). GET

was not different in POST (1.909 ± 0.388 l·min^{-1} and 63 ± 9% of $\dot{V}O_{2peak}$) *vs.* PRE (2.092 ± 0.425 l·min^{-1} and 61 ± 3% of $\dot{V}O_{2peak}$).

Vastus lateralis muscle oxygen extraction values obtained by NIRS (as indicated by Δ[deoxy(Hb + Mb)]) at exhaustion expressed as micromolar changes with respect to the initial value arbitrarily set equal to zero, significantly decreased after bed rest (~17%; P=0.02).

Work rate for MODERATE was 32 ± 7 % of peak work rate in PRE and 35 ± 6 % in POST. Mean values of $\dot{V}O_2$ and HR of MODERATE were 53 ± 5 % of $\dot{V}O_{2peak}$ and 69 ± 7 % of HR_{peak} in PRE and they did not change in POST. Skeletal muscle fractional O_2 extraction at the end of MODERATE was 18% of the physiological calibration in PRE and it did not change in POST.

Table 2. Main respiratory, cardiovascular, and metabolic end-exercise or steady state values, determined during incremental exercise (INCR) and constant work rate exercise (MODERATE) before (PRE) and after (POST) a 10-day horizontal bed rest.

	INCR			**MODERATE**		
	PRE	POST	P value	PRE	POST	P value
Work rate, W	251 ± 50	230 ± 41	0.02	81 ± 26	81 ± 26	---
$\dot{V}O_2$, l·min^{-1}	3.436 ± 0.673	3.039 ± 0.463	<0.001	1.795 ± 0.296	1.797 ± 0.286	0.96
$\dot{V}O_2$, ml·kg^{-1}·min^{-1}	44.4 ± 7.2	40.3 ± 6.1	<0.001	±	± 1	0.17
$\dot{V}CO_2$, l·min^{-1}	4.020 ± 0.761	3.527 ± 0.557	0.001	1.606 ± 0.269	1.597 ± 0.253	0.76
R	1.17 ± 0.07	1.16 ± 0.07	0.60	0.90 ± 0.02	0.89 ± 0.02	0.15
$\dot{V}E$, l·min^{-1}	150.5 ± 20.5	133.2 ± 20.3	0.01	46.4 ± 6.0	46.9 ± 7.6	0.67
fR, breaths·min^{-1}	57 ± 9	50 ± 7	0.003	26 ± 3	27 ± 4	0.23
HR, beats·min^{-1}	187 ± 8	189 ± 6	0.90	127 ± 11	131 ± 16	0.17
SV, ml	134 ± 28	101 ± 17	0.003	137 ± 23	135 ± 20	0.69
CO, ml·min^{-1}	25.2 ± 5.8	19.0 ± 0.9	0.004	17.4 ± 3.5	17.7 ± 2.9	0.40

Mean values ± SD. $\dot{V}O_2$, pulmonary oxygen uptake; $\dot{V}CO_2$, CO_2 output; R, gas exchange ratio; $\dot{V}E$, pulmonary ventilation; fR, breathing frequency; HR, heart rate; SV, stroke volume; CO, cardiac output. *P<0.05 different from PRE.

Peripheral vascular and endothelial responses

Baseline leg blood flow was not affected by bed rest (443 ± 100 ml·min^{-1} POST *vs.* and 429 ± 96 respectively; P=0.55), on the contrary baseline femoral arterial diameter was reduced after bed rest of about 5% (0.90 ± 0.11 cm in POST *vs.* 0.94 ± 0.12 in PRE; P=0.02). The PLM results are presented in **Figure 1**. Leg blood flow increased immediately after the onset of PLM, reaching a peak both in PRE (1310 ± 467 ml·min^{-1}) and POST (1107 ± 472) after about 10 s. There was not a significant difference in peak leg blood flow in POST *vs.* PRE (P=0.19). The area under the blood flow *vs.* time cure during PLM was lower (P=0.03) in POST (274 ± 233 mL) *vs.* PRE (427 ± 291).

