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Titolo della tesi

"DECREASE IN WORK RATE DURING EXERCISE AT A FIXED HEART RATE: NEW SYSTEMIC BIOMARKER OF EXERCISE (IN)TOLERANCE IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS"

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Table of contents

EXTENDED ABSTRACT

When exercise is carried out for periods of time longer than a 1-2 minutes oxidative metabolism is the highly prevalent mechanism for ATP resynthesis. Thus, oxidative metabolism constitutes the main determinant of exercise tolerance and work capacity for everyday life and work, and strongly influences the quality of life of the subjects/patients. A functional evaluation of oxidative metabolism is therefore of great importance for health status assessment as well as for the identification of possible preventive and/or therapeutic interventions aimed at enhancing exercise tolerance.

The work of our group is in general devoted to the identification of biomarkers of impaired exercise tolerance, in patient populations and in subjects exposed to reduced levels of physical activity. The studies presented in this PhD thesis are focused on the identification of non-invasive biomarkers of impaired exercise tolerance in subjects exposed to bed rest (taken as a model to study profound inactivity and to simulate microgravity exposure), as well as in patients with coronary artery disease. Specific attention was given to a new and simple approach of exercise (in)tolerance evaluation, recently identified by our group, that is the quantification of the decrease in work rate during exercise at a fixed heart rate (HR) slightly higher than that corresponding to the gas exchange threshold (GET).

In the first chapter of the thesis an overview on oxidative metabolism as well as on tools for its functional evaluation is provided. Thus, some "systemic" or more "peripheral" biomarkers related to oxidative function are presented, including both traditional and relatively new approaches. Functional impairments of oxidative metabolism following bed rest deconditioning are also briefly discussed in the introduction.

The first study presented in this thesis is mainly focused on the evaluation of oxidative metabolism following 10 days of bed rest, in order to identify possible "systemic" signs of functional impairments during exercise. The study was carried out during a bed rest campaign organized at the General Hospital of Izola (Slovenia) by the Koper Science and Research Center (ZRS Koper), and funded by the Italian Space Agency (ASI).

In the present study we proposed a new simple approach to evaluate exercise (in)tolerance to subjects exposed to microgravity/inactivity. This new method consists in the quantification of the work rate decrease necessary to keep HR constant (at a value slightly higher than corresponding to GET). This phenomenon (work rate decrease at a fixed HR) was previously demonstrated by our group both in healthy young subjects (Zuccarelli et al. 2018) and in obese adolescents (Zuccarelli et al. 2021) and interestingly, in the study with the obese patients, it was greatly attenuated after a 3-week exercise training program. This suggests that the phenomenon is influenced by aerobic fitness level, and that it represents a biomarker of exercise tolerance. Moreover, it is a well-accepted concept, present in textbooks (Astrand et al. 1986; McArdle et al. 1986; Wasserman et al. 1999; Clausen, 1977), that a

decreased HR for the same work rate is a sign of improved exercise tolerance; thus, a lower work rate for the same HR can be considered a sign of impaired exercise tolerance as well. Therefore, we hypothesized a significant more pronounced work rate decrease in order to keep HR constant following a 10-day horizontal bed rest period. Results of *Study 1* confirmed this hypothesis. The decrease in work rate necessary to keep HR constant at a value corresponding to GET+20% was significantly aggravated after (~40%) *vs.* before (~30%) bed rest, resulting in a shift from the heavy- (>GET) to the moderate-intensity (<GET) domain. Additionally, we found that the work rate decreases following bed rest were significantly correlated with the individual decreases in peak oxygen uptake ($\dot{V}O_{2peak}$), and negative effects on other traditional biomarkers (e.g., ventilatory thresholds, the kinetics of $\dot{V}O_2$ adjustment) of aerobic function were also reported. The greater work rate decrease at a fixed HR could then represent a "systemic biomarker" of the reduced exercise tolerance and of the impairment of oxidative metabolism following microgravity/inactivity. Besides this aspect, our data demonstrate the absence of a linear relationships between HR and work rate, which strongly affects exercise evaluation and exercise prescription, especially following inactivity.
The results of S*tudy 1*, mainly dealing with "systemic" biomarkers of impairment of oxidative metabolism, must be interpreted in association with those obtained by another study of our group on the same subjects (Zuccarelli et al. 2021). This study was focused on the identification of more "peripheral" biomarkers of impairment of oxidative metabolism following the same bed rest campaign of *Study 1*, to better understand the site(s) of limitation along the entire O_2 pathway from ambient air to the mitochondria of skeletal muscles. On this regard, I specifically dealt with the non-invasive evaluation of the microvascular function and of the skeletal muscle oxidative metabolism by near-infrared spectroscopy (NIRS). More particularly we looked for signs of impaired intramuscular matching between O_2 delivery and O_2 uptake in the early phase of a constant work rate exercise, by examining the profile of skeletal muscle O_2 extraction by NIRS (Quaresima & Grassi 2016). Whereas mitochondrial respiration was assessed *in-vivo*, inducing several transient arterial occlusions, by a rapid inflation and deflation of a pneumatic cuff during the recovery from moderate-intensity constant work rate cycling exercise (Zuccarelli et al. 2020). After bed rest we observed a greater transient "overshoot" of the deoxygenation signal by NIRS, suggesting an impaired spatial and temporal matching between O_2 delivery and O_2 uptake. This microvascular impairment was in agreement with the concomitant less pronounced blood flow determined by another recent non-invasive method, namely the passive leg movement (PLM) technique (Gifford & Richardson, 2017). As regards mitochondrial function, no impairments were detected by the *in-vivo* approach, more specifically the skeletal muscle $\dot{V}O_2$ recovery kinetics remained unchanged following bed rest. The lack of significant differences was confirmed also by *ex-vivo* assessment of mitochondrial respiration by high-resolution

respirometry (Pesta & Gneiger, 2012). Therefore, after 10 days of bed rest the impairment of oxidative metabolism seems to be "upstream" of skeletal muscle mitochondria.

Finally, in *Study 2*, we aimed to (i) quantify, in nineteen cardiac patients in stable conditions, the work rate decrease at a fixed HR and (ii) to verify if it would be attenuated by β-blockers administration, in order to gain insights into possible mechanistic causes of this phenomenon. We demonstrated that also in this population a decrease in work rate (and $\dot{V}O_2$) in order to keep a constant HR at a value slightly above GET occurs, thus this biomarker of exercise intolerance might be identified also in cardiac patients. Furthermore, confirming our hypothesis, the work rate decrease at a constant HR was attenuated by β-blockers, suggesting a causative role by β-adrenergic stimulation. These results as well as those from *Study 1* should also be relevant in terms of aerobic exercise prescription. Intensity prescription based on fixed HR values, is not associated with a specific work rate or $\dot{V}O_2$ making the approach quite questionable both in physiological and practical terms also in patients with cardiovascular diseases. However, the issue seems to be less relevant in patients treated with β-blockers.

Data presented in this thesis confirm that the work rate decrease at a fixed HR (slightly above GET) could then represent a "systemic biomarker" of the reduced exercise tolerance, which can be easily and reliably evaluated also in particular conditions, such as spaceflights, permanence on planetary habitats, or in patients with chronic diseases. However, reproducibility studies are clearly needed before the implementation of the proposed approach in practical terms, also to establish a threshold for the identification of a functionally relevant decrease. Furthermore, confirming previous observations by our group (Zuccarelli et al. 2018, 2021), our results demonstrate that exercise prescription at specific HR values is accompanied by progressive and significant work rate and $\dot{V}O_2$ decreases, which may result in a fall of exercise intensity in different domains (essentially from the heavy- to the moderate-intensity exercise domain), possibly altering the training stimulus and the resulting adaptations. This aspect remains be specifically evaluated by future studies, which will have to define the best modality for prescribing training intensity, on the basis of fixed HR or fixed work rate values.

1 CHAPTER I – INTRODUCTION

1.1 OXIDATIVE METABOLISM: ROLE AND RELEVANCE

To support physical activity and the work of any cell, energy is needed, and this energy derives from the hydrolysis of ATP (breakdown of high-energy phosphate bonds). The reserve of ATP in the cell is quite small relative to the needs, and in order to maintain [ATP] (squared brackets denote concentrations) relatively constant, as it occurs also during intense physical activity, ATP resynthesis must increase in proportion to ATP hydrolysis. ATP turnover in skeletal muscle fibers may increase hundreds of times from rest to exercise, therefore [ATP] must increase proportionally with the demand to sustain a given task. The bioenergetic processes for the regeneration of ATP in muscle substantially rely on three mechanisms (**Figure 1**). When intense and very short exercises (i.e., ~10 s) are performed, breakdown of phosphocreatine (PCr) is the main fuel for ATP resynthesis. The maximal power of this system is achieved almost immediately, but the mechanism rapidly fatigues and reaches a value close to zero in ~15 s. Another mechanism of energy production is the anaerobic glycolysis. Glycogen or glucose is catabolized into pyruvate. In conditions of low O_2 availability, the oxidation of NADH to NAD^+, which is necessary to sustain glycolysis, does not fully occur in mitochondria. In this case the reoxidation of NADH takes place in the cytoplasm through the transformation of pyruvate to lactate. However, lactate is now known to form continuously even under fully aerobic conditions fulfilling purposes of energy substrate production and distribution, as well as cell signalling, according to the "lactate shuttle" concept (Brooks, 2018).

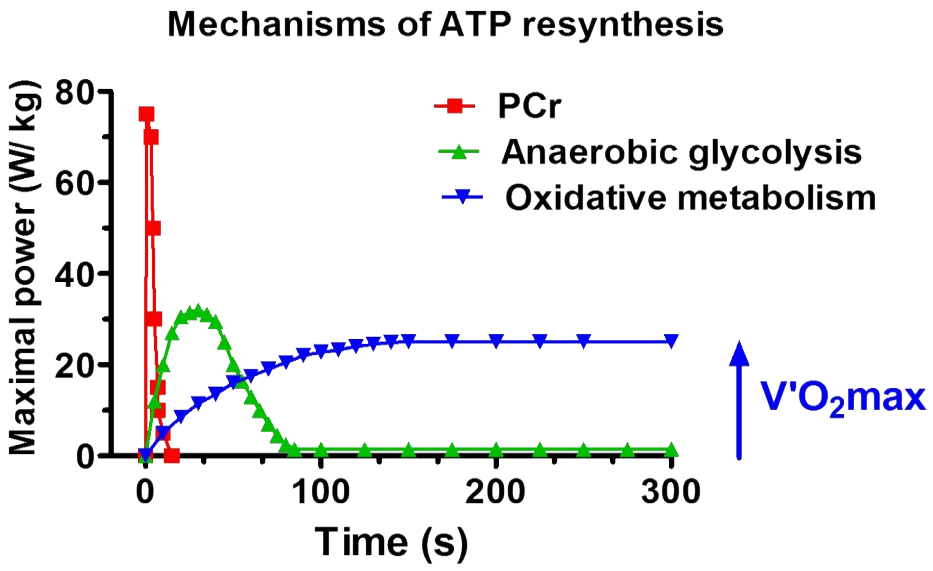

Figure 1. Schematic representation of the maximal power (expressed in watt/kg of body mass) of the three main mechanisms of ATP resynthesis (phosphocreatine [PCr] hydrolysis, anaerobic glycolysis and oxidative phosphorylation) as a function of time during intense exercise. The maximal power generated by oxidative phosphorylation is also shown ($\dot{V}O_{2max}$) (modified from Grassi 2003).

The anaerobic glycolysis generates lower power but for longer period (~60-90 s) compared to PCr hydrolysis. The third mechanism of energy supply is oxidative phosphorylation, which is the most important mechanism that supports life in mammals. In this case ATP resynthesis occurs in the mitochondria from the oxidation of metabolic substrates (primarily glycogen and fatty acids), and the presence of O_2 is crucial since it represents the key that unlocks the energy from substrates by serving as the final electron acceptor along the mitochondrial respiratory chain enzymes.

Oxidative phosphorylation has a relatively lower maximal power, represented by the maximal oxygen uptake ($\dot{V}O_{2max}$), and is significantly slower in getting into action (~2-3 min are needed to reach an asymptotic value even for transitions to submaximal work rates) compared to PCr hydrolysis and anaerobic glycolysis (see **Figure 1**). These are two important limitations of this system; however, oxidative metabolism also has some advantages: (i) the maximal power expressed by oxidative metabolism can be sustained for several minutes (7-10 minutes) (elevated capacity); (ii) a relatively large fraction of its maximum power can be maintained for relatively longer periods of time without incurring in significant fatigue. Thus, oxidative metabolism represents the main energy source for most activities of daily life, which generally lasts more than 1-2 minutes.

Physical activity provides the greatest stimulus to skeletal muscle energy metabolism; indeed the latter can increase hundreds of times during exercise compared to resting conditions. To sustain a given level of exercise, adequate interaction among the respiratory and cardiovascular systems is required to supply O_2 to the contracting muscles, in order to regenerate aerobically ATP, as well as to remove the metabolic carbon dioxide (CO_2) from the exercising muscles (**Figure 2**). The respiratory system allows the transfer of O_2 from the atmosphere to the alveoli and CO_2 from the alveoli to the ambient air, and permits gas exchanges by diffusion at the alveolar-capillary membrane.

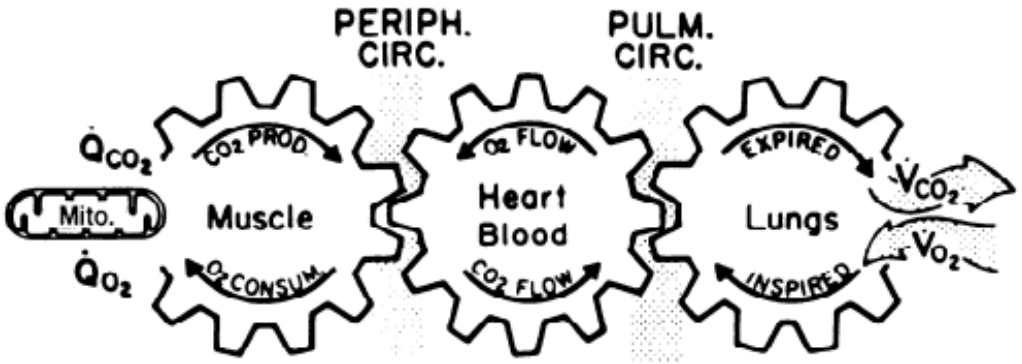

Figure 2. Gas transport mechanisms for coupling cellular (internal) to pulmonary (external) respiration. The gears represent the functional interdependence of the physiologic components of the system (from Wasserman 1994).

The heart pumps oxygenated blood to the exercising muscles and returns O_2-poor and CO_2-rich blood to the alveoli. Finally, downstream along the O_2 pathway, skeletal muscles extract oxygen from the capillary blood, thereby mitochondria can generate ATP in proportion to the amount of work being performed. Each of the systems mentioned above has a crucial role in terms of O_2 transport and utilization, and they are important determinants of the individual exercise tolerance (defined as the ability to produce force or power adequate to accomplish a task [Grassi et al. 2015]) during everyday life.

1.2 SYSTEMIC BIOMARKERS OF FUNCTIONAL EVALUATION OF OXIDATIVE FUNCTION DURING EXERCISE

Considering the importance of oxidative metabolism in affecting exercise tolerance and thus quality of life, functional evaluation tools of this metabolism during exercise are of utmost importance to identify and quantify possible impairments. Identification of these tools would be important also in terms of prescription of effective preventive and/or therapeutic interventions aimed at counteracting impairments. Some traditional "systemic" biomarkers for the functional evaluation of oxidative metabolism will be presented below.

Maximal oxygen uptake

The maximal oxygen uptake ($\dot{V}O_{2max}$) describes the highest oxygen consumption that can be attained following increases in exercise intensity (Hill & Lupon, 1923), and represents the greatest amount of oxygen a person can use to produce ATP aerobically per unit of time.

The $\dot{V}O_{2max}$ holds great physiologic significance as one of the main determinants of exercise tolerance, and since it is the result of the integration of respiratory, cardiovascular and muscular factors responsible for O_2 transport and utilization.

Mathematically, $\dot{V}O_{2max}$ can be defined as the product of the maximal cardiac output ($\dot{Q}_{max}$) and the maximal arterio-mixed venous O_2 concentration difference ($[CaO_2\text{-}C\bar{v}O_2]_{max}$) (Fick equation, see below).

$$\dot{V}O_{2max} = \dot{Q}_{max}\ (CaO_2\text{-}C\bar{v}O_2)_{max}$$

Although $\dot{V}O_{2max}$ is the result of an integration between "central" and "peripheral" factors along the O_2 pathway from the ambient air to skeletal muscle mitochondria, in healthy subjects, cardiovascular O_2 delivery (i.e. cardiac output and stroke volume) represents the main limitation to maximal oxidative power during exercise with large muscle groups (di Prampero & Ferretti 1990). $\dot{V}O_{2max}$ is one of the main variables in exercise physiology not only to assess aerobic fitness in endurance athletes but also exercise capacity and tolerance in diseased population. The higher the $\dot{V}O_{2max}$, the more effective the ventilatory, cardiovascular, and muscular systems are at performing their tasks and the greater the individual's exercise capacity. A typical $\dot{V}O_{2max}$ value for a sedentary individual might be ~30 $ml \cdot kg^{-1} \cdot min^{-1}$, which can move to ~40-45 $ml \cdot kg^{-1} \cdot min^{-1}$ in an untrained normally active young subject, and up to 70-85 $ml \cdot kg^{-1} \cdot min^{-1}$ in a highly trained endurance athlete (see **Figure 3**). On the other hand, patients with cardiovascular diseases may exhibit $\dot{V}O_{2max}$ values as low as 10-15 $ml \cdot kg^{-1} \cdot min^{-1}$, severely limiting the capacity to perform daily life activities and work, and thus worsening

the quality of life as well as the prognosis of this population (Myers et al. 2002). The peak exercise capacity ($\dot{V}O_{2max}$) expressed in metabolic equivalents (MET, namely multiples of resting $\dot{V}O_2$) has been demonstrated to represent both in healthy subjects and in patients with cardiovascular diseases a more powerful predictor of the risk of death than other clinical variables or established risk factors (Myers et al. 2002). However, reduced physical fitness is a modifiable risk factor, and improvements in fitness over time have been demonstrated to improve prognosis. Myers et al. (2002) reported that every 1-MET increase in exercise capacity was associated with a 12 percent improvement in survival. Additionally, this result confirms the presence of an inverse relation between exercise capacity and mortality from any cause.