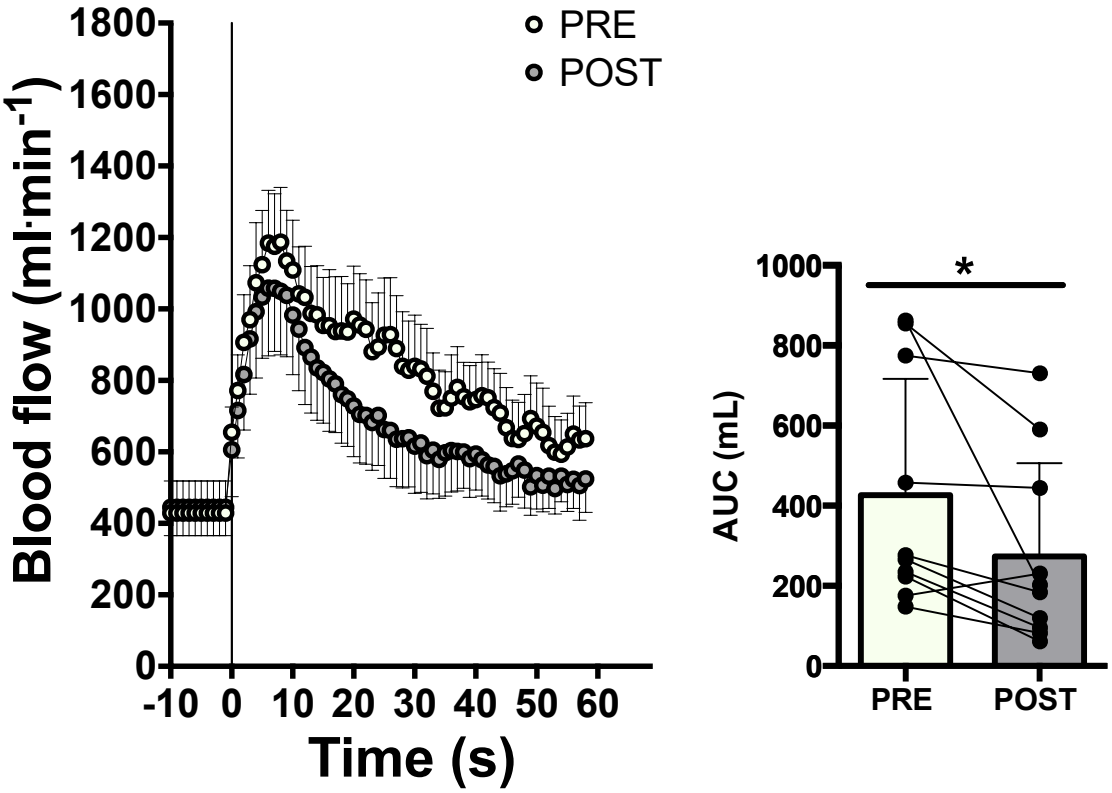

Figure 1. On the left, mean leg blood flow in response to passive leg movement (PLM) before (PRE) and after (POST) 10-day of bed rest is shown. Data are presented as mean ± SE. On the right, individual and mean values of the area under the curve (AUC) in response to PLM are given. Data are presented as mean ± SD. * indicates P<0.05 (paired t-test).

Mitochondrial function

In vivo

Representative $\dot{V}O_2m$ off- kinetics curves for a typical subject following MODERATE, is shown in **Figure 2**. A monoexponential decrease was observed for all participants before and after the intervention. For $\dot{V}O_2m$, individual values of the coefficient of determination (r^2) ranged between 0.90 and 0.95. In the panel with the $\dot{V}O_2m$ off- data, values obtained at rest before the exercise are also shown (dashed horizontal line). $\dot{V}O_2m$ at rest was reduced in POST (0.078 ± 0.02 μM*$^{s-}$1) *vs.* PRE (0.057 ± 0.02; P=0.006). Parameters of the $\dot{V}O_2m$ off- kinetics are also reported in **Figure 2**. $\dot{V}O_2m$ τ and *k* values were not significantly different in POST *vs.* PRE.

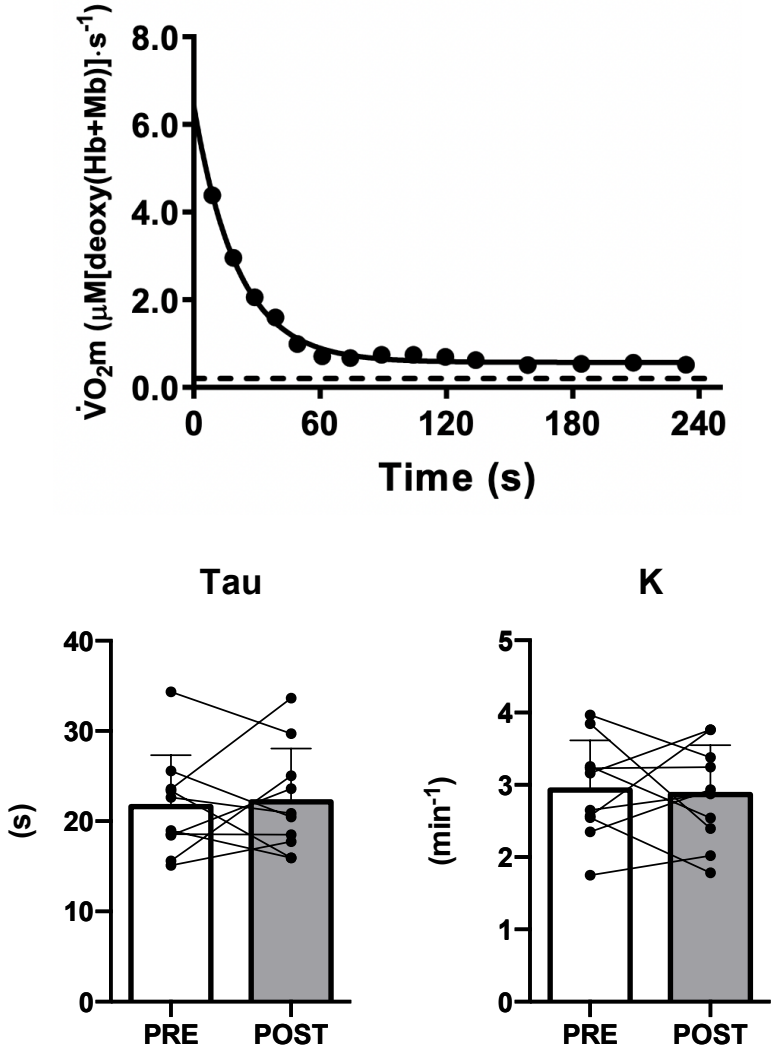

Figure 2. Assessments of *in-vivo* mitochondrial function. In the upper panel, muscle $\dot{V}O_2$ ($\dot{V}O_2m$) recovery kinetics for a representative subject following MODERATE exercise and fitted function are shown. The dotted horizontal line indicates the resting baseline value. In the lower panel, individual and mean (± SD) values of time constant (τ) and recovery rate constant (K) of muscle $\dot{V}O_2$ ($\dot{V}O_2m$) recovery kinetics before (PRE) and after (POST) bed rest are shown.

Ex vivo

The main data related to mitochondrial respiration *ex vivo* obtained by high-resolution respirometry are presented in **Figure 3**. The data were expressed per mg of wet weight. There were no significant changes in substrate-controlled mass-specific states in POST *vs.* PRE. Mitochondrial leak respiration, which represent the non-phosphorylating resting mitochondrial respiration sustained by Complex I and Complex II, was 19.2 ± 5.3 $pmol \cdot s^{-1} \cdot mg^{-1}$ in POST and 18.7 ± 5.7 $pmol \cdot s^{-1} \cdot mg^{-1}$ in PRE (P=0.85). Maximal ADP-stimulated mitochondrial respiration (OXPHOS), supported by complex I and Complex II, was 66.4 ± 17.5 $pmol \cdot s^{-1} \cdot mg^{-1}$ wet weight in POST and 72.3 ± 14.0 in PRE, P=0.41. High-resolution respirometry was also used to assess ADP sensitivity of respiration by ADP titrations from 12.5 to 10000 µM in the presence of glutamate, malate and succinate. The collected data were analysed according to a bi-exponential model (see statistical analysis). Titration of ADP showed that submaximal ADP-stimulated respiration was lower following bed rest (**Figure 4**). Km was indeed reduced of about 50% (P=0.04) following bed rest. The maximal capacity of electron transport system (ETS) did not change significantly after bed rest (63.7 ± 22.5 $pmol \cdot s^{-1} \cdot mg^{-1}$ in POST *vs.* 78.4 ± 17.4 in PRE), P=0.10. The degree of coupling of oxidative phosphorylation efficiency (respiratory control ratio, 4.10 ± 1.19 [PRE] *vs.* 3.59 ± 1.11 [POST], P=0.443) was not affected by bed rest. Skeletal muscle citrate synthase activity, an estimate of mitochondrial mass, was not different (P=0.12) in POST (131.2 ± 15.9 $mU \cdot mg^{-1}$ protein) *vs.* PRE (137.9 ± 18.8) (see **Figure 4**). When the respirometric data were normalized for CS activity, no change in any substrate-controlled state was observed.

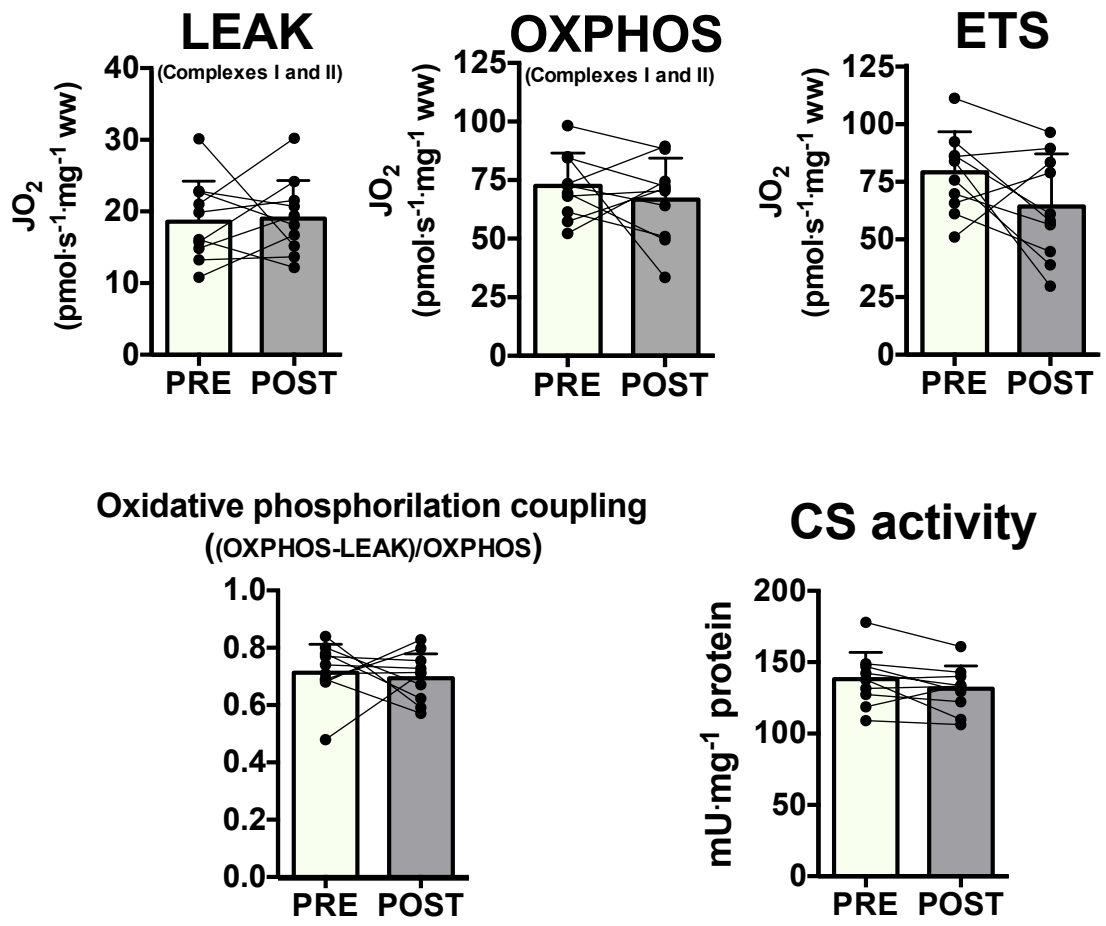

Figure 3. Variables of mitochondrial respiratory function and volume. Individual and mean values are shown ± SD.