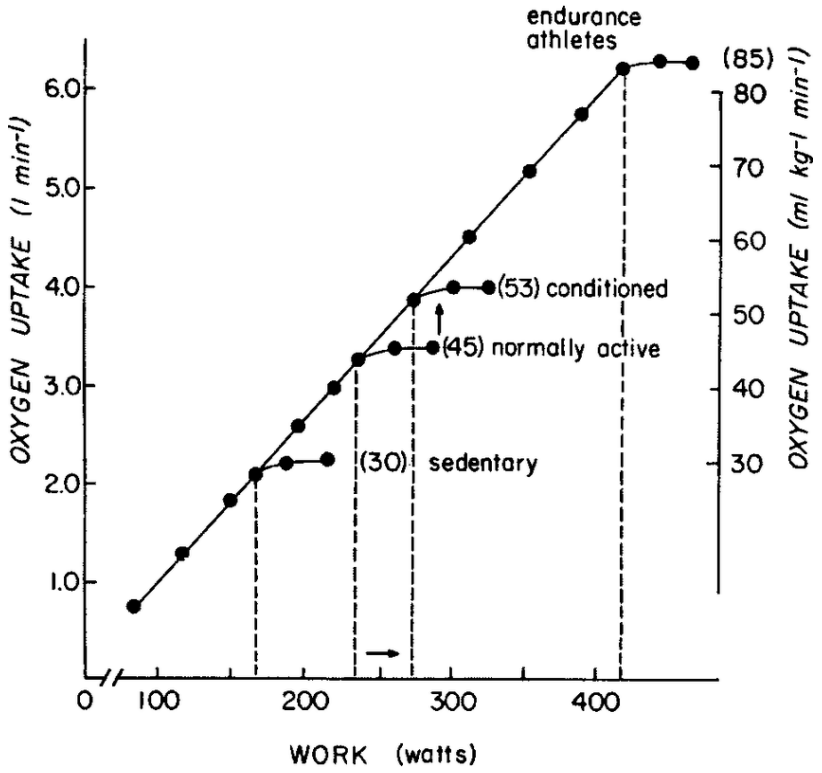

Figure 3. Oxygen uptake (expressed in $L \cdot min^{-1}$ and in $mL \cdot kg^{-1} \cdot min^{-1}$ on the left and right y-axis, respectively) as a function of exercise intensity (expressed in watts) in different populations. The relationship between the two variables is linear and is similar in human subjects with different levels of physical conditioning, but their maximal oxygen uptakes (values in brackets), revealed by the flattening of the oxygen uptake-work rate relationship, are quite different (from Rowell 1993).

$\dot{V}O_{2max}$ is usually assessed by the cardiopulmonary exercise test (CPET), which permits simultaneous evaluation of the respiratory and cardiovascular responses during exercise. The test is usually performed on either a treadmill or a cycle ergometer, and generally consists in a 8-12-min step or ramp incremental protocol during which the intensity (speed or work rate depending on the ergometer) is progressively increased over time until individual's voluntary exhaustion. During the test pulmonary ventilation ($\dot{V}E$), $\dot{V}O_2$ and carbon dioxide output ($\dot{V}CO_2$) are monitored non-invasively breath-by-breath, in addition to other variables such as heart rate, stroke volume, cardiac output, arterial blood oxygen saturation, fractional O_2 extraction, electrocardiogram (ECG) and blood pressure.

In an incremental test $\dot{V}O_2$ increases linearly in relation to the intensity of exercise since more ATP is required for muscle contraction but at a certain point $\dot{V}O_2$ fails to raise despite increases in work rate, and $\dot{V}O_{2max}$ is thus achieved (see **Figure 3**). However, this flattening of the $\dot{V}O_2$-work rate relationship is often not visible during this test because at peak work rates many subjects, especially patients, cannot endure the discomfort long enough to achieve a plateau in $\dot{V}O_2$, and maximal aerobic power could be underestimated. In this case the highest $\dot{V}O_2$ achieved during the incremental exercise protocol is called $\dot{V}O_{2peak}$ instead of $\dot{V}O_{2max}$, and fulfillment of some additional criteria, based on other metabolic and physiological variables (i.e., HR, respiratory exchange ratio and blood lactate levels), is needed for establishing true $\dot{V}O_{2peak}$ values. Another limitation of $\dot{V}O_{2max}$ assessment is that it requires the execution of a maximal effort exercise, which may expose patient populations or the elderly to risks. Therefore, during the test a continuous medical supervision with ECG monitoring is mandatory. An adequate incremental test protocol must also be selected according to the subject's cardiorespiratory fitness, in order to achieve exhaustion in 8-10 minutes. This is a crucial aspect considering that tests that are too brief or too long are likely to give lower $\dot{V}O_{2max}$ values. Moreover, during the incremental test, in order to determine $\dot{V}O_{2max}$ it's necessary the availability of a metabolic cart (relatively expensive instrumentation), in order to measure breath-by-breath $\dot{V}O_2$.

Ventilatory thresholds

$\dot{V}O_{2peak}$, the variable evaluating the maximal integrated (respiratory, cardiovascular, muscular) performance of oxidative metabolism, is classically considered associated with a mechanical power output which can be sustained for about 10 minutes. For exercises of longer duration (i.e., most everyday activities and works) other variables, corresponding to specific fractions of $\dot{V}O_{2max}$, are usually determined in order to identify the maximal sustainable work rate.

During the incremental exercise, $\dot{V}O_2$ increases linearly with time and work rate (**Figure 4**) and, for low exercise intensities, also pulmonary ventilation ($\dot{V}E$) and $\dot{V}CO_2$ follow the same pattern. In this intensity domain the CO_2 removed from the exercising muscles through the ventilation derives essentially from oxidative phosphorylation. The ratio $\dot{V}CO_2/\dot{V}O_2$ is called respiratory quotient (QR). This ratio reflects the metabolic exchange of the gases at tissues level and is dictated by the percentage of substrate (carbohydrates, fatty acids, and amino acids) used in energy production by the cells. If only fatty acids or carbohydrates are oxidized RQ equals respectively 0.71 or 1, however both during rest and exercise the organism utilizes a mixture of substrates. More specifically the resting RQ is ~0.8, with a greater proportion of fatty acids being consumed for energy production, but utilization of carbohydrates increases as a function of exercise intensity, rising RQ up to ~1. The ratio between $\dot{V}CO_2$ and $\dot{V}O_2$ measured at the mouth is called gas exchange ratio (R). R is equivalent to RQ, only

when there is a steady state in $\dot{V}CO_2$ and $\dot{V}O_2$; that is, when there is no CO_2 production deriving from bicarbonate buffering of H^+, and there are not changes in the body's CO_2 stores deriving from a respiratory compensation of metabolic acidosis.

From a certain point during incremental exercise $\dot{V}CO_2$ (and $\dot{V}E$) exceeds the linear relationship with $\dot{V}O_2$ (or work rate) (see **Figure 4**), resulting in R exceeding RQ until a new steady state in CO_2 stores is attained. This "excess" in $\dot{V}CO_2$ (which determines a proportional increase in $\dot{V}E$), comes from the buffering by bicarbonate (HCO_3^-) of H^+ resulting from the dissociation of lactic acid produced by anaerobic glycolysis. The metabolic rate corresponding to this "disproportionate" increase in $\dot{V}CO_2$ and $\dot{V}E$ is called ventilatory (VT) or gas exchange threshold (GET), and is identified by an increase in the $\dot{V}CO_2/\dot{V}O_2$ relationship (the "V-slope" plot) (Wasserman et al. 1973; Beaver et al. 1986).

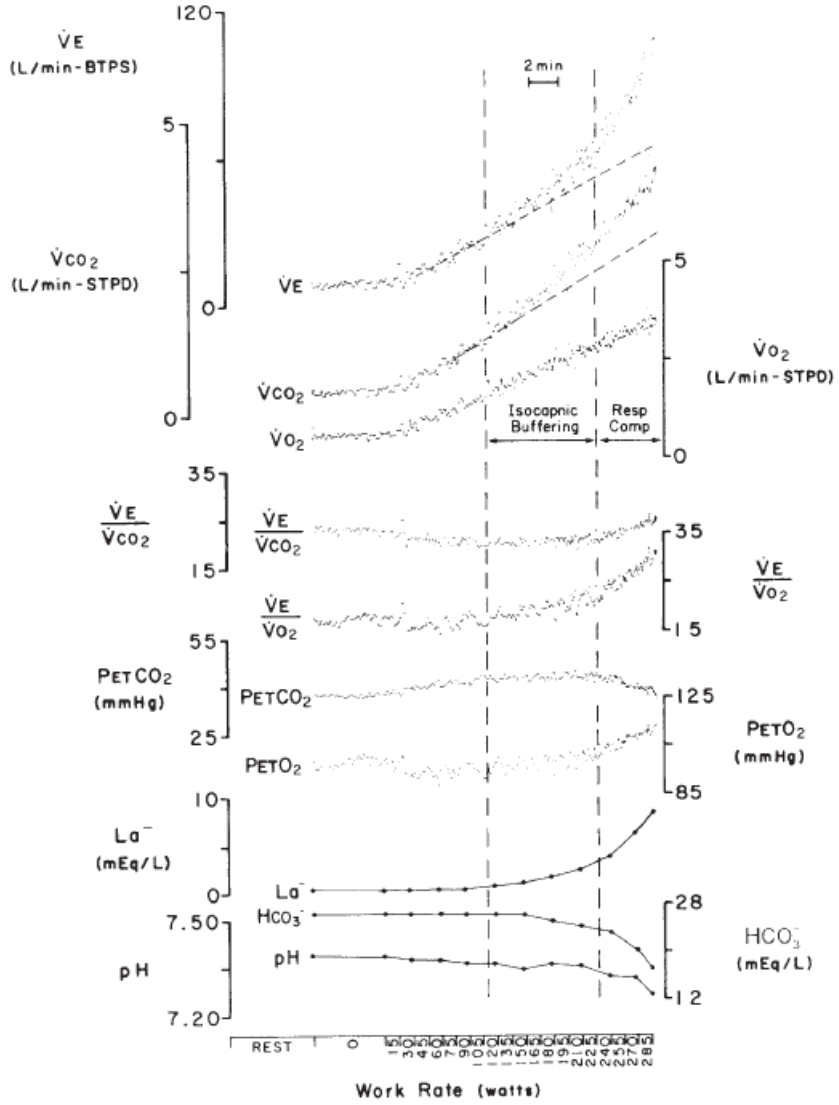

Figure 4. Pulmonary ventilation ($\dot{V}E$), oxygen uptake ($\dot{V}O_2$), carbon dioxide output ($\dot{V}CO_2$), ventilatory equivalent for CO_2 ($\dot{V}E/\dot{V}CO_2$) and for O_2 ($\dot{V}E/\dot{V}O_2$), end-tidal PCO_2 ($PETCO_2$) and PO_2 ($PETO_2$), blood lactate (La^-), pH and bicarbonate (HCO_3^-) during an incremental exercise with a step protocol. Vertical dashed lines indicate the two ventilatory thresholds (gas exchange threshold and respiratory compensation point). Isocapnic buffering refers to the period when both $\dot{V}E$ and $\dot{V}CO_2$ increase curvilinearly and more markedly than $\dot{V}O_2$. After it $\dot{V}E$ increases more rapidly than $\dot{V}CO_2$ to compensate the metabolic acidosis (Respiratory compensation) (modified from Wasserman 1994).

From this moment both $\dot{V}E$ and $\dot{V}CO_2$ increase curvilinearly and more markedly than $\dot{V}O_2$ resulting in increases in the ventilatory equivalents for O_2 ($\dot{V}E/\dot{V}O_2$) and in arterial-end-tidal PO_2 ($PETO_2$), and constant $\dot{V}E/\dot{V}CO_2$ and $PETCO_2$ (isocapnic buffering period). However, by further increasing the intensity of exercise beyond GET, and even before reaching $\dot{V}O_{2max}$, a second and more pronounced

increase in both $\dot{V}CO_2$ and $\dot{V}E$ can be identified, named second ventilatory threshold (VT2) or respiratory compensation point (RCP) (Wasserman et al. 1986). From this point the involvement of the anaerobic glycolysis is massive, H^+ buffering becomes incomplete, and a condition of metabolic acidosis (decrease in pH) is thus achieved. The metabolic acidosis would stimulate peripheral chemoreceptors, leading to an increased $\dot{V}E$ aimed at reducing CO_2 stores in blood. This ventilatory compensation for the exercise-induced lactic acidosis is therefore reflected by an increase in $\dot{V}E/\dot{V}CO_2$ and a decrease in $PaCO_2$ and $PETCO_2$, as well as by further increases in $\dot{V}E/\dot{V}O_2$ and $PETO_2$. However, exercise cannot be sustained at (and beyond) this intensity for long and, after a few tens of minutes, the subject is obligated to interrupt the test because of the onset of fatigue.

The identification of the ventilatory thresholds is very important to study skeletal muscle energy metabolism as well as to determine the limit of tolerance for relatively long submaximal exercises. The more the involvement of anaerobic glycolysis is delayed in relation to the intensity of exercise, the lower the metabolic perturbation at a given work rate, and the greater the work capacity to sustain exercise without incurring in fatigue. GET and RCP usually occur respectively at about 50-60% or 70-80% (~50% of the difference between $\dot{V}O_{2max}$ and GET) of $\dot{V}O_{2max}$ in healthy untrained subjects, whereas lower or higher percentages are generally observed in patients or aerobically fit subjects, respectively.

As for $\dot{V}O_{2max}$, ventilatory thresholds determination cannot be performed without the presence of a metabolic cart able to measure breath-by-breath pulmonary ventilation and gas exchanges, and requires to perform a maximal incremental exercise test as well. Furthermore, GET or RCP identification is not always straightforward as it usually requires the intervention of multiple independent and expert observers and the need for the often invoked "ancillary criteria" (i.e., analysis of ventilatory equivalents for O_2 and CO_2 and of end-tidal PO_2 and PCO_2 in additional to the "V-slope" method). These are important limitations of this method.

Oxygen uptake kinetics and the "slow component"

At the onset of exercise there is an immediate increase in the energetic demands by the contracting muscles and adequate ATP supply is consequently needed to sustain exercise. As previously discussed, oxidative phosphorylation is slow in getting into action and at least ~2-3 minutes are indeed needed before coupling the ATP demand (Cerretelli & di Prampero, 1987). More specifically, pulmonary $\dot{V}O_2$ response during the transition from rest to constant work rate exercise ($\dot{V}O_2$ kinetics) is characterized by an immediate rise (Phase I or cardiodynamic component) (**Figure 5**) which lasts about 15-20 s and is determined by a rapid increase in pulmonary blood flow deriving from

augmentations in heart rate and stroke volume. Phase I is then followed by an exponential increase in $\dot{V}O_2$ (Phase II, primary or fundamental component) which largely reflects $\dot{V}O_2$ kinetics in the exercising muscles (Grassi et al. 1996; Behnke et al. 2005). Finally, within 2-3 minutes $\dot{V}O_2$ achieves a steady-state (Phase III), at least during moderate-intensity (< GET) constant work rate exercises. This pulmonary $\dot{V}O_2$ response is best described as a time delay (TD, normally 15-20 s) followed by an exponential function (Whipp et al. 1981):

$$\Delta\dot{V}O_2(t) = \Delta\dot{V}O_2ss\ (1 - e^{-(t-TD)/\tau})$$

where t is the time elapsed from exercise onset, $\Delta\dot{V}O_2ss$ is the $\dot{V}O_2$ steady-state above baseline (expressed in $L \cdot min^{-1}$) and τ is the time constant (denoting the time to reach 63% $\dot{V}O_2ss$), which can vary by up to ~fourfold from the typical value of ~30 s in healthy young subjects (Rossiter, 2011).

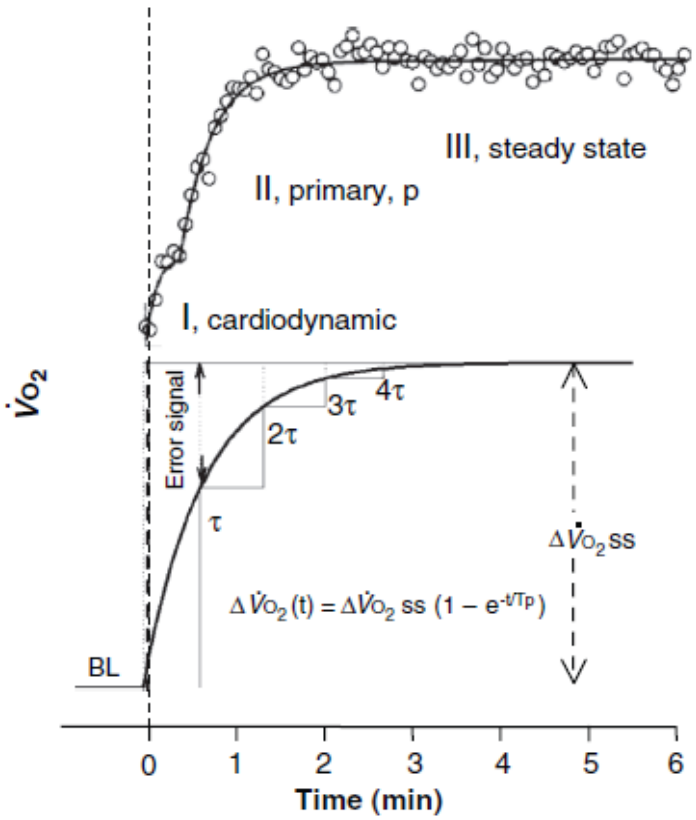

Figure 5. Top Breath-by-breath pulmonary $\dot{V}O_2$ response following the onset of moderate intensity exercise. Phases I (cardiodynamic), II (primary), and III (steady-state), fitted by an appropriate exponential model (see text). **Bottom:** schematic demonstrating fundamental properties of the single component exponential response. The rate of $\dot{V}O_2$ increase is quantified by the time constant (τ) of the exponential, where BL signifies baseline $\dot{V}O_2$ and Δ the amplitude of $\dot{V}O_2$ above baseline (right vertical arrows). For each multiple of τ $\dot{V}O_2$ increases by 63% of the difference between that value at the previous τ and the required steady state. τ_p designates the time constant of the primary component response. Also shown is the metabolic error signal (difference between $\dot{V}O_2(t)$ and Δ that drives the increase of $\dot{V}O_2$) which decreases with each increment of τ. The O_2 deficit is the area from exercise onset (time = 0) bounded by the actual $\dot{V}O_2$ profile and the asymptotic $\dot{V}O_2$ projected backward to time 0 (from Poole & Jones, 2012).

The rate of $\dot{V}O_2$ adjustment, quantified by τ, is a fundamental parameter of the functional capacity and integration of the pulmonary, circulatory and muscle bioenergetic systems and thus an important determinant of exercise (in)tolerance (Grassi et al. 2011). $\dot{V}O_2$ kinetics are inversely correlated to the magnitude of "O_2 deficit" at exercise onset, which indicates the total O_2 equivalent to the amount of energy that has been borrowed from both aerobic (dissolved O_2, oxymyoglobin, oxyhemoglobin, and O_2 stored in the lung) and anaerobic (PCr hydrolysis, and anaerobic glycolysis) sources (Cerretelli &

di Prampero, 1987). The $\dot{V}O_2$ kinetics or O_2 deficit, however, should not be considered per se direct causes of exercise (in)tolerance, but rather a marker of "metabolic stability". Disturbances in muscle metabolic stability can affect muscle function in various ways, whereas good metabolic stability is associated with higher exercise tolerance. Thus, a faster $\dot{V}O_2$ kinetics may simply be an "epiphenomenon" of a relatively higher metabolic stability, which would then represent the relevant variable in terms of fatigue and exercise tolerance (Grassi et al. 2011, 2015, 2021).