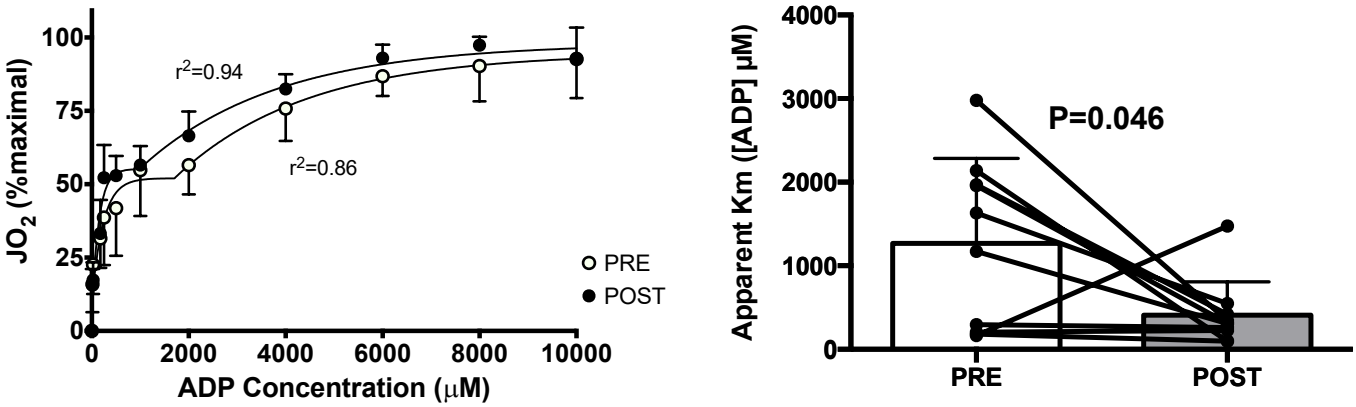

Figure 4. Assessments of mitochondrial ADP sensitivity. On the left, ADP-stimulated respiration before (PRE) and after (POST) 10-day of bed rest is shown. Data are presented as mean ± SD. On the right individual and mean values (± SD) of the estimated apparent ADP Km.

DISCUSSION

The main results observed in the present study after a 10-day horizontal bed rest can be summarized as follows: (*i*) a whole-body impairment *in vivo* oxidative function as evaluated by decreases in $\dot{V}O_{2peak}$, peak work rate, CO_{peak} and SV was observed; (*ii*) a less pronounced blood flow increase during passive leg movement and a reduction in the diameter of the common femoral artery was detected; (*iii*) skeletal muscle citrate synthase was not affected by bed rest; (i*v*) skeletal muscle mitochondrial function evaluated both non-invasively *in vivo* by near-infrared spectroscopy (NIRS) and invasively *ex vivo* by high-resolution respirometry (HRR) was unchanged.

Overall, these data suggest that the whole-body impairment of oxidative metabolism during exercise following 10 days of horizontal bed rest was associated with an impairment of cardiovascular function such as CO_{peak} and SV, but also with an impairment of peripheral vascular and endothelial function whereas mitochondrial mass and maximal mitochondrial respiratory function were unaffected.

As regards incremental exercises, $\dot{V}O_{2peak}$ and peak work rate decreased about 10% after bed rest. Considering the duration of the bed rest in the present study (10 days), the decreases are in line with other previous studies. For example, Porcelli et al. (2010) described a decrease of ~18% in both variables, after a 35-day bed rest, results almost equal to those observed by Ferretti et al. (1997) (~17 and ~19 % for $\dot{V}O_{2max}$ and peak work rate, respectively) during a 42-day bed rest; whereas Capelli et al. (2006) reported $\dot{V}O_{2peak}$ and peak work rate values of 14% and 22% lower, respectively, after a bed rest of 14 days. However, our results are in part in contrast with a recent review and meta-analysis of Reid-Larsen et al. (2017), where authors described a much lower rate of decline of $\dot{V}O_{2max}$ after BR (-0.3/-0.4 % per day). In this regard, a possible reason might be that our values have been overestimated because of the small sample size used (n = 10), as explained by the authors themselves (Reid-Larsen et al. 2017).

CO_{peak} was ~25 % lower in POST vs. PRE, whereas HR_{peak} remained unaffected. This almost twofold decrease in CO_{peak} compared to that in $\dot{V}O_{2peak}$, is in line with the concomitant and identical percentage wise decrease in SV observed in the present study, which can be essentially considered the only responsible for CO_{peak} decrease (Ferretti et al. 1997). These findings confirm results of Ferretti et al. (1997), who reported reductions in $\dot{V}O_{2peak}$, CO_{max} and SV of 16.6, 30.8 and 30.9%, respectively, after a 42-day head-down tilt BR. Moreover, also in the study of Capelli et al. (2006), CO_{max} and SV showed a faster average daily rate of decay than $\dot{V}O_2$ in the two shortest bed rests (14

and 42 days). More precisely, for the 14-day bed rest the decays amounted for 1.63, 1.61 and 0.99% per day, respectively, for CO_{max}, SV and $\dot{V}O_{2peak}$.

Whereas cardiovascular impairments associated with (or responsible for) the decreased $\dot{V}O_2$peak following bed rest have been well described (Ferretti et al. 1997; Capelli et al., 2006), more peripheral impairments have been relatively less investigated. For short term exposure to microgravity, some recent studies conducted by our group identified limitations at the peripheral level such as the level of microvascular supply of O_2, intramuscular matching between O_2 delivery and O_2 uptake, and peripheral O_2 diffusion (Porcelli et al. 2010, Salvadego et al. 2011, Salvadego et al. 2016).
For example, Salvadego et al. (2016) reported after a 10-day exposure to microgravity a significant impairment of oxidative performance as evaluated by $\dot{V}O_{2peak}$, peak skeletal muscle fractional O_2 extraction and profiles of O_2 extraction during CWR exercise. This occurred during cycle ergometer but also during one-leg knee extension exercises, in which central cardiovascular constraints are removed or significantly attenuated; suggesting that the impairments to oxidative function were downstream of cardiovascular O_2 delivery.
In this study with the aim of gaining more insights into the limitation in oxidative metabolism at the peripheral level, we have evaluated peripheral vascular and endothelial functions before and after a 10-day bed rest. We have utilized the method recently proposed by Gilford & Richardson (2017), evaluating by Eco-Doppler the blood flow increase in the femoral artery during a 1-min period of passive extension of a lower limb (PLM) which has been utilized to evaluate young untrained and trained subjects, untrained and trained older adults, patients with chronic heart failure (Gilford & Richardson, 2017) and patients with chronic obstructive pulmonary disease (Ives et al., 2020). The blood flow increase during PLM was higher in the trained vs. the untrained subjects, higher in the young vs. the old subjects, lower in the patients vs. healthy controls. Moreover, the blood flow increase during PLM, was well correlated with indices of nitric oxide availability and endothelial function (Gilford & Richardson, 2017), a critical index of general cardiovascular health. In our study we observed a less pronounced blood flow increase during PLM after 10 days of bed rest, suggesting an early impairment of peripheral/endothelial function following microgravity – inactivity. Interestingly, the blood flow increase during PLM observed in our young subjects after 10 days of bed rest was not substantially different from that described by Gilford & Richardson (2017) in subjects of 60-70 years of age. Moreover, the less pronounced increase in blood flow during PLM after bed rest was associated with an inward structural remodeling of the femoral common artery. In the present study, 10 days of bed rest led to a reduction in lumen diameter of 5%, a very similar decrease (i.e., 6%) was detected after 7 days of leg casting (Sugawara et al., 2004). This phenomenon

has also been reported across a spectrum of different modalities of physical inactivity, ranging from spinal cord injury patients to bed rest and unilateral lower limb suspension (Thijssen et al., 2011). Changes in femoral artery diameter are also associated with increases in wall thickness with the result of promoting atherosclerotic progression (Thijssen et al., 2011).

In the present study skeletal muscle mitochondrial function was evaluated both *ex vivo* in permeabilized vastus lateralis fibers by HRR (Pesta & Gnaiger 2012) and *in vivo*, non-invasively, by NIRS by calculating the skeletal muscle $\dot{V}O_2$ recovery kinetics following CWR exercise (Ryan et al., 2014; Adami & Rossiter 2018; Zuccarelli et al., 2020).

Mitochondrial function evaluated by HRR on isolated and permeabilized non-contracting skeletal muscle fibers obtained by biopsy on the vastus lateralis (Pesta & Gnaiger 2012; Perry et al., 2011), showed similarly to what observed Salvadego et al., (2016), that none of the respirometric parameters were modified in the present study (see Fig. 4). "Leak" respiration which represents the dissipation on H+ gradient across the inner mitochondrial membrane non associated with phosphorylation of ADP was not different following bed rest. Maximal ADP-stimulated mitochondrial respiration (OXPHOS) supported by complex I and complex II determined in presence of saturating ADP levels and unlimited substrates and O_2 availability, was unchanged. Also maximal capacity of electron transport system (ETS) uncoupled from the phosphorylating system, and oxidative phosphorylation coupling did not detect significant changes after 10 days of bed rest. The scenario could be different with prolonged exposure to microgravity where both respirometric (Salvadego et al., 2018) and proteomic data (Brocca et al., 2012) are in favor of an altered mitochondrial function and structure. However, the effects of short periods of bed rest on maximal ADP-stimulated mitochondrial respiration are somehow controversial. Whereas Miotto et al. (2019) and Dirks et al. (2020) described an impaired mitochondrial function following bed rest periods of 3 and 7 days, respectively, other authors (Larsen et al. 2018, Salvadego et al. 2016) did not see impairments following 4 and 10 days of bed rest exposure.

Free ADP concentration in skeletal muscle ranges between 25 and 250 μM (Howlett et al., 1998), way below the unlimited concentration of ADP utilized to evaluate OXPHOS. In order to put these measurements in a more "real" biological environment, ADP mitochondrial respiration sensitivity was evaluated. The apparent Km was estimated using a biexponential model which better fitted our data and which to our understanding might give us insights into cellular mechanisms regulating mitochondrial respiration (this hypothesis has to be verified). In the present study, while OXPHOS remained unchanged after bed rest, ADP sensitivity was enhanced, as expressed by the reduction in Km (see Fig. 4), indicating a greater sensibility of respiration to [ADP]. A conclusive interpretation

of these data is still lacking, but they confirm similar data obtained by Dirks et al. (2019) following a bed rest period of similar duration.

Mitochondrial content of the tissue, as estimated by CS activity, was not affected with bed rest, indicating that then no quantitative or functional changes occurred.

The data obtained *ex vivo* by HRR on isolated fibers, were also confirmed *in vivo*, and non-invasively, by the kinetics of muscle $\dot{V}O_2$ recovery following cycle ergometer exercise at moderate intensity (Ryan et al., 2012; Adami & Rossiter 2018; Zuccarelli et al., 2020). The values of the time constant (τ) muscle $\dot{V}O_2$ recovery kinetics were indeed not affected by bed rest intervention, thus confirming that skeletal muscle mitochondrial function after 10 days of bed rest was not compromised.