The O_2 deficit is traditionally computed as the difference between the actual $\dot{V}O_2$ during an exercise bout and the product of the $\dot{V}O_2$ss and the exercise duration (see **Figure 5**). Substrate-level phosphorylation processes are limited in their capacity and challenge cellular and organ homeostasis (Allen et al. 2008), therefore minimization of their use is potentially beneficial for the ability to sustain subsequent exercise. The more rapid the $\dot{V}O_2$ kinetics (i.e., the shorter the τ) the better, because this would result in a smaller O_2 deficit, and thus higher metabolic stability (lower degradation of PCr and glycogen stores, lower accumulation of lactate and H^+), with positive consequences on exercise tolerance and muscle fatigue (Grassi et al. 2006, 2011).

During the recovery phase after exercise, $\dot{V}O_2$ does not return immediately to baseline, but has a slow exponential decay (Hill et al. 1924). This excess $\dot{V}O_2$ above resting values is called "O_2 debt" or "excess post-exercise O_2 consumption (EPOC)" (Gaesser & Brooks, 1984) and reflects the ATP credit from anaerobic sources (mainly PCr stores [Rossiter et al. 1999]) in the O_2 deficit period that must be repaid by oxidative phosphorylation. For moderate intensity exercises (<GET) O_2 debt is repaid within 5 minutes of recovery and its size approximates that of the O_2 deficit (Whipp et al. 1970). Therefore, also $\dot{V}O_2$ recovery kinetics analysis may be a good and reliable tool for function evaluation of oxidative metabolism and exercise tolerance.

The profile of $\dot{V}O_2$ following the onset of constant work rate exercise may be defined with respect to the exercise-intensity domain in which the exercise is performed (**Figure 6**). When work rate is situated below GET (moderate-intensity domain), pulmonary $\dot{V}O_2$ rises relatively rapidly and attains a steady state within a few minutes after exercise onset. This response is best modelled by a simple exponential profile, whose amplitude is ~9-11 mL $O_2 \cdot min^{-1} \cdot W^{-1}$ for cycle ergometry (Whipp et al. 1981). In the heavy-intensity domain, which encompasses all metabolic rates between GET and critical power (CP) (see paragraph critical power), the attainment of a steady state is delayed due to a slowly developing increase in $\dot{V}O_2$, traditionally termed "slow component" of $\dot{V}O_2$ kinetics (Jones et al. 2011; Poole & Jones, 2012; Rossiter, 2011) (see **Figure 6**). The slow component of $\dot{V}O_2$ arises ~1-3 min after exercise onset and is superimposed on the fundamental kinetics observed during

moderate-intensity exercise, and therefore the fitting of the $\dot{V}O_2$ kinetics requires a more complex model (Whipp et al. 2002):

$$\Delta\dot{V}O_2(t) = \Delta\dot{V}O_2ss\ (1 - e^{-(t-TD)/\tau}) + \Delta\dot{V}O_2sc\ (1 - e^{-(t-TDsc)/\tau sc})$$

where TD_{sc} and τsc are respectively the time delay and time constant related to $\dot{V}O_2$ slow component ($\dot{V}O_2sc$). This "excess" $\dot{V}O_2$ represents an additional O_2 cost which increases the gain (i.e., $\Delta\dot{V}O_2/\Delta$work rate >11 $mLO_2 \cdot min^{-1} \cdot W^{-1}$) effectively lowering work efficiency and delaying achievement of a steady-state condition (Whipp & Wasserman, 1986; Poole et al. 1988). However, for even higher intensities, which exceed CP (i.e., severe-intensity domain) $\dot{V}O_2$ fails to attain a steady-state and projects to $\dot{V}O_{2max}$, leading to task failure (Poole et al. 1988; see **Figure 6**). CP therefore represents the highest submaximal metabolic rate that can be stabilized as a function of time (Jones et al. 2008).

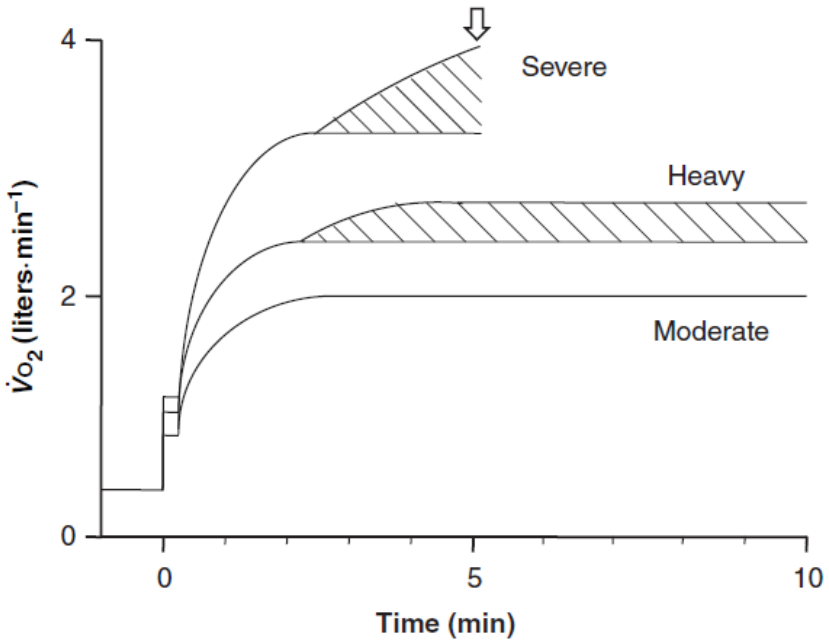

Figure 6. Schematic representation of the $\dot{V}O_2$ response to constant-work rate exercise in the moderate- (<GET), heavy- (>GET to <CP), and severe-intensity (>CP to $\dot{V}O_{2max}$) domains. Hatched areas denote $\dot{V}O_2$ slow component during heavy and severe-intensities exercises, whereas the arrow depicts point of exhaustion during severe-intensity exercise, and attainment of $\dot{V}O_{2max}$ (from Poole et Jones, 2012).

For both heavy- and severe-intensity exercises, where a slow component of $\dot{V}O_2$ kinetics manifests, $\dot{V}O_2$ changes as a function of time and not work rate, therefore the "traditional" linearity between $\dot{V}O_2$ and work rate is not preserved for intensities above GET (Zoladz et al. 1995; Majerczak et al. 2012; Grassi et al. 2015). From a more functional viewpoint the slow component of $\dot{V}O_2$ kinetics heralds a progressive loss of efficiency and muscle fatigue (Burnley & Jones, 2007; Jones et al. 2011),

which represent the major causes of exercise intolerance and thus task failure (Grassi et al. 2015). Studying the $\dot{V}O_2$ slow component may therefore provide important insights into the determinants of exercise (in)tolerance in both healthy and diseased populations. It has been demonstrated that the majority (>80%) of the magnitude of the slow component of the pulmonary $\dot{V}O_2$ kinetics originates in skeletal muscles (Poole et al. 1991), however the factors responsible for this phenomenon are still debated. Rossiter et al. (2002) reported that the $\dot{V}O_2$ slow component was associated with a slow component of intramuscular PCr breakdown, indicating that the slow component is linked to a greater ATP cost of force generation (reduced muscle mechanical efficiency, increased P/w, unchanged P/O_2), rather than an elevated $\dot{V}O_2$ cost of ATP production (reduced P/O_2). However, growing evidence (Cannon et al. 2014; Rasmussen et al. 2001; Bartlett et al. 2020) suggest that there is some effect on the side of ATP synthesis (i.e., reduced efficiency of oxidative ATP synthesis by mitochondrial uncoupling). Several studies have shown that the slow component of $\dot{V}O_2$ kinetics is associated with aerobically less-efficient type II muscle fibers content or recruitment (Krustrup et al. 2008; Majerczak et al. 2014). It is becoming increasingly evident, however, that the progressive recruitment of muscle fibers per se is not necessary for the development of $\dot{V}O_2$ slow component but, rather, may be driven by metabolic processes occurring within already recruited fibers that are fatiguing (Vanhatalo et al. 2011; Zoladz et al. 2008; Woledge, 1998; Barclay, 1996).

The analysis of $\dot{V}O_2$ kinetics for exercise (in)tolerance evaluation presents the advantage of not requiring a maximal incremental test to task failure, thus it could be particularly suitable for patient population. However, the use of a metabolic cart is still necessary. Moreover, given the breath-to-breath fluctuations ("noise") in pulmonary gas exchange, superimposition of breath-by-breath data obtained from several submaximal constant work rate repetitions is necessary for adequate precision (Whipp et al. 1982; Lamarra et al. 1987). This makes the procedure time-consuming, and difficult to perform in patients, voluntary subjects or athletes. Finally, in order to determine the main parameters of $\dot{V}O_2$ kinetics more or less complex mathematical analysis must be performed to fit data.

Critical power

Another important parameter of aerobic function is the critical power (CP), which describes the tolerable duration of high-intensity exercises (Poole et al. 2016). When the time of exhaustion during severe-intensity constant work rate tasks is plotted against speeds or power outputs, the relationship is not linear, but hyperbolic, with the ability to sustain exercise decreasing more rapidly at higher compared to lower exercise powers (Hill, 1925). The power-asymptote of this curvilinear relationship is defined as CP whereas the curvature constant is termed W′ (measured in units of work done, i.e.,

J), and is equivalent to the maximum amount of work that can be performed above CP (see **Figure 7**). When work done is plotted as a function of the reciprocal of time, the hyperbolic relationship can be transformed into a linear one, such that the slope of the line equals CP, whereas the intercept equals W'. In healthy, physically active but untrained young subjects, CP typically occurs at ~50% of the difference between $\dot{V}O_{2max}$ and GET (Poole & Jones, 2012), and at ~60-80% of $\dot{V}O_{2max}$ (similarly to RCP), and represents the highest metabolic rate at which variables associated with exercise intolerance and fatigue such as $\dot{V}O_2$, blood lactate, as well as intramuscular [PCr], [Pi] and [H^+] stabilize (Poole et al. 1988; Pringle & Jones 2002; Jones et al. 2008, 2011; Grassi et al. 2015).

During exercises carried out at a constant power even slightly above CP, all the above-mentioned variables change as a function of time (Jones et al. 2010, 2011; Grassi et al. 2015). More specifically, intramuscular [PCr] fails to reach a steady state and keeps decreasing as a function of time (Jones et al. 2011). A similar scenario is seen for [blood lactate] that conversely keeps increasing during exercise as well as $\dot{V}O_2$ who inexorably projects to $\dot{V}O_{2max}$ (Jones et al. 2011; Poole & Jones 2012). The slow components of $\dot{V}O_2$, [PCr], and [blood lactate] (which may be considered as tools for functional evaluation of the contributions of the three main bioenergetic systems) during severe-intensity exercises are strongly associated with the development of muscular fatigue and exercise intolerance (Grassi et al. 2015).

The actual time until exhaustion (T_{lim}) for exercise performed at a constant power output (P) above CP can be closely predicted (Whipp et al. 1981), and can range from 20-30 min to just a few minutes depending on the proximity of the exercise power output being sustained to CP. Conversely, for intensities equal to or below CP, exercises can be performed for longer periods as steady-state responses of fatigue-inducing factors, and thus metabolic stability are attained. Therefore, CP represents the highest rate of oxidative metabolism that can be sustained without a progressive loss of homeostasis and occurs at a similar work rate to the second ventilatory threshold (RCP).

The power–time curve can be reconstructed by plotting data (power or velocity *vs.* T_{lim}) obtained from four or more independent severe-intensity constant-power exercise bouts for which the tolerable duration is 2–15 min (Poole et al. 1988).

This approach has the advantage that can be applied also to other modes of human locomotion including running and swimming, and it can be performed even on the field (Jones & Vanhatalo, 2017). For CP estimation the use of a metabolic cart is no longer required, since the two main variables that must be collected during the tests are power output or speed and duration of exercise. However, in order to select the appropriate work rate for the several severe-intensity constant-power bouts, $\dot{V}O_{2max}$ should be known in advance. Although determination of the power-duration

relationship constitutes an important and reliable tool for exercise tolerance evaluation both in healthy individuals and patient populations, it is not a widespread practice during either physiological research or diagnostic exercise testing mainly because of the complexity of the approach (Jones et al. 2010). Indeed, as highlighted above, several submaximal, maximal and supramaximal tests to task failure, are necessary to determine CP.

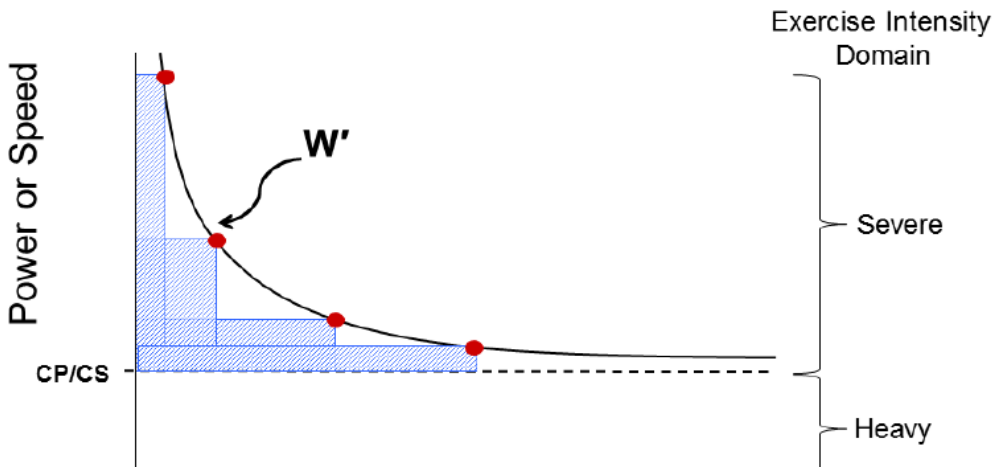

Figure 7. The hyperbolic Power/Speed-duration curve that defines the limit of exercise tolerance. The curve is constructed by the subject performing at least four bouts at a constant power or speed to the point of exhaustion (red dots). The hyperbolic relationship is defined by two parameters: the asymptote for power (critical power, CP, or critical speed, CS) and the curvature constant W′ (denoted by the rectangular boxes above CP and expressed in kJ). Note that CP/CS defines the upper and lower boundary of the heavy- and severe-intensity domain, respectively (modified from Poole et al. 1988).

Decrease in work rate at a fixed heart rate: a new systemic biomarker of exercise (in)tolerance

For submaximal aerobic exercise, training intensity is usually prescribed at specific heart rate (HR) values (Riebe et al. 2018; Powers et al. 2004) mainly because of the practicality of this approach, since HR can be easily measured and recorded. This common practice, however, is based on the concept of a linear relationship between HR, $\dot{V}O_2$ and work rate (see e.g., Astrand et al. 1986), which does not always hold true. A progressive increase in HR (traditionally termed "slow component") has been indeed reported also for 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). Recently, HR kinetics and its slow component in relation to different intensity domains and $\dot{V}O_2$ kinetics were systematically analyzed by Zuccarelli et al. (2018). In this study (Zuccarelli et al. 2018), the slow component of HR kinetics was described during constant work rate moderate-intensity exercise (70% GET), therefore at a lower work rate, compared to the slow component of pulmonary

$\dot{V}O_2$ kinetics (Jones et al. 2011; Grassi et al. 2015) (**Figure 8**). Furthermore, above GET (heavy-[45% ΔGET-$\dot{V}O_{2peak}$] and severe-[95% ΔGET-$\dot{V}O_{2peak}$] intensity domains) the amplitude of the HR kinetics resulted of greater amplitude than the slow component of pulmonary $\dot{V}O_2$ kinetics (Jones et al. 2011; Grassi et al. 2015). As a consequence of this phenomenon, during exercise carried out for 15 minutes at a HR slightly above that corresponding to GET (GET +20%) (an intensity often recommended for aerobic exercise prescription [Riebe et al. 2008]), both work rate and $\dot{V}O_2$ had to decrease by approximately 14% and 10%, respectively, in order to maintain HR constant (Zuccarelli et al. 2018).

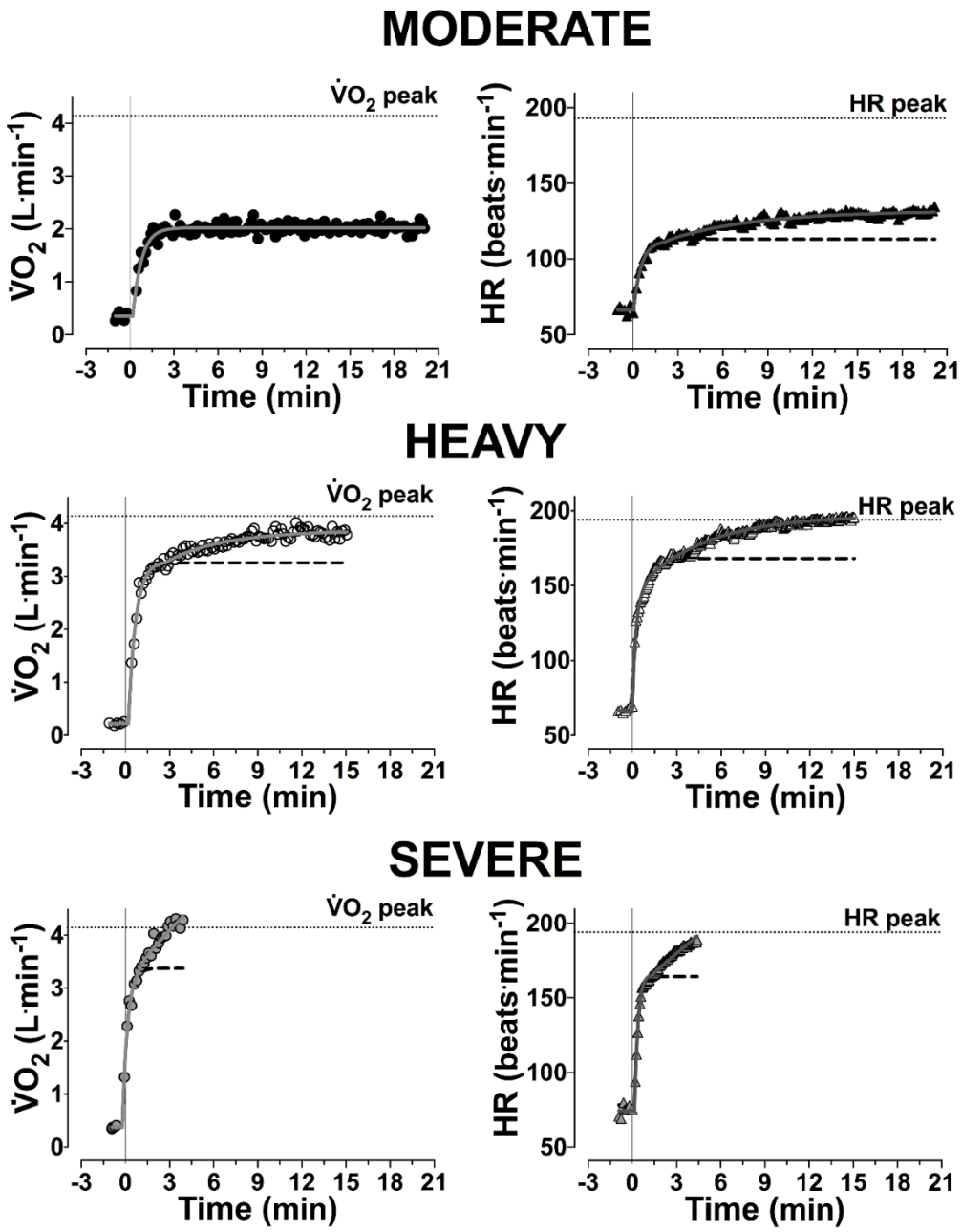

Figure 8. Pulmonary O_2 uptake ($\dot{V}O_2$) and heart rate (HR) kinetics for a representative young healthy subject during constant work rate exercises in the moderate- (<GET), heavy- (>GET, <CP), severe-intensity (>CP) domains. The dashed lines indicate the asymptotes of the fundamental component (from Zuccarelli et al. 2018).

This observation represents a sort of a "mirror image" of the slow component of the HR kinetics. Overall, these data suggest that exercise prescription at a fixed submaximal HR is not associated with a specific metabolic rate or intensity, making the approach questionable in physiological and practical terms. The presence of the above-mentioned phenomenon was confirmed also in a group of obese adolescents (Zuccarelli et al. 2021). More specifically, a slow component of HR kinetics was observed during both moderate- (80% GET) and heavy-intensity (120%) constant work rate exercises, whereas both work rate (~-20%) and $\dot{V}O_2$ (~-10%) had to decrease in order to keep HR constant (at a value slightly higher than that corresponding to GET [120% GET]) during a 15-min task (Zuccarelli et al. 2021) (**Figure 9**). An interesting result of the present study was that after a 3-week multidisciplinary body mass reduction program (including moderate-intensity exercise), the HR slow component during exercise <GET was eliminated, whereas the amplitude of the HR (and $\dot{V}O_2$) slow component during exercise >GET or conversely the decrease in work rate (and $\dot{V}O_2$) at a fixed HR corresponding to value slightly higher than GET were significantly reduced (Zuccarelli et al. 2021). These results suggest that the phenomenon (slow component of HR kinetics or decrease in work rate for the same fixed HR) is influenced by aerobic fitness level, and may represents a biomarker of exercise tolerance and oxidative metabolism. It is indeed a well-accepted concept, present in textbooks (Astrand et al. 1986; McArdle et al. 1986; Wasserman et al. 1999; Clausen, 1977), that a decreased HR for the same

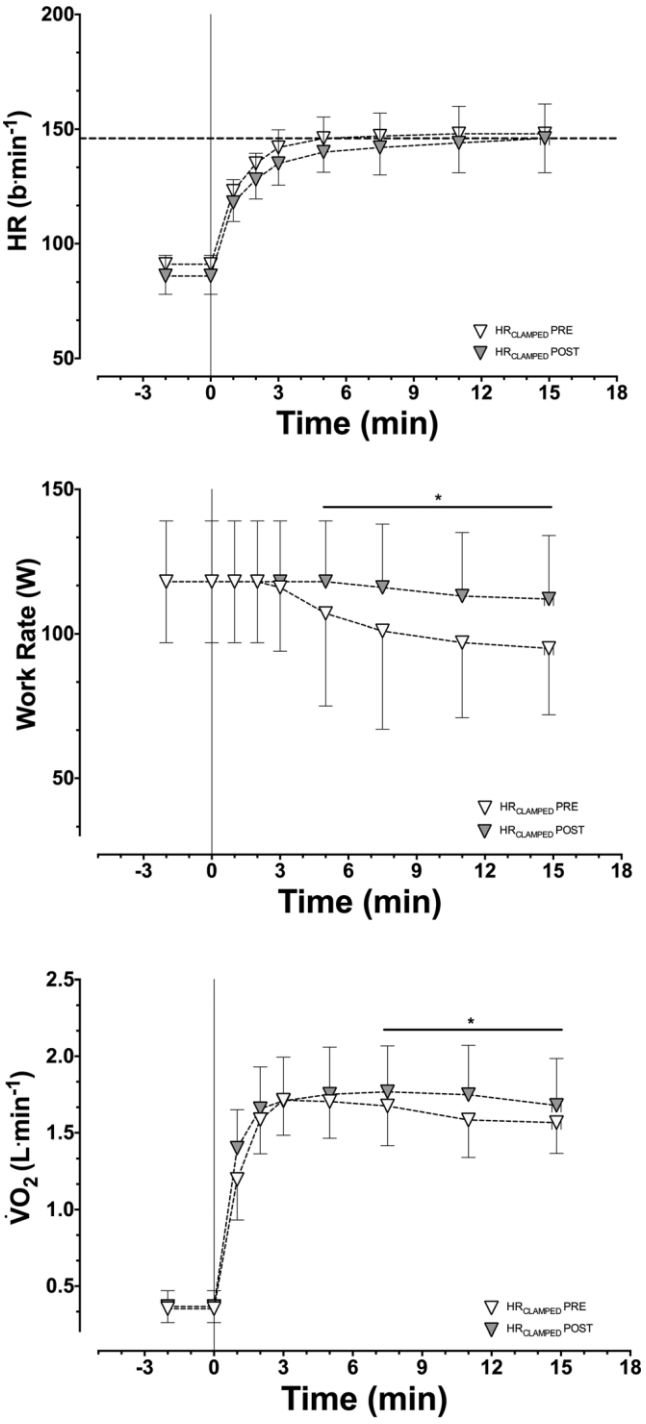

Figure 9. Mean (±SD) values of HR, work rate, and $\dot{V}O_2$ during exercise at a fixed HR ($HR_{CLAMPED}$) exercise before (PRE) and after (POST) the 3-wk multidisciplinary body mass reduction intervention in obese patients. Vertical lines indicate that exercise started at time 0. The horizontal dashed line indicates the mean HR target value. *Significantly different from PRE ($P < 0.05$) (from Zuccarelli et al. 2021).

work rate is a sign of improved exercise tolerance; conversely, a lower work rate for the same HR can be considered a sign of impaired exercise tolerance as well. Moreover, given the ease and reliability of this approach, it could be potentially useful to evaluate exercise tolerance also in particular conditions, such as spaceflights, permanence on planetary habitats, or in patients with chronic diseases. This topic will specifically be addressed in the present thesis (*Study 1* and *3*).

The mechanism(s) responsible for the decrease in work rate during exercise at a fixed HR (or for the HR slow component during constant work rate exercise) have not been elucidated yet, and it's not clear if the slow component of HR kinetics is also somehow related to fatigue. However, considering the different behaviours between HR and $\dot{V}O_2$ kinetics, their slow components likely recognize different mechanistic bases, and effects of blood catecholamines (Zuccarelli et al. 2021; Orizio et al. 1988) or body temperature (Zuccarelli et al. 2021, González-Alonso et al. 1997) on HR were hypothesized.

1.3 PERIPHERAL BIOMARKERS OF FUNCTIONAL EVALUATION OF OXIDATIVE FUNCTION

Microvascular / endothelial function

The measurement of the increase in blood flow (i.e., hyperemia) in the common femoral artery, by Doppler ultrasound, during 1 minute of passive knee flexion–extension (PLM), has been recently established as a useful method to assess peripheral vascular and endothelial functions (Gilford & Richardson, 2017). A great deal of evidence indicates that the PLM response is primarily a product of changes in peripheral arterial diameter/tone, and subsequent studies have revealed an important role for endothelium (Trinity et al. 2012; Mortensen et al. 2012). More specifically, endothelial nitric oxide (NO) bioavailability has been demonstrated to be up to 80% responsible for the vasodilatory response after PLM in healthy young subjects (Trinity et al. 2012; Mortensen et al. 2012). Thus, it seems that NO release and other unknown vasodilatory mechanisms are initiated in response to the mechanical perturbation of the leg associated with the passive movement, which quickly results in the dilation of the vascular bed. Moreover, an additional stimulus for further vasodilation is likely determined by the concomitant increases in perfusion pressure and shear stress.

The parameters obtained with the PLM technique were also well correlated with those obtained with invasive or more technically challenging methods considered as "gold standard" for the evaluation of peripheral vascular function, such as intra-arterial infusion of acetylcholine (ACh, a molecule with an endothelium-dependent vasodilatory action [Mortensen et al. 2012]), or flow-mediated vasodilation by Doppler ultrasound (FMD) (Rossman et al. 2016).

The hyperemic response during PLM is accentuated following training, whereas it is blunted in elderly subjects and in patients with various pathological conditions, mainly cardiovascular, in which it seems to represent an early sign of endothelial and cardiovascular impairment (Gilford & Richardson, 2017). More recently, we have also successfully used the PLM method in healthy volunteers undergoing a 10-day bed rest period, aimed at simulating microgravity conditions (Zuccarelli et al. 2021). In these subjects, after bed rest, a reduction and attenuation of increased blood flow in the common femoral artery was observed during PLM, suggesting an early impairment of microvascular/endothelial function following simulated microgravity (Zuccarelli et al. 2021) (**Figure 10**). This allowed to identify, in the measurement of blood flow during PLM, a useful biomarker of functional impairment of oxidative metabolism also following inactivity/exposure to microgravity.

Interestingly, the increases in blood flow during PLM observed in our young subjects after 10 days of bed rest were not substantially different from that described by Gifford & Richardson (2017) in subjects of 60–70 years of age.

Thus, the PLM test provides a simple, non-invasive method to measure *in-vivo* peripheral vascular and endothelial functions, which could be easily used both in healthy and diseased populations.

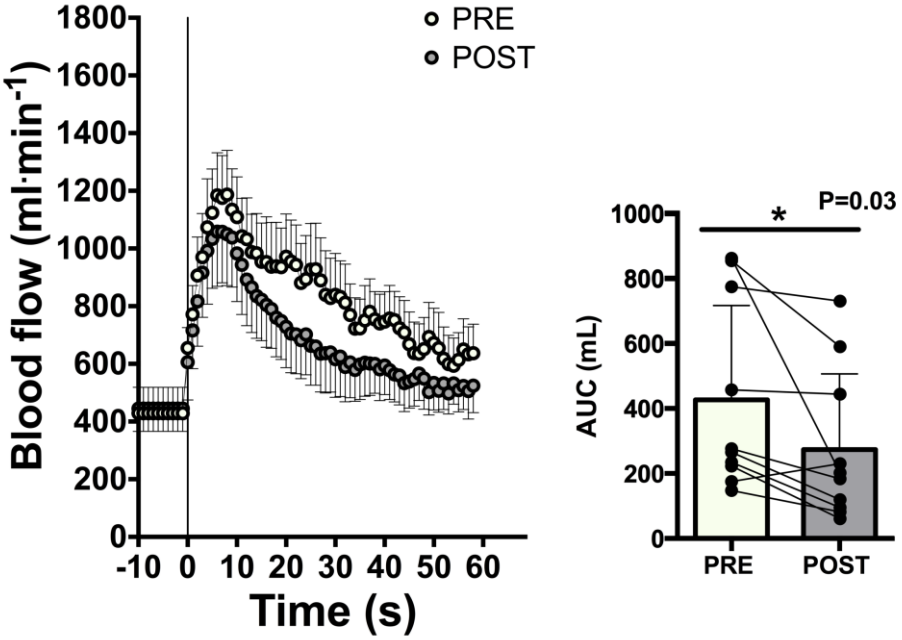

Figure 10. In the left panel, mean values of blood flow in the common femoral artery at rest and in response to 1-min passive leg movement (PLM), before (PRE) and after (POST) 10 days of bed rest are shown. The vertical line indicates the onset of PLM. Data are presented as means ± SEM. In the right panel, individual and mean values of the area under the curve (AUC) in response to PLM are given. Data are presented as means ± SD. * indicates the presence of a statistically significant difference (from Zuccarelli et al. 2021).

Near infrared spectroscopy: skeletal muscle fractional O_2 extraction, intramuscular matching of O_2 delivery / O_2 uptake, muscle O_2 uptake recovery kinetics

Near-infrared (NIR) spectroscopy (NIRS) is a powerful non-invasive tool which allows to gain insights into skeletal muscle oxidative metabolism during exercise, both in health and disease (Grassi & Quaresima, 2016; Barstow, 2019). NIRS utilizes the oxygen-dependent absorption of NIR light (~700-900 nm) in small vessels (small arterioles, capillaries and venules) by chromophores (i.e., hemoglobin [Hb] and myoglobin [Mb]), to determine their concentrations, and thus tissue oxygenation. When the probe is attached to the skin overlying the muscle of interest, NIRS instruments can interrogate only a relatively small (2 to 6 cm^3) and superficial volume of skeletal muscle tissue. Generally, the depth of penetration of the NIR light in tissues approximately corresponds to half of the distance between the light source and the detector (~3-4 cm). Therefore, a thick subcutaneous adipose tissue layer would preclude or at least reduce the relative contribution of the underlying skeletal muscle to the NIRS signals (Grassi & Quaresima, 2016). Several types of NIRS devices, based on different NIRS methods and with different characteristics, are commercially

available (Barstow, 2019). The earliest and still most common device is the single-distance continuous wave (CW) NIRS, where the light source is of constant intensity, and the transmitted light intensity is detected, providing only changes in light attenuation. It is important to appreciate that CW, do not provide absolute concentrations of chromophores in the interrogated tissue but rather relative micromolar (μM) changes in deoxygenated (Hb + Mb) concentrations (Δ[deoxy(Hb + Mb)]), oxygenated (Hb + Mb) concentrations (Δ[oxy(Hb + Mb)]), and total (Hb + Mb) (Δ[deoxy(Hb + Mb) + oxy(Hb + Mb)]), with respect to an initial value arbitrarily set equal to zero. This represents a limitation, which can be at least mitigated by performing a "physiological calibration" at the end of a test, by a transient ischemia of the investigated limb, obtained by applying for a few minutes a suprasystolic pressure (~250-300 mmHg) by a cuff, "upstream" of the region of investigation (Grassi & Quaresima, 2016).

An improvement to the original CW NIRS is represented by the spatially-resolved spectroscopy (SRS), where photons are measured at multiple distances from the source, and a quantitative absolute measure of tissue oxygenation (i.e., TSI, expressed as percent, %) is thus provided. Absolute values of the above-mentioned variables can be obtained by the more technologically sophisticated (and more expensive) time-resolved (TSR) / time domain (TD), or frequency-domain (FD) instruments (Grassi & Quaresima, 2016; Barstow, 2019).

Oxygenation variable obtained by NIRS allow the evaluation of the dynamic balance between O_2 extraction and O_2 delivery in the investigated volume of tissue, namely skeletal muscle fractional O_2 extraction, a proxy of $[CaO_2\text{-}C\bar{v}O_2]_m$ (Grassi & Quaresima, 2016). An increased Δ[deoxy(Hb + Mb)] or a decreased Δ[oxy(Hb + Mb)], would indicate an increased fractional O_2 extraction only when Δ[total (Hb + Mb)] is constant, which is unlikely in exercising muscles. The problem could be, at least in part, bypassed by taking as an index of deoxygenation the Δ[deoxy(Hb + Mb)] variable, which is relatively insensitive to changes in blood volume compared to changes in Δ[oxy(Hb+Mb)] (Adami et al. 2015).

The highest Δ[deoxy(Hb + Mb)] observed during an incremental exercise ($\Delta[deoxy(Hb+Mb)]_{peak}$) can be considered an estimate of the maximal capacity of O_2 extraction during exercise and is often utilized as an important variable of functional evaluation of skeletal muscle oxidative metabolism (Grassi & Quaresima, 2016).

NIRS can also be used to examine the intramuscular temporal and spatial matching between oxygen delivery ($\dot{Q}O_2$) and O_2 utilization ($\dot{V}O_2$) in the early phase of a constant work rate exercise (Grassi & Quaresima, 2016; Barstow, 2019). More specifically, a transient "overshoot" in Δ[deoxy(Hb+Mb)] following exercise onset would indicate a transient mismatch between $\dot{Q}O_2$ and $\dot{V}O_2$ (Koga et al. 2014), a phenomenon which has been repeatedly demonstrated by NIRS in diseased populations

(Sperandio et al. 2012; Porcelli et al. 2014) as well as in healthy population exposed to bed rest (Porcelli et al. 2010; Salvadego et al. 2016, 2018; Zuccarelli et al. 2021) (see **Figure 11**). The transient and sharp increase in fractional O_2 extraction would represent a mirror image of a sudden decrease in microvascular O_2 partial pressure (PO_{2mv}), which would reduce the driving pressure for peripheral O_2 diffusion and impair skeletal muscle oxidative metabolism (Poole et al. 2012).