Another interesting finding of the present study is related to the resting muscle $\dot{V}O_2$, measured non-invasively on the basis of change in muscle oxygenation determined by NIRS. A decreased resting muscle $\dot{V}O_2$ was observed following 10 days of bed rest. A decrease in whole body basal $\dot{V}O_2$ of about 7% after 7 weeks of immobilization has already been reported by Deitrick *et al.* (1948) and confirmed also by other studies (Teasell & Dittmer, 1993; Downs et al. 2020). Disuse/immobilization is known to decrease muscle protein synthesis and increase protein degradation leading to skeletal muscle atrophy (Crossland et al., 2019; Degens et al., 2019). As far as we know, we have reported for the first time that immobilization induced by bed rest decreases the relative basal muscle $\dot{V}O_2$. A reduction of muscle protein synthesis decreases the energy needed in the muscle, which can lead to an attenuation of global as well as relative (per muscle unit) basal $\dot{V}O_2$. The reduction in basal muscle $\dot{V}O_2$ observed in the present study could be the result of (*i*) the decreased energy demand expressed per muscle mass of the immobilized muscle or (*ii*) a higher muscle oxidative phosphorylation efficiency of at rest (i.e., lower oxygen cost of ATP synthesis). At this regard, Kang & Ji, (2013) have shown that a 2-week period of immobilization significantly downregulated the PGC-1α signaling and the mitochondrial biogenesis pathway in mice skeletal muscles with a concomitant suppression of mitochondrial transcription factor A and cytochrome-c content by 57 and 63%, respectively and cytochrome-c oxidase activity by 58%. Furthermore, the immobilization-induced direction of the transformation of type I towards the less efficient type IIA and IIX muscle fibers, suggests that immobilization plays a lore in decreasing the energy needed per muscle mass, rather than increasing muscle mitochondrial efficiency.

In conclusion, the main limitations to oxidative metabolism after a 10-day horizontal bed rest study were “upstream” of mitochondria function, at the level of central and peripheral O_2 delivery. Substantial impairments to oxidative function were observed at the peripheral vascular and endothelial function (see PLM) whereas mitochondrial content and maximal respiration were unaffected.

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4 CONCLUSIONS

The research work presented in this PhD thesis deals, in general terms, with the implementation of new methods for the functional evaluation of oxidative metabolism during exercise, which could be ultimately utilized in normal subjects, athletes, patients and subjects exposed to environmental stressors. The proposed methods allow to identify biomarkers of functional impairment (or improvement) which would substantially increase our capacity to evaluate exercise tolerance and to prescribe exercise as a therapeutic / rehabilitation intervention.

In the first set of studies, we proposed a new and simple method to evaluate exercise (in)tolerance, that is the work rate decrease at a fixed HR, slightly above that corresponding to the gas exchange threshold (GET), which demarcates the moderate-intensity exercise domain (below GET) and the high-intensity exercise domain (above GET). Exercise at a HR value slightly above that at GET is often utilized for moderate-intensity exercise training in healthy subjects and particularly in patients (Lansley et al., 2011; Iannetta et al., 2020). This approach is based on the assumption of a linear relationship between HR and work rate. In our study we demonstrated that this approach is substantially flawed. Both in healthy subjects (*Study 1*), in obese patients (*Study 2*), and in subjects exposed to a 10-day bed rest (*Study 3*) exercise at a fixed HR value slightly higher than that at GET is associated with a substantial decrease in work rate, ranging from about -15-20% (healthy subjects, obese patients before exercise training) to -40% (healthy subjects after a 10-d bed rest) over a 15–20-minute period. What happens during exercises of longer duration has not been investigated and should be evaluated in future studies. Textbook physiology states that a lower work rate for the same HR indicates a reduced exercise tolerance. In other words, the proposed method could represent a new tool for evaluating exercise tolerance, more sensitive than other variables such as $\dot{V}O_2$peak and GET (see *Studies 1, 2, 3*). This is further confirmed by the observations that the work rate decrease at a fixed HR was substantially reduced after exercise training in obese patients (*Study 2*), in the absence of significant changes of $\dot{V}O_2$peak and GET, and was substantially aggravated after 10 days of bed rest (*Study 3*), in the absence of changes of GET.

Interestingly, the work rate decrease at a fixed HR was associated with *decreases* of variables whose increase during a constant work rate exercise ($\dot{V}O_2$, R, [La]b, muscle deoxygenation) would represent a sign of fatigue. One could expect (see Jones et al. 2012) that a work rate decrease during fatiguing exercise would be aimed at preventing increases of the above-mentioned variables, and in particularly of $\dot{V}O_2$, whose increase during constant work rate exercise ("slow component") is a warning sign for a reduced efficiency of oxidative metabolism and of impending fatigue (Jones et al. 2012; Grassi et al. 2015). In our studies (*Studies 1, 2, 3*), on the other hand, the work rate decrease was so pronounced

that it actually determined a decreased $\dot{V}O_2$. That is to say, the work rate decrease was more pronounced than that expected to prevent the $\dot{V}O_2$ slow component. What drives this apparently excessive work rate decrease is not clear. Muscle or whole-body temperature increases, or blood catecholamine levels may be involved.

In any case, the work rate decrease at a fixed HR is inevitably linked with "slow components" of HR during constant work rate exercise, which were anecdotally described in the past. In our *Study 1* we demonstrated the presence of a HR slow component also during constant work rate exercise below GET, i.e. in an exercise domain in which no $\dot{V}O_2$ slow component is present. In the same study we also demonstrated that during constant work rate exercise above GET the HR slow component is more pronounced (percentage wise) than the $\dot{V}O_2$ slow component.

The observations described above have profound implications on exercise prescription. Let's imagine an astronaut during a spaceflight or living in a planetary station. She/he receives an exercise prescription to perform 30 minutes of exercise per day on a cycle ergometer at a HR slightly above that previously determined at GET during an incremental exercise. After 10 days of exposure to microgravity, the work rate corresponding to that HR has decreased by 40%, moving from a heavy-intensity exercise domain to a moderate-intensity exercise domain. Is the training still effective? What happens to the work rate decrease after 30 minutes of exercise? What happens after 20 days of microgravity exposure? Or after 1 or 2 months? All questions that need to be answered, and that represent a direct consequence of our studies.