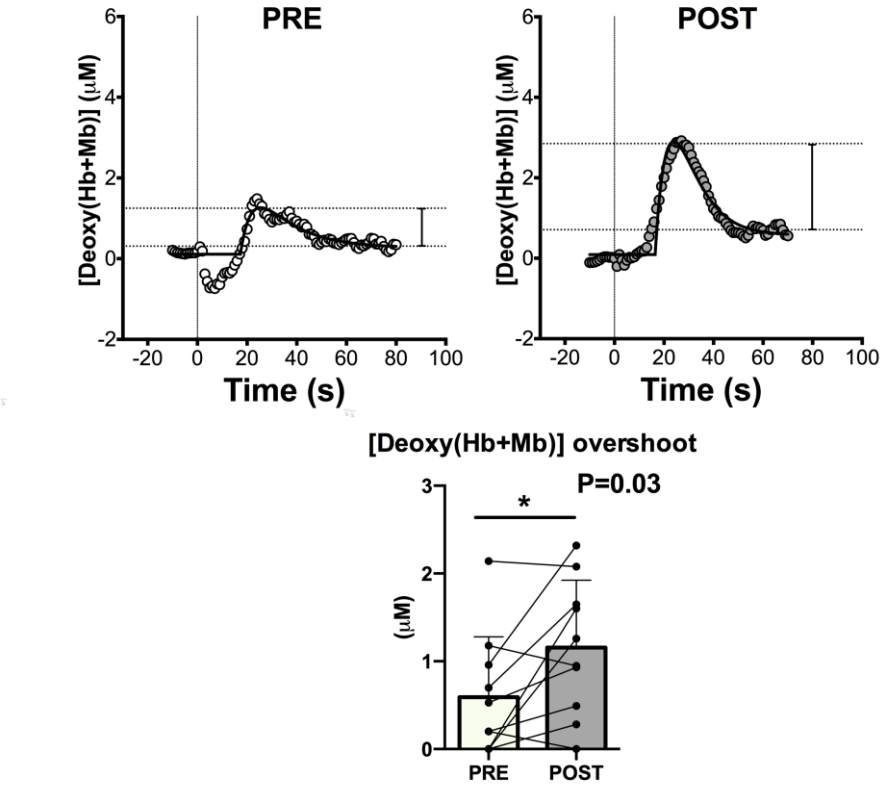

Figure 11. In the upper panels, typical examples of the dynamics of [deoxy(Hb+Mb)] during the first 60 s of a low-intensity constant work rate cycling exercise are shown before (PRE, left panel) and after (POST, right panel) bed rest in 10 young healthy subjects. The presence of a transient deoxygenation overshoot was checked by fitting the responses by two exponential equations (solid line). The amplitude of the overshoot is indicated by vertical distance between the asymptotes of the two equations (indicated by the horizontal dashed lines). In the lower panel, individual and mean (± SD) values of the overshoot data before (PRE) and after (POST) bed rest are shown. * indicates the presence of a statistically significant difference (from Zuccarelli et al. 2021)

Another important evaluation that can be performed with NIRS is the assessment of skeletal muscle O_2 uptake ($\dot{V}O_{2m}$). $\dot{V}O_{2m}$ measurement needs to be carried out during ischemic conditions at rest or immediately after submaximal exercise, because during a transient cuff occlusion the linear rate of increase of [deoxy(Hb+Mb)], or the linear rate of decrease of [oxy(Hb + Mb)] represents an index of $\dot{V}O_{2m}$ (Hamaoka et al 1996; Sako et al. 2001). Based on this concept, Ryan et al. (2012) proposed a method to estimate the skeletal muscle oxidative function (mitochondrial respiration) by determining

by NIRS the $\dot{V}O_{2m}$ recovery kinetics following muscle contractions with a series of repeated transient occlusions. The kinetics of recovery of $\dot{V}O_{2m}$, determined by the above-mentioned protocol, has been validated against two well-established variables of functional evaluation of skeletal muscle oxidative metabolism, namely the kinetics of recovery of [PCr], by ^{31}P-MRS and (Ryan et al. 2013) the maximal ADP-stimulated mitochondrial respiration, by high-resolution respirometry in isolated and permeabilized skeletal muscle fibers (Ryan et al. 2014). Moreover, our group recently demonstrated 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 (Zuccarelli et al. 2020). As far as we know, our group was the first one to successfully apply this non-invasive methodology in young healthy subjects undergoing a 10-d horizontal bed rest (Zuccarelli et al. 2021). What we observed was that the main parameters which describe the $\dot{V}O_2$m recovery kinetics, namely the time constant (τ) and the rate constant (k), were not affected by bed rest (see **Figure 12**), suggesting that skeletal muscle mitochondrial function was not compromised following a 10-d bed rest.

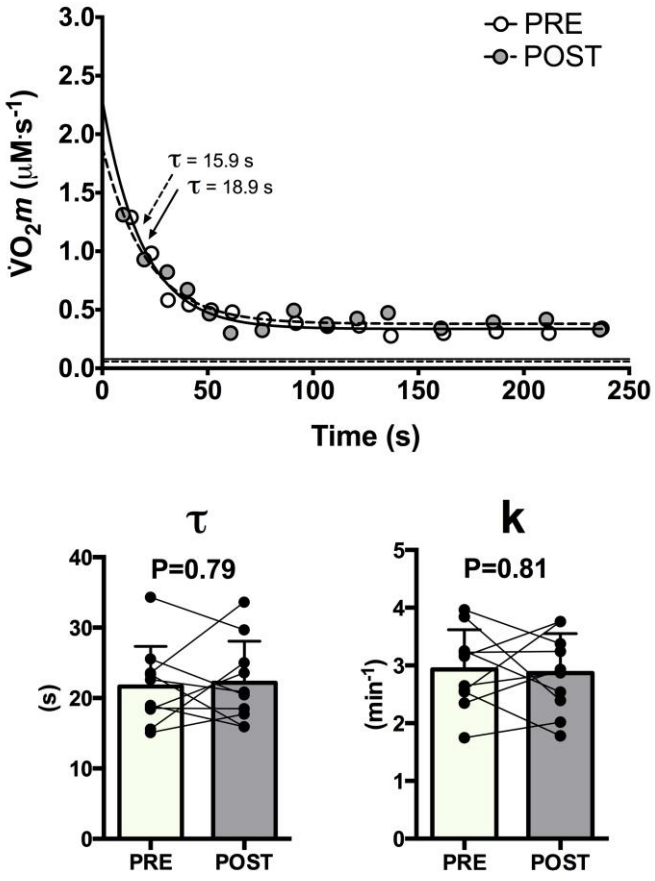

Figure 12. In the upper panel, muscle $\dot{V}O_2$ ($\dot{V}O_{2m}$) data during the recovery from moderate-intensity exercise are shown for a representative subject before (PRE) and after (POST) bed rest. The fitted functions and the calculated time constant (τ) values are also shown. In the lower panel, individual and mean (± SD) values of the time constant and rate constant (k) of muscle $\dot{V}O_2$ ($\dot{V}O_{2m}$) recovery kinetics before (PRE) and after (POST) bed rest are shown. No statistically significant differences were observed.

High-resolution respirometry and mitochondrial function

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 "gold standard" for the functional evaluation of mitochondrial respiration is high-resolution respirometry (HRR) (Pesta & Gneiger, 2012). With HRR small needle biopsies of skeletal muscle are exposed, in a chamber of the instrument, to a multiple substrate-uncoupler-inhibitor titration (SUIT) protocol in the presence of saturating levels of O_2, in order to avoid experimental oxygen limitation of respiration. Some of the variables that can be determined by HRR are: "leak respiration", which represents the non-phosphorylating resting mitochondrial respiration mainly driven by the back leakage of protons through the inner mitochondrial membrane; maximal ADP-stimulated O_2 flux, supported by respiratory complex I or by respiratory complexes I and II (OXPHOS capacity); maximal uncoupled respiration (electron transport system [ETS] capacity); oxidative phosphorylation coupling (ratio between OXPHOS and LEAK); and others more depending on the selected protocol (Pesta & Gneiger, 2012). Mitochondrial respiration variables are generally 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. 2012), subjects undergoing short-term bed rest (Zuccarelli et al. 2021) 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).

1.4 IMPAIRMENTS OF OXIDATIVE METABOLISM FOLLOWING INACTIVITY / MICROGRAVITY (BED REST)

Bed rest (BR) studies are widely used as experimental models for evaluating the effects of prolonged muscle disuse and unloading (Fortney et al. 1996; Pavy-Le Traon et al. 2007), a condition commonly experienced by astronauts exposed to microgravity or patients with chronic diseases.

In a recent meta-analysis by Reid-Larsen et al. (2017), which included ~80 strict BR studies, a linear decrease in $\dot{V}O_{2max}$ (index of the "cardiorespiratory fitness") as a function of BR duration (ranging from 1 to 90 days) was reported, resulting in a rate of decrease of ~0.3-0.4% per day (**Figure 13**). A considerably similar result was observed by Krogh-Madsen et al. (2010), who described a ~7% reduction in $\dot{V}O_{2max}$ (-0.5% per day) after 2 weeks of daily step reduction from ~10.000 to ~1500. These results are alarming considering the clinical relevance of $\dot{V}O_{2max}$ in relation to the risk of morbidity and mortality (Myers et al. 2002; Booth et al. 2017).

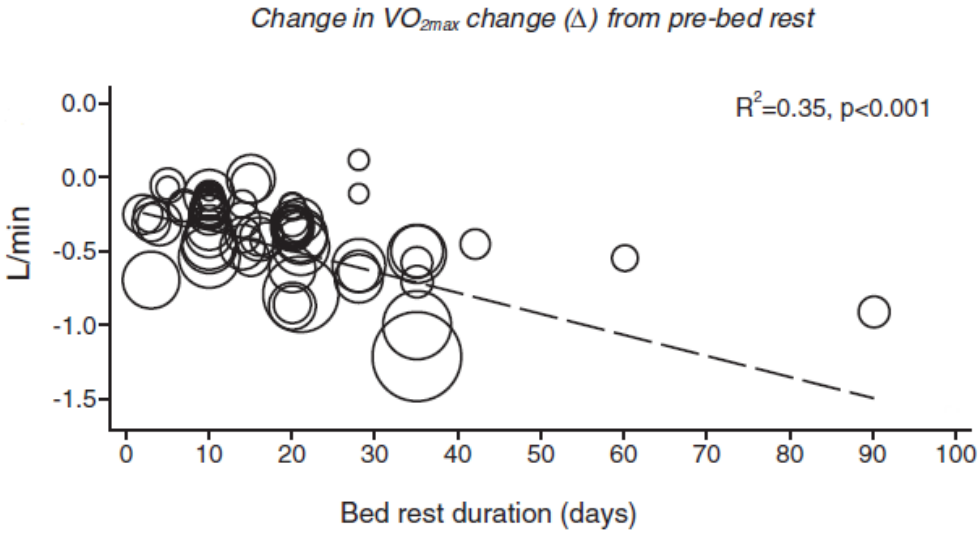

Figure 13. Changes in $\dot{V}O_{2max}$ (expressed in L/min) over bed rest duration. The area of each circle is inversely proportional to the variance of the estimate. The linear regression is also shown (modified from Reid-Larsen et al. 2017).

The reduction in $\dot{V}O_{2max}$ after BR (lasting for up to ~40 days) is associated with a higher percent decrease in $\dot{Q}_{max}$, essentially due to reductions in maximal stroke volume (SV_{max}), and substantially unchanged maximal capacity of O_2 extraction by skeletal muscle ($C(a\text{-}\bar{v})O_{2max} = \dot{V}O_{2max} / \dot{Q}_{max}$) (see e.g., Ferretti et al. 1997; Convertino, 1997; Saltin et al. 1968; Capelli et al. 2006). Therefore, the cardiovascular system plays the main role in limiting $\dot{V}O_{2max}$ also after BR.

A decreased SV was also reported during submaximal workloads (Capelli et al. 2006), accompanied by an increased HR (Saltin et al. 1968), resulting in a substantially constant $\dot{Q}$ (Porcelli et al. 2010).

The maximal sustainable metabolic rate for prolonged period (i.e., anaerobic threshold or GET) was reported to decrease after BR, and similarly (percentagewise) to $\dot{V}O_{2max}$ (Convertino et al. 1986; Porcelli et al. 2010), such that GET expressed as a percentage of $\dot{V}O_{2max}$ was unchanged before *vs.* after BR (Porcelli et al. 2010). Therefore, the involvement of anaerobic glycolysis occurs earlier following bed rest, at lower work rates, reducing the work capacity to sustain prolonged exercise without incurring in fatigue.

The $\dot{V}O_2$ kinetics during moderate intensity (<GET) constant work rate exercise were significantly slower after a 35-d bed rest (Porcelli et al. 2010), results in agreement with the larger O_2 deficit described by Convertino et al. (1984) after only 7 day of bed rest. A slower $\dot{V}O_2$ kinetics and thus greater O_2 deficit is associated with a lower metabolic stability (higher PCr and glycogen breakdown, and higher accumulation of lactate and H^+), which negatively affects exercise tolerance (Grassi et al. 2006, 2011).

In addition to central factors (mainly cardiovascular), other aspects localized at a more distal level along the O_2 transport pathway may play a role in impairing oxidative function and thus exercise tolerance. More specifically, it seems that the impact of peripheral factors increases as the duration of microgravity exposure is extended (Capelli et al. 2006; Ferretti & Capelli, 2009; Ade et al. 2015, 2017). Previous studies by our group confirmed the presence of peripheral limitations to oxidative metabolism following BR. Salvadego et al. (2016, 2018), for instance, observed, after both 10 and 21 days of BR, a decreased $\dot{V}O_{2max}$ during dynamic knee-extension incremental exercise, during which central cardiovascular constraints are reduced because of the recruitment of a relatively small muscle mass (i.e., the quadriceps femoris muscle) (Richardson et al. 1993). Also, following BR of different duration (from 10 to 35 days) our group reported indirect signs of impaired microvascular function as demonstrated by the presence of a more pronounced transient "overshoot" of muscle O_2 fractional extraction, evaluated by NIRS, in the early phase of constant work rate exercises (Porcelli et al. 2010; Salvadego et al. 2016, 2018; Zuccarelli et al. 2021). This phenomenon represents an inadequate matching between intramuscular O_2 delivery and O_2 uptake (Koga et al. 2014) which is associated with a reduced PO_{2mv} and thus impaired peripheral O_2 diffusion (Poole et al. 2012).

The negative impact of BR/disuse on peripheral vascular function was also confirmed by a blunted blood flow response during the PLM method (Zuccarelli et al. 2021, see paragraph above "Microvascular / endothelial function") or after intra-arterial infusion of acetylcholine (an endothelial-dependent vasodilator) (Hesse et al. 2005; Rytter et al. 2020) and by a decreased reactive hyperemia after blood flow occlusion (Hamburg et al. 2007; Kamiya et al. 2000; Birk et al. 2013; Shoemaker et al. 1998).

As regard skeletal muscle mitochondrial respiration, which represents the last step along the O_2 pathway of oxidative metabolism, results following short BR exposures are controversial. An impaired mitochondrial function (determined *ex-vivo* in permeabilized vastus lateralis by high-resolution respirometry [Pesta & Gnaiger, 2012]) was described by Miotto et. (2019) and Dirks et al. (2020) after bed rest periods of 3 and 7 days, respectively; however, no changes were described by other authors after 10 (Salvadego et al. 2016; Zuccarelli et al. 2021) and 4 days (Larsen et al. 2018) of BR exposure. On the contrary, reduced maximal ADP-stimulated mitochondrial respiration was seen after longer BR period (i.e., 21 days) (Salvadego et al. 2018).

The absence of impairments of mitochondrial respiration, observed *ex-vivo* by HRR after relatively short bed rest period (10 days), was also confirmed *in-vivo*, by Zuccarelli et al. (2021) by the analysis of the kinetics of $\dot{V}O_{2m}$ recovery following cycle ergometer exercise, carried out by NIRS with the repeated occlusions method (Ryan et al. 2012; Zuccarelli et al. 2020; see paragraph above "Near infrared spectroscopy: skeletal muscle fractional O_2 extraction, intramuscular matching of O_2 delivery / O_2 uptake, muscle O_2 uptake recovery kinetics).

An unexpected and interesting finding of the same study (Zuccarelli et al. 2021) was related to the resting $\dot{V}O_{2m}$, measured non-invasively by calculating the linear slope of muscle deoxygenation (determined by NIRS) during a transitory limb ischemia induced by the rapid inflation of a pneumatic cuff. A significant decrease (of about 25%) in resting $\dot{V}O_{2m}$ was observed after bed rest. This result could represent an adaptive phenomenon in response to simulated microgravity/inactivity, suggesting that catabolic processes induced by bed rest/inactivity are less energy-consuming than anabolic ones (Zuccarelli et al. 2021).

Overall, the presented data clearly demonstrate that BR/inactivity determines a significant impairment of oxidative metabolism at different levels along the O_2 cascade, from the cardiovascular system to the mitochondria at skeletal muscle level. Thus, an early identification of the "sites" of impairment would be of utmost importance not only from a "basic science" point of view, but also for a correct identification of countermeasures and rehabilitation interventions.

2 CHAPTER II – AIM OF THE STUDIES

The main aim of the studies included in the present thesis was to evaluate functional biomarkers of impaired oxidative metabolism and exercise (in)tolerance in condition of simulated microgravity (bed rest) and in patients with cardiovascular disease. Particular attention was paid to "systemic" biomarkers, and specifically to a new variable recently identified by our group (Zuccarelli et al. 2018, 2021), namely the quantification of the decrease in work rate during cycle ergometer exercise at a fixed heart rate (HR), set at a level usually utilized for training purposes. The approach seems to be physiologically "sound", is non-invasive, simple and easy to administer, moreover it offers the advantage of being based on the measurement of variables (work rate, HR) which can be determined with precision also on the field. Another significant advantage is that the measurements could be performed, in a simplified procedure, by utilizing instruments of low economic cost. The new proposed approach could represent a significant advantage compared to some methods presently utilized, and could be of significant impact both on exercise evaluation and exercise prescription (Zuccarelli et al. 2018, 2021).

In two previous studies we applied the proposed approach (work rate decrease at a fixed HR) on young physically active healthy subjects (Zuccarelli et al. 2018) and on obese adolescents (Zuccarelli et al. 2021). The two studies discussed in the present thesis, on the other hand, deal with healthy young subjects exposed to profound inactivity (bed rest, a condition simulating microgravity exposure) (*Study 1*) and patients with coronary artery disease (*Study 2*). In both experimental models both central and peripheral factors may contribute to the impairment of oxidative metabolism, increasing the risk of mortality and negatively affecting the subjects/patients' quality of life. In both populations identification and quantification of the functional impairment would be of utmost importance for the evaluation of the health status as well as for the definition of possible therapeutic or rehabilitative interventions. *Study 1* was associated with another study (Zuccarelli et al. 2021) which concentrated its attention on "peripheral" biomarkers (endothelial/microvascular function, skeletal muscle oxidative metabolism and mitochondrial respiration), thereby allowing a comprehensive evaluation of oxidative metabolism at different levels along the pathway of O_2 from ambient air to skeletal muscle mitochondrial respiration. *Study 2* (coronary artery disease patients) specifically compared patients treated or not treated with beta-blockers, thereby allowing insights into one of the mechanisms (beta-adrenergic stimulation) potentially involved in determining the phenomenon of work rate decrease at a fixed heart rate.

3 CHAPTER III – EXPERIMENTAL STUDIES

3.1 DECREASE IN WORK RATE IN ORDER TO KEEP A CONSTANT HEART RATE: BIOMARKER OF EXERCISE INTOLERANCE FOLLOWING A 10-DAY BED REST – *STUDY 1*

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Running head: Decrease in work rate at a fixed heart rate after bed rest

ABSTRACT

Aerobic exercise prescription is often set at specific heart rate (HR) values. Previous studies demonstrated that during exercise carried out at a HR slightly above that corresponding to the gas exchange threshold (GET), work rate (WR) has to decrease in order to maintain HR constant. We hypothesized a greater WR decrease at a fixed HR following simulated microgravity/inactivity (bed rest, BR). Ten male volunteers (23±5 yr) were tested before (PRE) and after (POST) a 10-day horizontal BR, and performed on a cycle ergometer: a) incremental exercise; b) 15-min $HR_{CLAMPED}$ exercise, in which WR was continuously adjusted to maintain a constant HR, corresponding to that at 120% of GET determined in PRE; c) two moderate-intensity constant WR (MOD) exercises. Breath-by-breath $\dot{V}O_2$, HR and other variables were determined. After BR, $\dot{V}O_{2peak}$ and GET significantly decreased, by about 10%. During $HR_{CLAMPED}$ (145±11 $b \cdot min^{-1}$), the decrease in WR needed to maintain a constant HR was greater in POST *vs.* PRE (-39±10 *vs.* -29±14%, $p<0.01$). In 6 subjects the decreased WR switched from the heavy- to the moderate-intensity domain. The decrease in WR during $HR_{CLAMPED}$, in PRE *vs.* POST, was significantly correlated with the $\dot{V}O_{2peak}$ decrease ($R^2=0.52$; $p=0.02$). A greater amplitude of the slow component of the HR kinetics was observed

during MOD following BR. Exercise at a fixed HR is not associated with a specific WR or WR domain; the problem, affecting exercise evaluation and prescription, is greater following BR. The WR decrease during $HR_{CLAMPED}$ is a biomarker of exercise intolerance following BR.

NEW & NOTEWORTHY

During a 15-min exercise carried out at a heart rate (HR) slightly above that corresponding to the gas exchange threshold, in order to keep HR constant work rate significantly decreased, and the decrease was more pronounced after a 10-day horizontal bed rest. The work rate decrease at a fixed HR can be considered a systemic biomarker of exercise intolerance during microgravity/inactivity, and could be easily and reliably determined also during spaceflights, or in patients.

Key words: EXERCISE PRESCRIPTION, EXERCISE TOLERANCE, MICROGRAVITY, DECREASED PHYSICAL ACTIVITY, BED REST.

INTRODUCTION

In order to achieve the best results in terms of health status and physical performance, exercise prescription must be personalized in terms of intensity, volume and frequency (Riebe et al. 2018). For submaximal "aerobic" exercise, training intensity is usually prescribed at specific heart rate (HR) values (Riebe et al. 2018; Powers & Howley, 2004) mainly because of the practicality of this approach, since HR can be easily measured and recorded. This common practice, however, is based on the notion of a linear relationship between HR and work rate (Astrand et al. 1986), which represents an erroneous oversimplification. In young physically active adults, a slowly developing increase in HR, which delays the attainment of a steady state (traditionally termed ''slow component'') is present during constant work rate moderate-intensity exercise (below the gas exchange threshold [GET]) (Zuccarelli et al. 2018). Zuccarelli et al. (2018) demonstrated that the slow component of HR kinetics occurs at a lower work rate, and for the same work rate is of greater amplitude than the slow component of pulmonary O_2 uptake ($\dot{V}O_2$) kinetics (Jones et al. 2011; Grassi et al. 2015). This HR slow component translated into the observation that during exercise carried out for 15 minutes at a HR slightly above that corresponding to GET (GET +20%) (an intensity often recommended for aerobic exercise prescription [Riebe et al. 2018]), work rate had to decrease by approximately 14% in order to maintain HR constant (Zuccarelli et al. 2018). Thus, exercise prescription at a fixed submaximal HR is not associated with a specific work rate, making the

approach questionable in physiological and practical terms. A similar phenomenon to that described by Zuccarelli et al. (2018) was observed in obese adolescents (Zuccarelli et al. 2021). Interestingly, in obese adolescents the decrease in work rate was greatly reduced after a 3-week body mass reduction training program (Zuccarelli et al. 2021). This suggests that the phenomenon is influenced by aerobic fitness level, and confirms that it represents a biomarker of exercise tolerance. It is indeed a well-accepted concept, present in textbooks (Astrand et al. 1986; McArdle et al. 1986; Taylor & Groeller, 2008; Wasserman et al. 1999), that a decreased HR for the same work rate is a sign of improved exercise tolerance; conversely, a lower work rate for the same HR can be considered a sign of impaired exercise tolerance as well.

In the present study we aimed to apply the same approach utilized in our previous studies (Zuccarelli et al. 2018, 2021) to subjects exposed to bed rest (BR), in whom we have been aiming to identify peripheral (Zuccarelli et al. 2021) and "systemic" (present study) biomarkers of exercise intolerance. The working hypothesis of the present study is that the work rate decrease needed to keep HR constant at a value corresponding to 120% of GET, being related to exercise tolerance, would be more pronounced following a 10-day (BR) period. BR studies are widely utilized to simulate exposure to microgravity, but at the same time they allow to study the effects of prolonged and profound inactivity (Pavy-Le Traon et al. 2007). It is well documented that BR determines a significant impairment of exercise tolerance and oxidative metabolism at several levels along the O_2 pathway from ambient air to the mitochondria of skeletal muscles (Zuccarelli et al 2021; Saltin et al. 1968; Ferretti et al 1997; Porcelli et al, 2010; Ade et al. 2015; Salvadego et al. 2011, 2016, 2018; Ried-Larsen et al. 2017; Dirks et al. 2019). Several studies by our group (Procelli et al. 2010; Salvadego et al. 2011, 2016, 2018) specifically focused on biomarkers of impaired skeletal muscle oxidative metabolism, confirming the presence of impairments distal to cardiovascular O_2 delivery.

The work rate decrease at a fixed HR could then represent a "systemic biomarker" of the impaired exercise tolerance and of the impairment of oxidative metabolism following microgravity (and inactivity). The approach would be based on variables (work rate, HR) which can be easily determined with precision also in particular conditions, such as spaceflights, permanence on planetary habitats, or in patients with chronic diseases. Moreover, if confirmed also following microgravity/inactivity, the phenomenon mentioned above (work rate decrease at a fixed HR) would stress the need to reconsider the whole approach to exercise prescription and exercise evaluation in these conditions.

MATERIALS AND METHODS

Subjects

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, 23.5 ± 2.5 $kg \cdot m^{-2}$. None of the participants was engaged in competitive sports activities or followed specific training programs before the study. Subjects were informed about the aims, procedures and possible risks of the investigations before giving their written informed consent to participate. The study was approved by the National Committee for Medical Ethics at the Ministry of Health of the Republic of Slovenia (No. 0120-304/2019/9, 17th of July 2019), and conformed to the Declaration of Helsinki (2000).

Experimental protocol

The present study was part of the Italian Space Agency (ASI) Project "MARS-PRE", 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 impairments or of an altered state of health.

Subjects were tested before (PRE) and after (POST) a 10-day horizontal BR without countermeasures, carried out at the General Hospital of Izola, Slovenia. During the BR neither deviations from the lying position nor muscle stretching or static contractions were allowed. Adherence to the assigned protocol was ensured using continuous closed-circuit television surveillance and constant supervision by researchers and medical staff. 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 the reambulation.

All tests were conducted under continuous medical supervision and 12-lead electrocardiography (Quark C12x, Cosmed, Rome, Italy). Before data collection, participants were allowed enough time to become familiar with the researchers and with the setup environment, as well as with the experimental protocol, by performing short preliminary practice runs. During the first day the participants completed an incremental exercise on an electronically braked cycle ergometer (Monark 818E; Stockholm, Sweden) to determine $\dot{V}O_{2peak}$, GET and RCP. Pedaling frequency was digitally displayed to the subjects, who were asked to keep a constant cadence throughout the tests between 70 and 80 rpm. The protocol consisted of 20-30 W increases every minute, preceded by an initial minute at 0-40 W (depending on the participant's fitness level), aiming to allow the subjects to reach voluntary exhaustion in ~10-15 minutes. Voluntary exhaustion was defined as the incapacity to

maintain pedaling frequency for 5 s at the imposed work rate despite vigorous encouragement by the researchers. During the second day the subjects performed, after an initial 2-min of very-low intensity cycling exercise (about 30 W), a 15-min ‘‘HR-controlled’’ exercise ($HR_{CLAMPED}$), in which work rate was continuously adjusted to maintain a constant HR, equivalent to about 120% of GET in PRE (4). During the exercise 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 by decreasing/increasing 2 W every 5 s in order to maintain HR constant throughout the remaining part of the exercise (4). On the same day participants performed also two repetitions of a 10-min constant work rate (CWR) exercise at a moderate intensity corresponding to 80% of GET determined in PRE, preceded by 2-min of a very low-intensity (about 30 W) exercise. Each repetition was separated by a 15-min recovery period.

Measurements

Pulmonary ventilation ($\dot{V}E$), O_2 uptake ($\dot{V}O_2$), and CO_2 output ($\dot{V}CO_2$) were assessed 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 three different flow rates. Calibration of O_2 and CO_2 analyzers was performed before each experiment by utilizing gas mixtures of known composition (O_2: 16%, CO_2: 4%). Gas exchange ratio (R) was calculated as $\dot{V}CO/\dot{V}O_2$. GET and RCP were determined using both the “V-slope” method and “secondary criteria” (Beaver et al. 1986). To identify the work rate and HR corresponding to $\dot{V}O_2$ 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 (Whipp et al. 1981) previously obtained by our group in a similar population of subjects exposed to bed rest (Porcelli et al. 2010).

Net mechanical efficiency for oxidative metabolism (ME_{OX}, %) was calculated for each minute during $HR_{CLAMPED}$ exercise as the ratio between average external mechanical power output (work rate, expressed in watt) and oxidative energy output above resting level (E, expressed in watt) (Lafortuna et al. 2006), which was in turn computed as follows:

$$E = (4.94 \cdot R + 16.04) \cdot \dot{V}O_{2net} / 60 \quad \text{(Equation 1) (Garby et al. 1987)}$$

Net $\dot{V}O_2$ ($\dot{V}O_{2net}$, $mL \cdot min^{-1}$) was obtained by subtracting resting $\dot{V}O_2$ from the total $\dot{V}O_2$ measured during each minute. The functional gain ($\Delta\dot{V}O_2/\Delta$work rate) was calculated for each minute of exercise as an additional estimate of the energy cost of exercise. HR was recorded by a chest band (S610i; Polar, Kempele, Finland).

Stroke volume (SV) was estimated by impedance cardiography (PhysioFlow; Manatec, Paris, France) and cardiac output (CO) was calculated by multiplying SV and HR. Arterialized blood O_2 saturation (SpO_2) was monitored by pulse oximetry ($MicrO_2$; Siemens Medical Systems, Danvers, MA, USA) at the earlobe.

At the end of the incremental and the moderate CWR exercises, and at specific time points (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 and the moderate CWR exercises, and during the $HR_{CLAMPED}$ exercise (at 5, 10 and 15 minutes), 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 electroenzymatic analyzer (Biosen C-line; EKF, Cardiff, United Kingdom).

Oxygenation changes in 4 different sites of the anterior compartment of the right thigh were determined by a portable continuous-wave near infrared spectroscopy (NIRS) instrument (OctaMon M; Artinis Medical System, Elst, The Netherlands) (Barstow, 2019; Grassi & Quaresima, 2016), thereby allowing an evaluation of variables in different portions of the same muscle. The light transmitters-channels (which emitted 2 wavelengths at 760 and 850 nm) were separated by 35 mm from the receiving optode. The instrument non-invasively measures 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, obtained during the resting condition preceding the test. The sampling frequency was set at 10 Hz. The sum of the two variables (Δ[total(Hb+Mb)]) indicates 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)] indicate an increased fractional O_2 extraction in the tissue under consideration (Grassi & Quaresima, 2016). The Δ[deoxy(Hb+Mb)] signal was considered in the present study since it is usually less affected (with respect to the Δ[oxy(Hb+Mb)] signal) by changes in blood volume in the tissue (Grassi & Quaresima, 2016). The probe was firmly attached to the skin overlying the distal portion of the quadriceps femoris muscle, so that two light transmitters were on the vastus lateralis and the other two on the rectus femoris muscle. The skin overlying the investigated muscle regions was carefully shaven and cleaned before the experiments, and the place where the probe was attached was recorded using a skin marker, thereby allowing to position the probe in the same place during different experimental sessions. Adipose tissue thickness (ATT) at the site of application of the NIRS probe was measured by a caliper (Gima, Milan, Italy). Black bandages were put around the probe and the skin to prevent contamination from ambient light.

Mean values of ventilatory, pulmonary gas exchange, cardiovascular and muscle oxygenation variables were calculated during the last 20 s of each minute of exercise; values obtained during the exhausting work rate were considered peak values. Mean steady-state values of the main variable obtained during very low- and moderate-intensity CWR exercises were determined during the last 20 s of exercise. During $HR_{CLAMPED}$ exercise, mean values of measured variables were calculated during each minute of the exercise. To confirm the absence of changes in HR as a function of time ("$HR_{CLAMPED}$"), a linear regression from the second minute to the end of exercise was calculated. A slope not significantly different from zero would confirm that the variable was effectively kept constant throughout the exercise, as planned by the experimental protocol.

Kinetics analysis

$\dot{V}O_2$ kinetics were mathematically evaluated during transitions from the very low- to the moderate-intensity CWR exercise. Breath-by breath $\dot{V}O_2$ values obtained during exercise were time aligned and then superimposed for each subject (Lamarra et al. 2002). 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:

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

and parameter values (TD_f, τ_f) were determined that yielded the lowest sum of squared residuals. In equation 2, *t* is 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 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:

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

In equation 3, A_s, TD_s, and τ_s 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 using the F-test (see Statistical analysis). That is to say, when equation 3 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 (equation 4) was also used, with an exponential function for the fundamental component and a linear function for the slow component (exponential + linear fitting) (Linnarsson, 1974):

$$y(t) = y_{BAS} + A_f[1 - e^{-(t-TD_f)/\tau_f}] + S[t - TD_s] \qquad \text{(Equation 4)}$$

where S (slope) is the angular coefficient of the linear regression of $\dot{V}O_2$ versus 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 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 then calculated.

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 earlier for $\dot{V}O_2$.

Statistical analysis

Results are expressed as mean ± SD values. Statistical significance of differences between the two conditions (PRE *vs.* POST) was checked 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. When significant differences were found, a Bonferroni *post hoc* test was used to determine the exact location of the difference. Linear regression, correlation analysis and data fitting by exponential functions were carried out by the least-squared residuals method. Correlation analyses were expressed as Pearson coefficient (r). The goodness of fit and precision of the regression equation were evaluated using multiple coefficient of determination (R^2). Comparisons between fitting with different models were carried out using the F-test. 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

Body mass and body mass index significantly decreased ($p < 0.05$) during BR, by ~2% and ~3%, respectively.

Peak values of the main respiratory, cardiovascular and metabolic variables obtained during the incremental exercises are shown in **Table 1**. Peak work rate, $\dot{V}O_{2peak}$, CO_{peak} and SV_{peak} were significantly lower in POST *vs.* PRE. GET and RCP expressed as absolute $\dot{V}O_2$ values and as a percentage of $\dot{V}O_{2peak}$ are also shown in Table 1.

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**
Peak work rate (W)	251 ± 50	230 ± 41*
$\dot{V}O_{2peak}$ ($L \cdot min^{-1}$)	3.436 ± 0.673	3.039 ± 0.463***
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	44.4 ± 7.2	40.3 ± 6.1***
$\dot{V}CO_{2peak}$ ($L \cdot min^{-1}$)	4.020 ± 0.761	3.527 ± 0.557**
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*
V_{Tpeak} (L)	2.68 ± 0.45	2.71 ± 0.49
fR_{peak} ($breaths \cdot min^{-1}$)	57 ± 9	50 ± 7**
$PetO_{2peak}$ (mmHg)	118.9 ± 2.8	119.5 ± 3.1
$PetCO_{2peak}$ (mmHg)	31.9 ± 3.5	31.3 ± 3.8
HR_{peak} ($b \cdot min^{-1}$)	187 ± 8	189 ± 6
$\dot{V}O_{2GET}$ ($L \cdot min^{-1}$)	2.035 ± 0.373	1.764 ± 0.350*
$\dot{V}O_{2GET}$ ($\%\dot{V}O_{2peak}$)	59 ± 4	58 ± 7
Work $rate_{GET}$ (W)	102 ± 33	88 ± 31
HR_{GET} ($b \cdot min^{-1}$)	130 ± 10	136 ± 10
$\dot{V}O_{2RCP}$ ($L \cdot min^{-1}$)	2.967 ± 0.538	2.612 ± 0.460**
$\dot{V}O_{2RCP}$ ($\%\dot{V}O_{2peak}$)	87 ± 4	86 ± 5
SV_{peak} (mL)	134 ± 28	101 ± 17**
CO_{peak} ($L \cdot min^{-1}$)	25.2 ± 5.8	19.0 ± 3.2**
SpO_{2peak} (%)	98 ± 1	98 ± 1
$[La]_{b\ peak}$ (mM)	11.9 ± 2.7	11.2 ± 3.1
RPE_{peak} (6–20)	19 ± 1	19 ± 1

Data are means ± SD. $\dot{V}O_2$, 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; $\dot{V}O_{2GET}$, pulmonary oxygen uptake at GET; work $rate_{GET}$, work rate at GET; HR_{GET}, heart rate at GET; $\dot{V}O_{2RCP}$, pulmonary oxygen uptake at RCP; SV, stroke volume; CO, cardiac output; SpO_2, arterialized blood O_2 saturation by pulse oximetry; $[La]_b$, blood lactate concentration; RPE, rate of perceived exertion. Asterisks denote differences from PRE by means of Student's paired t-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

GET and RCP ($L \cdot min^{-1}$ of $\dot{V}O_2$) were significantly lower in POST *vs.* PRE; no significant differences in POST *vs.* PRE were observed when the two variables were expressed as a percentage of $\dot{V}O_{2peak}$.

Percentage-wise the GET and RCP decreases were very similar to that observed for $\dot{V}O_{2peak}$ (about -10%). HR_{peak} [corresponding to 94 and 96% of the age-predicted maximum values (calculated as 208 - 0.7 · 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 subjects exercised until the limit of tolerance. SpO_{2peak} values were normal and not significantly different in POST *vs.* PRE.

Adipose tissue thickness (ATT) at the site where NIRS probe was positioned was significantly ($p = 0.03$) lower in POST (4.7 ± 0.7 mm) *vs*. PRE (5.4 ± 1.3 mm). $\Delta[deoxy(Hb+Mb)]_{peak}$ of quadriceps femoris muscle, calculated as a mean of the values obtained at the 4 sites, was not different ($p = 0.70$) in POST (6.3 ± 2.0 μM) *vs*. PRE (6.1 ± 2.5 μM).

In **Figure 1** mean values of HR and work rate obtained during the $HR_{CLAMPED}$ exercises are shown. Both in PRE and POST, HR mean target value (set at 145 ± 11 $b \cdot min^{-1}$ [see horizontal dashed line], corresponding to 120% of GET in PRE) was reached within the first 2-3 minutes of exercise and remained constant throughout the test. The slopes of the individual linear regression lines drawn from the second to the last minute of exercise were not significantly different from zero ($p > 0.05$). Both in PRE and in POST work rate decreased in order to maintain HR constant, and reached a plateau after about 6 (in PRE) and 4 (in POST) minutes of exercise. The work rate decrease, in respect to the value obtained in the first minute of exercise, was more pronounced ($p < 0.01$) and occurred earlier in POST (-53 ± 13 W, corresponding to -39 ± 10 %) *vs.* PRE (-38 ± 15 W, corresponding to -29 ± 14%). The area under the curve of the work rate *vs.* time relationships (total amount of work performed) calculated from the first minute to the end of the $HR_{CLAMPED}$ exercise was significantly ($p < 0.001$) lower in POST (80 ± 22 kJ) *vs.* PRE (95 ± 32 kJ).