In the second set of studies (*Study 4* and *5*), we concentrated on the search of new biomarkers of impaired oxidative metabolism during exercise following exposure to microgravity. To perform these studies (as well as *Study 3* discussed above) our group has participated, together with several other research groups, to a 10-day horizontal bed rest campaign organized in the Summer of 2019, by the Koper Science and Research Center (ZRS Koper), at the Izola General Hospital, in Slovenia. The campaign was financed within the "MARS-PRE Bed Rest SBI 2019" project by the Agenzia Spaziale Italiana (ASI). Besides allowing to investigate on Earth conditions of microgravity, bed rest studies offer a unique opportunity to evaluate the effects of profound deconditioning.

Whereas cardiovascular impairments associated with (or responsible for) the decreased $\dot{V}O_2$peak following bed rest have been well described, more peripheral impairments have been relatively less investigated. Over the last 10-15 years, our group has significantly contributed to the study of skeletal muscle impairments of oxidative metabolism following bed rest (Porcelli et al 2010, Salvadego et al 2011, Salvadego et al. 2016, Salvadego et al 2018). In the present PhD work we focused on the search

of biomarkers of impairment related to peripheral vascular and endothelial function, the intramuscular matching between O_2 delivery and O_2 uptake and mitochondrial function.

In a preliminary study (*Study 4*) we implemented a new method for the non-invasive evaluation of skeletal muscle oxidative function by the analysis of the kinetics of muscle $\dot{V}O_2$ during the recovery phase following cycle ergometer exercise of different intensities. The method is based on the general concept that the rate of muscle deoxygenation determined non-invasively by near-infrared spectroscopy (NIRS) (Grassi & Quaresima 2015) during a transient ischemia of the limb allows to determine muscle $\dot{V}O_2$. A series of brief ischemic periods during the recovery from exercise allows to the evaluate muscle $\dot{V}O_2$ kinetics, a classic variable of functional evaluation of oxidative metabolism.

In the last study (*Study 5*) of the present PhD we first evaluated, before and after the 10-day bed rest, peripheral vascular and endothelial functions. To this aim, we utilized the method recently proposed by Gilford & Richardson (2017), evaluating by Eco-Doppler the blood flow increase in the femoral artery during a 1-min period of passive extension of a lower limb (passive leg movement, PLM). The method has been utilized to evaluate young untrained and trained subjects, untrained and trained older adults, patients with chronic heart failure (Gilford & Richardson, 2017) and patients with chronic obstructive pulmonary disease (Ives et al., 2020). The blood flow increase during PLM was higher in the trained vs. the untrained subjects, higher in the young vs. the old subjects, lower in the patients vs. healthy controls. Moreover, the blood flow increase during PLM, moreover, was well correlated with indices of nitric oxide availability and endothelial function (Gilford & Richardson, 2017), a critical index of general cardiovascular health. In our study we observed a less pronounced blood flow increase during PLM after 10 days of bed rest (see *Study 5* and Zuccarelli et al. 2020), suggesting an early impairment of peripheral/endothelial function following microgravity – inactivity. Interestingly, the blood flow increase during PLM observed in our young subjects after 10 days of bed rest was not substantially different from that described by Gilford & Richardson (2017) in subjects of 60-70 years of age. It would of course be of interest to follow this variable during longer bed rest periods, following the adoption of countermeasures, or following the termination of the bed rest exposure.

On the other hand, the evaluation of classical variables of mitochondrial function ("leak" respiration, maximal ADP-stimulated mitochondrial respiration, maximal uncoupled respiration, oxidative phosphorylation coupling, and others), carried out by high-resolution respirometry (HRR) on isolated and permeabilized skeletal muscle fibers obtained by biopsy, did not detect significant changes after 10 days of bed rest (see *Study 5* and Zuccarelli et al. 2020). The same was true for the activity of citrate synthase, taken as an estimate of mitochondrial mass. In other words, mitochondrial mass and

function were not significantly affected by the relatively short bed rest period, confirming previous observations by our group (Salvadego et al. 2016) following a similar bed rest period. The main limitations to oxidative metabolism, in other words, would be "upstream" of mitochondria, at the level of central and peripheral O_2 delivery. The data obtained *ex vivo* by HRR on isolated fibers, were also confirmed *in vivo*, and non-invasively, by the approach described above (*Study 4*), which was applied also on the subjects exposed to the bed rest (*Study 5*). In these experiments, as discussed above, mitochondrial oxidative function was evaluated by the kinetics of muscle $\dot{V}O_2$ recovery following cycle ergometer exercise at different intensities.

Interestingly, further analyses carried out by HRR on the biopsies obtained before and after the bed rest, allowed us to evaluate another variable: the sensibility of mitochondrial respiration to submaximal (and physiological) ADP concentrations ([ADP]). This variable was slightly but significantly *enhanced* following bed rest, indicating a greater sensibility of respiration to [ADP]. A conclusive interpretation of these data is still lacking, but they confirm similar data obtained by Dirks et al. (2019) following a bed rest period of similar duration.

Our data of a substantially preserved mitochondrial function following 10 days of bed rest may seem in contradiction with data obtained by other groups during the same bed rest campaign. Sandri et al. (personal observations), for example, observed a substantial impairment of the "trascriptome" of mitochondrial genes after as early as 5 days of bed rest. However, the contradiction with our results, however, could be only apparent, in the sense that changes at the level of the trascriptome may become evident before functional changes are observed. In a previous study by our group carried out by HRR we indeed observed impairments of mitochondrial function after 21 days of bed rest (Salvadego et al. 2018).