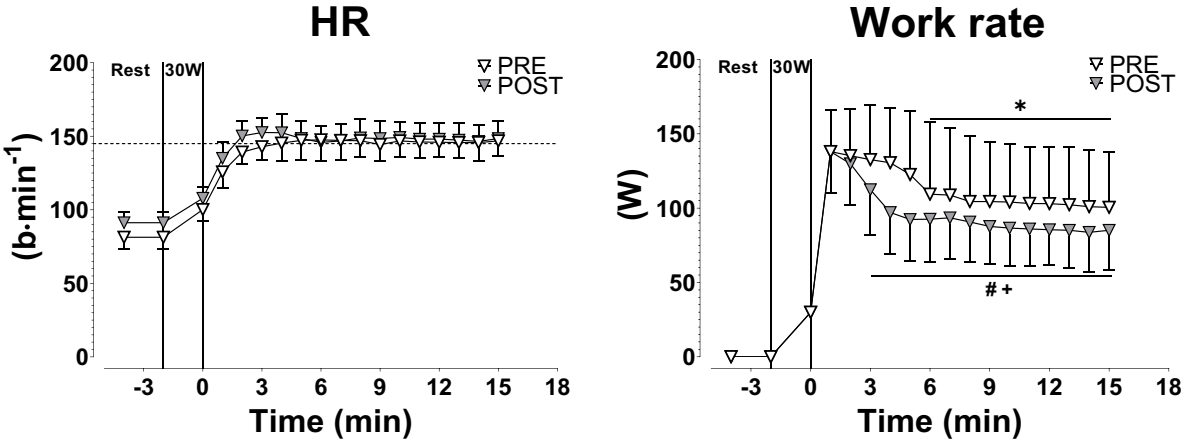

Figure 1. Mean (± SD) values of heart rate (HR) and work rate during $HR_{CLAMPED}$ exercises, before (PRE) and after bed rest (POST). The horizontal dashed line indicates the mean HR target value. *,# Statistically different from respective 1-min value ($p < 0.05$). + Statistically different from respective values in PRE ($p < 0.05$). See text for further details.

As shown in **Figure 2**, mean values of work rate decreased from a value above GET at the beginning of the $HR_{CLAMPED}$ exercise to a value corresponding to GET at the end of $HR_{CLAMPED}$, both in PRE and in POST. For about half of the subjects a change in work rate domain, from heavy- (above GET) to moderate- (below GET) occurred during the 15 minutes of $HR_{CLAMPED}$ exercise, both in PRE and in POST.

Mean values of other variables determined during the $HR_{CLAMPED}$ exercise are shown in **Figure 3**. The work rate decrease was accompanied by a significant decrease of $\dot{V}O_2$, R, $[La]_b$ and ME_{OX}, and by a significant increase of the functional gain (variable inversely proportional to ME_{OX}) during the 15-min exercise, both in PRE and in POST. In muscles of the anterior compartment of the thigh, a significant decrease of fractional O_2 extraction (mean values calculated over the 4 sites) was observed in both conditions. RPE remained substantially constant at a value of ~12 throughout the test both in PRE and POST (data not shown in the figure). Also SV and CO did not significantly change during the exercise, in both conditions; these values were slightly but not statistically lower in POST *vs*. PRE.

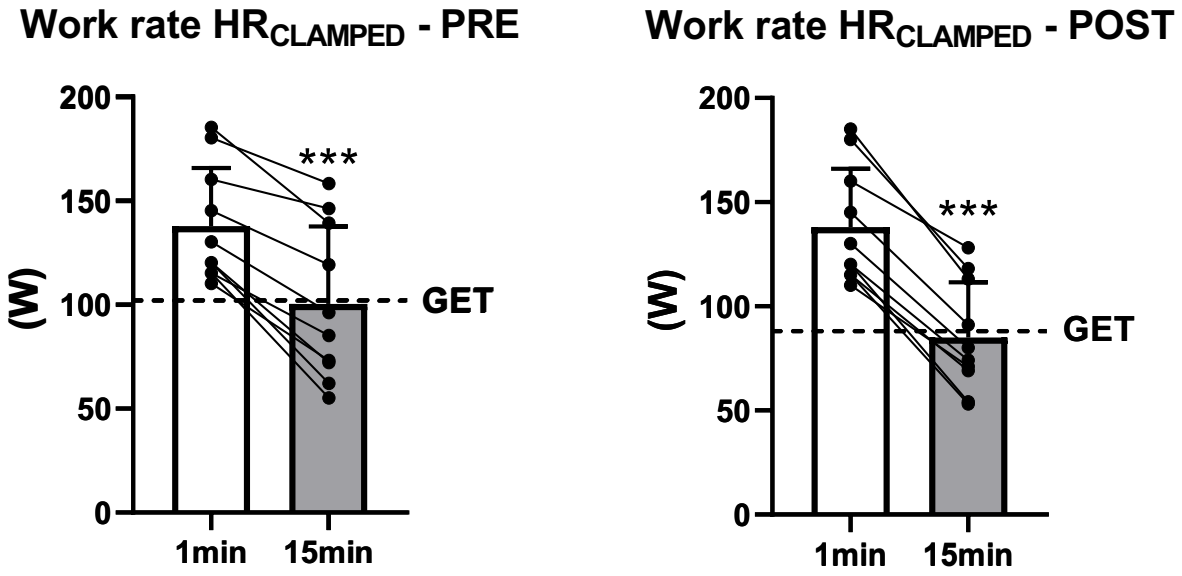

Figure 2. Individual and mean (± SD) values of work rate at the 1st (1min) and 15th minute (15min) of the $HR_{CLAMPED}$ exercise, before (PRE) and after bed rest (POST). The horizontal dashed line indicates the respective mean value of the work rate at the gas exchange threshold (GET). *** $p < 0.001$. See text for further details.

Mean (± SD) steady-state values of the main variables obtained at the end of the very low- and moderate-intensity CWR exercises are given in **Table 2**. HR was significantly higher and SV was significantly lower after BR, whereas no significant difference was observed for CO. No differences were observed in POST *vs.* PRE for the other variables.

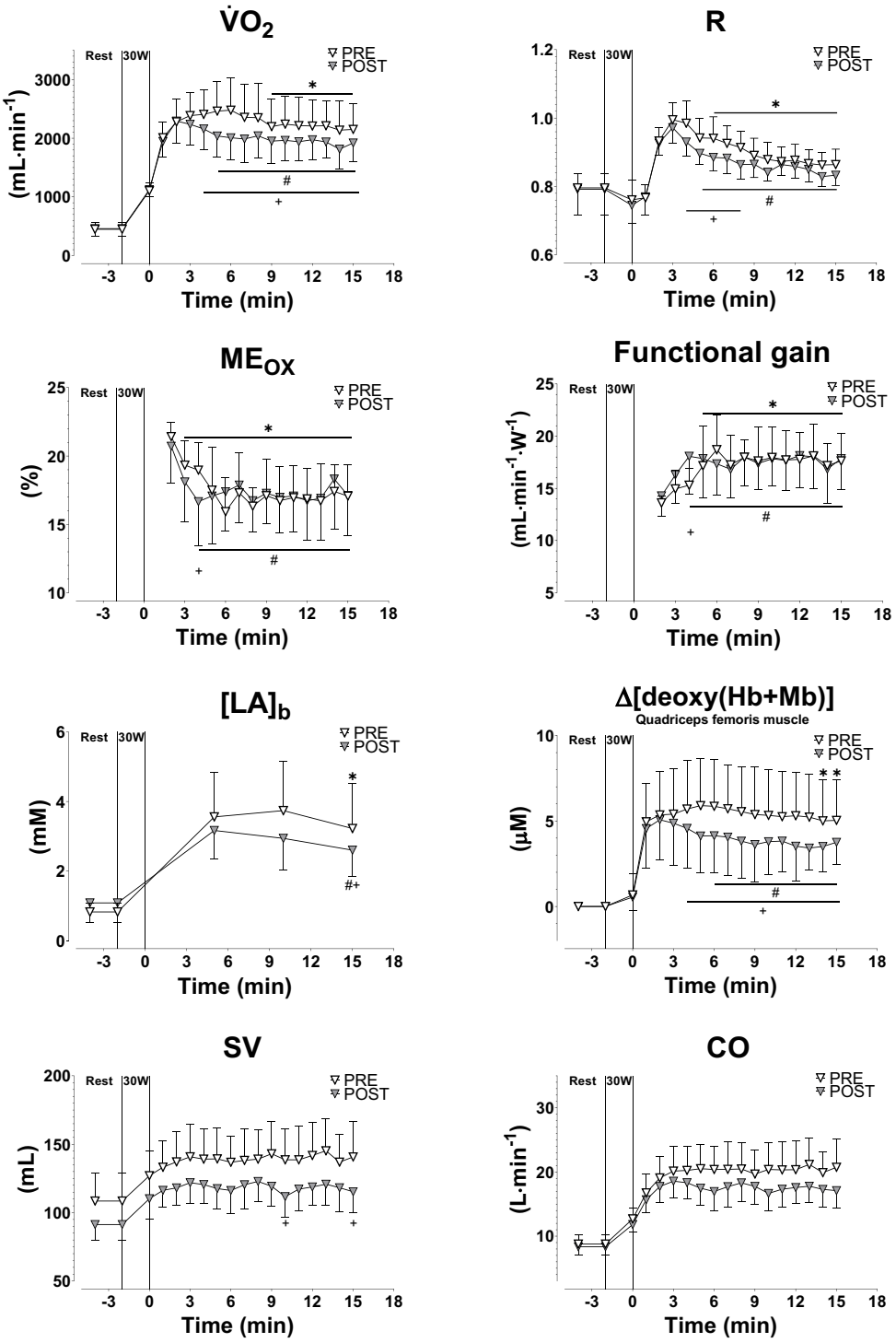

Figure 3. Mean (± SD) values of pulmonary oxygen uptake ($\dot{V}O_2$), gas exchange ratio (R), net mechanical efficiency for oxidative metabolism (ME_{OX}), functional gain, blood lactate concentration ($[La]_b$), deoxygenated Hb+Mb concentrations in quadriceps femoris muscle (Δ[deoxy(Hb+Mb)]), stroke volume (SV) and cardiac output (CO) during $HR_{CLAMPED}$ exercises, before (PRE) and after bed rest (POST). ME_{OX} and functional gain values were calculated from the 2nd min of exercise. *,#Statistically different from respective highest value ($p < 0.05$). +Statistically different from respective values in PRE ($p < 0.05$). See text for further details.

Individual examples of $\dot{V}O_2$ and HR kinetics in PRE and POST during the moderate-intensity CWR exercise are shown in **Figure 4.** Mean values of $\dot{V}O_2$ and HR kinetics parameters are presented in **Table 3**. $\dot{V}O_2$ and HR kinetics during the transition from the very low- (about 30 W) to the moderate-intensity CWR exercise were slower in POST *vs.* PRE (see **Table 3** and individual and mean ± SD values in the left panel of **Figure 5**). The relatively high τ_f values of $\dot{V}O_2$ kinetics are typical for a very low-intensity to moderate-intensity exercise transition (Brittain et al. 2001). No slow component of $\dot{V}O_2$ kinetics was detected in both conditions, whereas a slow component of HR kinetics was observed both in PRE (in 6 subjects of 10) and POST (in all subjects). The amplitude of the slow component ($b \cdot min^{-1}$) was relatively small in both conditions, but it was significantly greater in POST *vs.* PRE, both when expressed in absolute (A'_s, $b \cdot min^{-1}$) and in relative values (A'_s/A_{tot}, %) (see **Table 3** and individual and mean ± SD values in the right panel of **Figure 5**). A significant correlation was observed between the individual decreases in work rate during the $HR_{CLAMPED}$ exercise (PRE and POST values were merged) and the corresponding individual values of A'_s/A_{tot} HR ($r = 0.56$; $R^2 = 0.31$; $p = 0.01$).

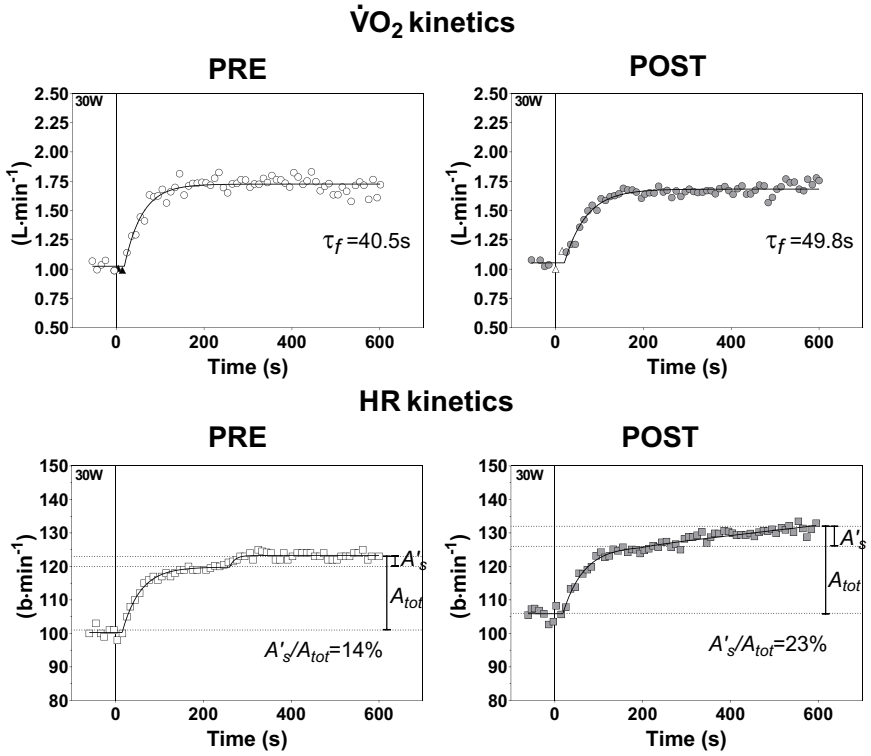

Figure 4. Individual examples of pulmonary O_2 uptake ($\dot{V}O_2$) and heart rate (HR) kinetics before (PRE) and after bed rest (POST) during the moderate-intensity constant work rate exercise. Each data point indicates breath-by-breath or beat-to-beat values averaged every 10 s. The first two $\dot{V}O_2$ data points (cardiodynamic phase) were excluded from the fitting. τ_f is the time constant of the fundamental component. The vertical line indicates the transition from the very-low (about 30 W) to the moderate-intensity exercise. Horizontal dashed lines indicate the amplitudes of the total response (A_{tot}) and the actual amplitude of the slow component (A'_s). See text for further details.

In **Figure 6** mean (± SD) values of the changes of the main variables after BR, expressed as a percentage of values obtained in PRE, are shown. Changes were of similar extent for all the variables (~10%), except for CO and τ_f in which changes were about two-fold greater (~20%).

A positive and significant correlation ($r = 0.72$; $R^2 = 0.52$; $p = 0.02$) was found between the decreases in work rate during $HR_{CLAMPED}$ (calculated from the 1st to the 15th minute), in PRE *vs.* POST, and the corresponding decreases in $\dot{V}O_{2peak}$. On the other hand, the PRE *vs.* POST decreases in peak work rate, GET and RCP, and the PRE *vs.* POST increases in the τ_f of pulmonary $\dot{V}O_2$ kinetics were not significantly correlated with the decreases in $\dot{V}O_{2peak}$.

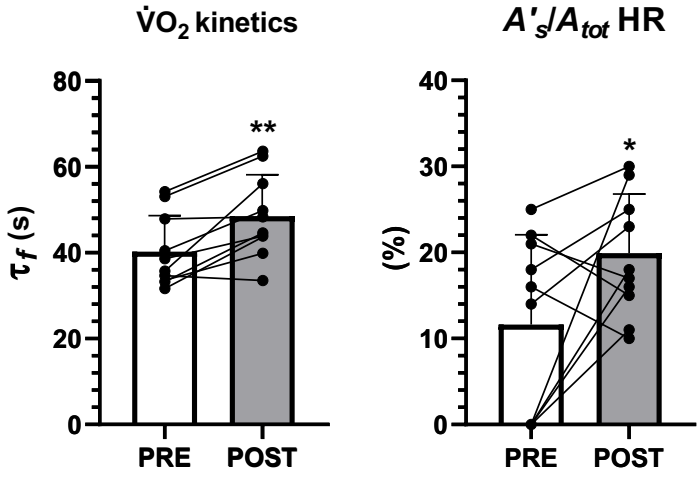

Figure 5. Individual and mean (± SD) values of the time constant of the fundamental component of the $\dot{V}O_2$ kinetics (τ_f) (left panel), and of the percentage contribution of the slow component of HR kinetics to the total amplitude of the response (A'_s/A_{tot} HR) (right panel) before (PRE) and after bed rest (POST). * $p < 0.05$; ** $p < 0.01$. See text for further details.

Table 2. Steady-state values of the main variables obtained at the end of the very low- (CWR_{30W}) and moderate-intensity ($CWR_{80\%GET}$) CWR exercises, before (PRE) and after (POST) bed rest.

	Work rate (W)	HR ($b \cdot min^{-1}$)	$\dot{V}O_2$ ($L \cdot min^{-1}$)	$\dot{V}CO_2$ ($L \cdot min^{-1}$)	R	$\dot{V}E$ ($L \cdot min^{-1}$)	SV (mL)	CO ($L \cdot min^{-1}$)	$[La]_b$ (mM)	RPE (6–20)	Δ[deoxy(Hb+Mb)] (μM)
CWR_{30W}											
PRE	30 ± 0	97 ± 10	1.034 ± 0.152	0.847 ± 0.093	0.79 ± 0.04	26.9 ± 3.2	124 ± 16	12.0 ± 1.9	-	-	0.7 ± 1.3
$CWR_{80\% GET}$											
PRE	81 ± 26	127 ± 11	1.795 ± 0.296	1.606 ± 0.269	0.90 ± 0.02	46.4 ± 6.0	137 ± 23	17.4 ± 3.5	2.0 ± 0.7	10 ± 2	2.3 ± 1.9
POST	81 ± 26	140 ± 12***	1.797 ± 0.286	1.597 ± 0.253	0.89 ± 0.02	46.9 ± 7.6	116 ± 13**	16.3 ± 2.6	2.3 ± 0.7	10 ± 2	2.5 ± 2.0

Data are means ± SD. HR, heart rate; $\dot{V}O_2$ pulmonary oxygen uptake; $\dot{V}CO_2$, CO_2 output; R, gas exchange ratio; $\dot{V}E$, pulmonary ventilation; SV, stroke volume; CO, cardiac output; $[La]_b$, blood lactate concentration; RPE, rate of perceived exertion; (Δ[deoxy(Hb+Mb)]), deoxygenated Hb+Mb concentrations in quadriceps femoris muscle obtained before (PRE) and after (POST) bed rest during the very low- (CWR_{30W}) and moderate-intensity ($CWR_{80\%GET}$) constant work rate (CWR) exercises. Asterisks denote differences from PRE by means of Student's paired t-test: ** $p < 0.01$; *** $p < 0.001$.