An aspect of skeletal muscle oxidative metabolism which showed a significant change following the 10 days of bed rest was represented by the resting muscle $\dot{V}O_2$, measured non-invasively on the basis of the change in muscle oxygenation determined by NIRS during a transient limb ischemia (rapid inflation of a pneumatic cuff). We observed a significant decrease (by about 15%) of resting muscle $\dot{V}O_2$ following bed rest (see *Study 5*). A decreased resting muscle $\dot{V}O_2$ following bed rest is, to the best of our knowledge, a novel finding. It could represent an adaptive (or maladaptive) phenomenon in response to microgravity – inactivity, attributable to the fact that muscle catabolic processes within muscles (the subjects underwent a decrease in muscle mass of about 5% during the period, Narici et al personal observations) are less expensive, in terms of energy, than anabolic ones.

The concepts mentioned above, besides being of interest from a basic science point of view, may be of interest also for other pathological conditions characterized by relatively short periods of profound inactivity, and it could affect the definition of countermeasures or of rehabilitative interventions.

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mitochondria. *67th Annual Meeting, American College of Sports Medicine*. *San Francisco, California (USA)* May 26-30, 2020.

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6 LIST OF STUDIES INCLUDED IN THIS THESIS

Study 1

Zuccarelli L, Porcelli S, Rasica L, Marzorati M, Grassi B. Comparison between Slow Components of HR and $\dot{V}O_2$ Kinetics: Functional Significance. *Medicine and science in sports and exercise*. 2018 Aug;50(8):1649-57.

Study 2

Zuccarelli L, De Micheli R, Tringali G, Sartorio A, Grassi B. Obese patients decrease work rate in order to keep a constant target heart rate. *Medicine and science in sports and exercise.* 2020 *Epub ahead of print*

Study 3

Zuccarelli L, do Nascimento Salvador PC, Del Torto A, Fiorentino R, Grassi B. Skeletal muscle $\dot{V}O_2$ kinetics by the NIRS repeated occlusions method during the recovery from cycle ergometer exercise. *Journal of Applied Physiology*. 2020 Mar 1;128(3):534-44.

7 OTHER PUBLICATIONS DURING CANDIDATURE

Research articles

Mogilever NB, **Zuccarelli L**, Burles F, Iaria G, Strapazzon G, Bessone L, Coffey EB. Expedition cognition: a review and prospective of subterranean neuroscience with spaceflight applications. *Frontiers in Human Neuroscience*. 2018 Oct 30;12:407.

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Narici M, De Vito G, Franchi M, Paoli A, Moro T, Marcolin G, Grassi B, Baldassarre G, **Zuccarelli L**, Biolo G, Di Girolamo FG. Impact of sedentarism due to the COVID-19 home confinement on neuromuscular, cardiovascular and metabolic health: Physiological and pathophysiological implications and recommendations for physical and nutritional countermeasures. *European Journal of Sport Science*. 2020 May 8:1-22.

Congress communications

Zuccarelli L, Porcelli S, Rasica L, Marzorati M and Grassi B. " Comparison between Slow Components of HR and VO_2 Kinetics: Functional Significance" § Poster, American College of Sports Medicine (ACSM), Minneapolisn, Minnesota, USA (30/05/2018)

Pedrali M, **Zuccarelli L**, Biasutti L, Azzini V, Rasica L, Marzorati M, Porcelli S, Sechi A, Grassi B, “Exercise tolerance in late-onsetPompe disease patients: positive effects of physical training and high-protein diet” § Poster presentation 23th annual Congress of the European College Sport Science (ECSS), Dublin, Ireland (05/07/2018)

Zuccarelli L, Do Nascimento Salvador PC, Del Torto A, Fiorentino R, Grassi B. "Skeletal muscle $\dot{V}O_2$ kinetics by NIRS "repeated occlusions method" during recovery from cycle ergometer exercise". § Poster, American College of Sports Medicine (ACSM), Orlando, Florida, USA (30/05/2019)

Zuccarelli L, Magnesa B, Degano C, Comelli M, Gasparini M, Manferdelli G, Marzorati M, Mavelli I, Pilotto A, Porcelli S, Rasica L, Šimunič B, Pišot R, Narici M, Grassi B. "The impairment of oxidative metabolism after 10-day of bed rest is upstream of skeletal-muscle mitochondria". § Poster, American College of Sports Medicine (ACSM), San Francisco, California, USA (26/05/2020)

Porcelli S, Rasica L, **Zuccarelli L**, Magnesa B, Degano C, Comelli M, Manferdelli G, Marzorati M, Mavelli I, Pilotto A, Burleigh M, Šimunič B, Pišot R, Narici M, Grassi B. "Effects of 10-day bed-rest on nitric oxide metabolites and microvascular function assessed by near-infrared spectroscopy". § Poster, American College of Sports Medicine (ACSM), San Francisco, California, USA (27/05/2020)

Baldassarre G, **Zuccarelli L**, Manferdelli G, Manfredini V, Rasica L, Pilotto A, Marzorati M, Porcelli S, Šimunic B, Pišot R, Narici M, Grassi B. "Decrease in work rate in order to keep a constant heart rate: effects of a 10-day bed rest". § Oral presentation 25th annual Congress of the European College Sport Science (ECSS), Sevillia, Spain (05/07/2020)

Zuccarelli L, Baldassarre G, Magnesa B, Degano C, Comelli M, Gasparini M, Manferdelli G, Marzorati M, Mavelli I, Pilotto A, Porcelli S, Rasica L, Šimunič B, Pišot R, Narici M, Grassi B. "Peripheral impairments of oxidative metabolism after 10-day bed rest are upstream of mitochondrial respiration". § Oral presentation, 5th Human Physiology Workshop, Cologne, Germany, (05/12/2020)