Table 3. Pulmonary O_2 uptake ($\dot{V}O_2$) and HR (heart rate) kinetics parameters determined during the moderate-intensity constant work rate exercise, before (PRE) and after (POST) bed rest.

	TD_f (s)	τ_f (s)	MRT (s)	TD_s (s)	y_{BAS} ($L \cdot min^{-1}$)	A_f ($L \cdot min^{-1}$)	A'_s ($L \cdot min^{-1}$)	A'_s/A'_{tot} (%)
$\dot{V}O_2$								
PRE	12.7 ± 5.0	40.3 ± 8.4	53.5 ± 11.1	-	1.053 ± 0.091	0.759 ± 0.313	-	-
POST	19.0 ± 2.6*	48.5 ± 9.7**	68.1 ± 11.5**	-	1.067 ± 0.110	0.699 ± 0.247	-	-
	TD_f (s)	τ_f (s)	MRT (s)	TD_s (s)	y_{BAS} ($b \cdot min^{-1}$)	A_f ($b \cdot min^{-1}$)	A'_s ($b \cdot min^{-1}$)	A'_s/A'_{tot} (%)
HR								
PRE	6.1 ± 14.9	47.7 ± 14.2	53.8 ± 22.5	295.5 ± 108.4	97 ± 10	27 ± 9	4 ± 3	11.6 ± 10.4
POST	10.3 ± 9.4	65.6 ± 15.1*	75.9 ± 20.7*	274.9 ± 75.1	105 ± 9**	29 ± 9	7 ± 4**	19.9 ± 6.9*

Data are Means ± SD. TD_f, time delay; τ_f, time constant; MRT, mean response time; TD_s, time delay slow component; y_{BAS}, oxygen uptake baseline; A_f, amplitude of the fundamental component; A'_s, actual amplitude of the slow component; A'_s/A'_{tot}, total amplitude of the response. Asterisks denote differences from PRE by means of Student's paired t-test: * $p < 0.05$; ** $p < 0.01$.

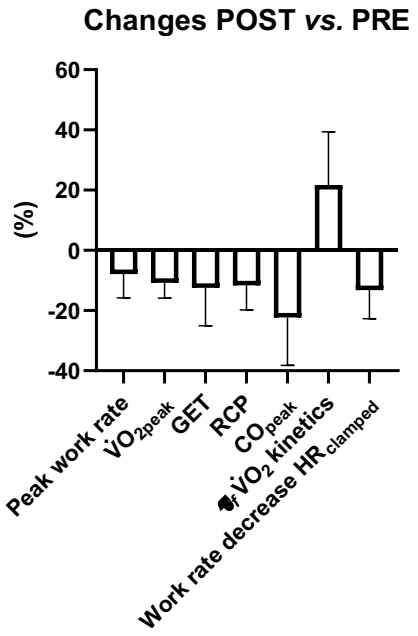

Figure 6. Mean (± SD) values of the changes of the main variables after bed rest (POST), expressed as a percentage of values obtained in PRE. $\dot{V}O_2$, pulmonary oxygen uptake; GET, gas exchange threshold; RCP, respiratory compensation point; CO, cardiac output; τ_f, time constant of the fundamental component of $\dot{V}O_2$ kinetics. See text for further details.

DISCUSSION

Confirming our hypothesis, the work rate decrease necessary to keep HR constant at a value slightly above that corresponding to GET was significantly greater (~40%) after *vs*. before (~30%) 10 days of horizontal bed rest (BR). This observation was associated with a greater amplitude of the HR slow component during moderate-intensity exercise after BR. The greater work rate decrease at a fixed HR can be considered a "systemic biomarker" of fatigue and impaired exercise tolerance in conditions of simulated microgravity/inactivity.

The present data demonstrate, also in conditions of simulated microgravity/inactivity, the absence of a linear relationships between HR, $\dot{V}O_2$ and work rate, and have profound implications on exercise evaluation and prescription. The results confirm the concept, put forward by recent studies by our group (Zuccarelli et al. 2018, 2021), that exercise prescription at fixed submaximal HR slightly above that determined at GET (as it is frequently done when submaximal "aerobic" training is involved) is not associated with a specific work rate or work rate domain. We demonstrate that the problem is more relevant following exposure to simulated microgravity/inactivity. The issue may be relevant also for exercise intensities below GET, since, as confirmed in the present study, the slow component of HR kinetics occurs also in the moderate-intensity domain, at lower work rates compared to those associated with a slow component of $\dot{V}O_2$ kinetics (Zuccarelli et al. 2018, 2021).

A higher HR for the same work rate is a sign of reduced exercise tolerance. This represents a widely accepted concept, mentioned in physiology textbooks (Astrand et al. 1986; McArdle et al. 1986; Taylor & Groeller, 2008; Wasserman et al. 1999). In the present study (as well as in previous studies carried out in normal subjects [Zuccarelli et al. 2018] and in obese patients [Zuccarelli et al. 2021]) we observed a "mirror image" of the above-mentioned phenomenon, that is a lower work rate for the same HR. We postulate that this finding is a sign of impaired exercise tolerance as well. In the present study we demonstrate that the decrease in work rate at a fixed HR is aggravated after 10 days of bed rest, identifying an impairment of exercise tolerance occurring after a period of simulated microgravity/inactivity. A similar concept was put forward in a previous study by our group (Zuccarelli et al. 2021), carried out in obese patients, in which the work rate decrease at a fixed HR was substantially attenuated after a 3-wk body mass reduction intervention, including exercise training, which improved the patients' exercise tolerance (Zuccarelli et al. 2021).

Other systemic biomarkers of exercise intolerance were investigated in the present study. Peak work rate, $\dot{V}O_{2peak}$, GET and RCP were about 10% lower in POST *vs.* PRE. $\dot{V}O_{2peak}$, the variable evaluating the maximal integrated (respiratory, cardiovascular, muscular) performance of oxidative metabolism, is classically considered associated with a mechanical power output which can be sustained for about

10 minutes. For exercises of longer duration other variables, such as GET or the RCP are usually determined in order to identify the maximal sustainable work rate. In the present study also GET and RCP were significantly lower in POST *vs.* PRE. Another variable associated with exercise tolerance (Grassi et al. 2011; Goulding et al. 2021) evaluated in the present study was the kinetics of adjustment of $\dot{V}O_2$ during transitions to moderate-intensity exercise. Confirming previous studies (Porcelli et al. 2010; Convertino et al. 1984), the $\dot{V}O_2$ kinetics was slower after BR. A slower $\dot{V}O_2$ kinetics mandates a greater O_2 deficit, lower metabolic stability and is then negatively associated with exercise tolerance (Grassi et al. 2006; Poole & Jones, 2012).

Taking $\dot{V}O_{2peak}$ as a standard for the evaluation of exercise tolerance, among those determined in the present study, we found that the work rate decreases during the $HR_{CLAMPED}$ exercise following BR were significantly correlated with the individual decreases in $\dot{V}O_{2peak}$. On the other hand, no significant correlations were observed for the individual decreases in $\dot{V}O_{2peak}$ and decreases in peak work rate, GET, RCP, or with the increases of τ_f of the $\dot{V}O_2$ kinetics. This should confirm the validity of the proposed approach (work rate decrease at a fixed HR) as a functional evaluation tool. Work rate decreases by 30% (before BR) and by 40% (after BR) during $HR_{CLAMPED}$ seem substantial and functionally relevant, particularly since they were associated, in about half of the subjects, with a switch from the heavy- to the moderate-intensity domain (see Figure 2). Our data, however, do not allow to identify a threshold for a functionally relevant decrease. Also age- and sex-specific issues have not been investigated yet. Future studies are needed to elucidate these aspects.

The proposed approach offers also other advantages. The variables to be determined (HR, work rate) could be easily and reliably measured also in experimental conditions such as spaceflights or planetary habitats, or in patients with chronic conditions. The approach would obviate the need to perform several submaximal, maximal and supramaximal tests to task failure, necessary to determine variables such as the critical power (Grassi et al. 2015; Jones et al. 2010), often considered the "gold standard" in the evaluation of exercise tolerance. The proposed test would also obviate the need to perform several repetitions of the same exercise, necessary for a reliable evaluation of the $\dot{V}O_2$ kinetics (Lamarra et al. 1987), or the methodological uncertainties associated with GET or RCP determination, requiring the intervention of multiple observers and the need for the often invoked "ancillary criteria" (Beaver et al. 1986). Reproducibility studies are obviously needed before the implementation of the proposed approach in practical terms.

The present results should also be relevant in terms of exercise prescription. During aerobic exercise, training intensity is often prescribed at a fixed HR value, slightly higher than that corresponding to GET. In other words, the training intensity is chosen in the lower boundaries of the heavy-intensity domain (Riebe et al. 2018). The present study, as well as our previous ones (Zuccarelli et al. 2018,

2021) demonstrate that this approach is inevitably associated with a progressive and substantial work rate decrease during the training session, at least in untrained subjects. In the present study the work rate decrease was steep early during $HR_{CLAMPED}$, and steady-state conditions were then achieved from the 4th (in POST) and 6th minute (in PRE) to the end of exercise. During 15-min task the work rate decrease amounted to -30% before BR and to -40% after BR, and resulted in a shift from the heavy- to the moderate-intensity domain in 6 subjects out of 10 (see Figure 2). The notion that methods of exercise prescription based on fixed percentages of maximal values poorly conform to exercise intensity domains, and thus do not adequately control the metabolic stimulus and the subsequent adaptations to exercise training has also been recently raised by Iannetta et al. (2020). The relationships between the percentages of the "reserve values" of HR and either $\dot{V}O_2$ or $\%\dot{V}O_{2max}$ have been recently questioned by Ferri Marini et al. (2021), who showed that both relationships were slightly but significantly different from the identity line. These authors confirmed that the utilization of fixed percentages of HR during a training session may cause a fall of work rate into different intensity domains, potentially leading to major errors in exercise intensity prescription. According to a recent study by Teso et al. (2022), performed on post-menopausal women, the HR slow component inevitably introduces a mismatch between the prescribed HR and the resulting $\dot{V}O_2$/metabolic load.
In our opinion the issue has been overlooked in the past. Future studies will have to take into account this issue, particularly when the work rate decrease at a fixed HR is modified following an intervention. In the present study we evaluated the effects of a 10-day exposure to simulated microgravity/inactivity. What happens after exposures of significantly longer duration, as during spaceflights? This issue clearly needs to be investigated in detail. The perspective changes also in terms of exercise evaluation, since a decreasing $\dot{V}O_2$ (see Figure 3) may lead to an overestimation of the metabolic cost associated with the exercise.
The mechanism(s) responsible for the decrease in work rate during the $HR_{CLAMPED}$ exercise are at least in part unclear. A factor is likely represented by the "slow component" of the HR kinetics, mirror image of the work rate decrease at a fixed HR. Confirming our previous observations (Zuccarelli et al. 2018, 2021), in the present study we observed that a slow component of this variable occurs also during moderate-intensity exercise. At higher exercise intensities the amplitude of the HR slow component is greater than the amplitude of the $\dot{V}O_2$ slow component (Zuccarelli et al. 2018, 2021; Teso et al. 2022). In the present study we demonstrated that the amplitude of this slow component was greater after BR. It appears reasonable to hypothesize that the more pronounced decrease in work rate for the same HR observed after BR is attributable to the more pronounced amplitude of the HR slow component. In support of this hypothesis we observed a significant correlation between the individual decreases in work rate during the $HR_{CLAMPED}$ exercise and the corresponding values of the

amplitude of the HR slow component. Thus, investigating what determines the work rate decrease during $HR_{CLAMPED}$ should be equivalent to investigating the mechanism(s) responsible for the HR slow component during a constant work rate exercise. Whereas the slow component of the $\dot{V}O_2$ kinetics has been widely studied (Jones et al. 2011; Grassi et al. 2015; Poole & Jones, 2012; Rossiter, 2011), and it seems to be related to a decreased efficiency of oxidative metabolism and to muscle fatigue, for the HR slow component mostly anecdotal observations are present in the literature (Linnarsson, 1974; Engelen et al. 1996; Wasserman et al. 1967; Orizio et al. 1988; Hebestreit et al 1988; Grassi et al. 1997; Bearden & Moffatt, 2001). If we suppose, at a first approximation, that the mechanisms responsible for the HR and $\dot{V}O_2$ slow components are at least in part the same, and that both phenomena are associated with a reduced efficiency of oxidative metabolism and fatigue, then the work rate decrease observed in the present study could be aimed (via a "metaboreflex"?) at preventing excessive inefficiency and premature fatigue (Jones et al. 2011; Grassi et al. 2015). Some evidence in the literature points to such phenomenon. Ribeiro et al. (1986) and Stoudemire et al. (1996), for example, documented that during exercises carried out for several minutes at work rate greater than or equal to GET, in order to keep pulmonary $\dot{V}O_2$ constant, and thus avoid muscle fatigue, subjects decreased work rate or running speed. A similar observation was made by Herman et al. (2003): keeping HR constant at 75% HR_{max}, by decreasing work rate, reduced the $\dot{V}O_2$ slow component. In the present study the substantial work rate decrease during $HR_{CLAMPED}$ was accompanied by a significant (-15-20%) decrease in net mechanical efficiency of oxidative metabolism (see Figure 3), considered harbinger of fatigue (Grassi et al. 2015). Conversely, the functional gain, increased from minute 2 to minutes 4-6 of the $HR_{CLAMPED}$ exercise, and then reached a steady-state, values (about 17 $mL \cdot min^{-1} \cdot W^{-1}$) significantly higher than normal (Poole & Jones, 2012; Rossiter, 2011), confirming a substantial loss of oxidative efficiency during the trial.

In the present study, however, the work rate decrease was more pronounced than that needed to prevent a $\dot{V}O_2$ slow component. During $HR_{CLAMPED}$ exercise, indeed, not only $\dot{V}O_2$ did not increase, but it actually decreased both in PRE and in POST (Figure 3). The same was observed for other variables (R, $[La]_b$, muscle deoxygenation), whose progressive increase would be directly or indirectly associated with fatigue. All these variables actually decreased during $HR_{CLAMPED}$ exercise (Figure 3). Which mechanism(s) could be held responsible for the more pronounced decrease of work rate, compared to that necessary to keep $\dot{V}O_2$, R, $[La]_b$, muscle deoxygenation constant (that is to say, to prevent "slow components" of this variables)? No answer to this question can derive from the present results. Effects of blood catecholamines (Orizio et al. 1988) or body temperature (Zuccarelli et al. 2021; González-Alonso et al. 1997) on HR could be hypothesized. The mechanisms, in any case, 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 minutes of moderate-intensity exercise (Coyle & González-Alonso, 2000). This phenomenon, often termed "cardiovascular drift", is usually associated with hyperthermia and dehydration (González-Alonso et al. 1997). In the present study we did not measure core body temperature or indices of dehydration. Our data, however, did not show a decrease in SV during $HR_{CLAMPED}$ exercise (see Figure 3). Moreover, in the present study the work rate decrease occurred well before the 10th minute of exercise, differently from the HR increase associated with the cardiovascular drift. It seems very unlikely that a substantial increase in body temperature and dehydration would occur after 2-3 minutes of exercise slightly above GET.

In conclusion, confirming our hypothesis, during a 15-min exercise initially set at a work rate corresponding to a HR slightly above that corresponding to GET (as frequently done for aerobic exercise prescription), in order to keep HR constant work rate substantially decreased (by 30-40%), and the decrease was greater after a 10-day horizontal BR. The observation was associated with a greater amplitude of the HR slow component during moderate-intensity exercise following BR. The work rate decrease was accompanied by decreases of $\dot{V}O_2$, of metabolic efficiency and of other variables (R, $[La]_b$ and muscle deoxygenation) for which progressive increases would be associated with fatigue. The work rate decrease at a fixed HR can be considered a systemic biomarker of exercise intolerance, which could be easily and reliably determined also during spaceflights or permanence on planetary habitats. The work rate decrease at a fixed HR may significantly affect exercise evaluation (for example in terms of its metabolic cost) and exercise prescription, with the impossibility to identify, on the basis of HR, a specific $\dot{V}O_2$, work rate or exercise intensity domain during a training session. Future studies should evaluate the effects of longer periods of exposure to simulated microgravity/inactivity.

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3.2 IN CARDIAC PATIENTS BETA-BLOCKERS ATTENUATE THE DECREASE IN WORK RATE DURING EXERCISE AT A CONSTANT SUBMAXIMAL HEART RATE – *STUDY 2*

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Running head: Decrease in work rate at a fixed heart rate in cardiac patients

ABSTRACT

Purpose. Exercise prescription based on fixed heart rate (HR) values is not associated with a specific work rate (WR) during prolonged exercise. This phenomenon has never been evaluated in cardiac patients, and might be associated with a slow component of HR kinetics and β-adrenergic activity. The aim was to quantify, in cardiac patients, the work rate decrease at a fixed HR, and to test if it would be attenuated by β-blockers. **Methods.** 17 patients with coronary artery disease in stable conditions (69±9 yr) were divided into two groups according to the presence (BB) or absence (no-BB) of a therapy with β-blockers, and performed on a cycle ergometer: an incremental exercise (INCR); a 15-min ''$HR_{CLAMPED}$'' exercise, in which WR was continuously adjusted to maintain a constant HR, corresponding to the gas exchange threshold (GET) +15%. HR was determined by the ECG signal, and pulmonary gas exchange was assessed breath-by-breath. **Results.** During INCR HR_{peak} was lower in BB *vs.* no-BB ($p<0.05$), whereas no differences were observed for other variables. During $HR_{CLAMPED}$ the decrease in WR needed to maintain HR constant was less pronounced in BB *vs.* no-BB (-16±10% *vs.* -27±10, $p=0.04$), and was accompanied by a decreased $\dot{V}O_2$ only in no-BB (-13±6%, $p<0.001$). **Conclusions.** The decrease in WR during 15-min exercise at a fixed HR (slightly higher than that at GET) was attenuated in BB, suggesting a causative role by β-adrenergic stimulation. The phenomenon may represent, also in this population, a sign of impaired exercise tolerance, and interferes with aerobic exercise prescription.

Key words: EXERCISE PRESCRIPTION, EXERCISE TOLERANCE, CARDIOVASCULAR DISEASE, HR KINETICS, β-BLOCKERS.

INTRODUCTION

Compelling evidence indicates that physical inactivity is implicated in the etiology of numerous chronic diseases which impact negatively on the health status (Lee et al. 2012; Booth et al. 2017). On the contrary, regular physical activity improves the quality of life by increasing exercise tolerance and by reducing the risk of all-cause mortality in a dose-response manner (Lee & Skerrett 2001). Although some physical activity is better than none, an individually tailored exercise prescription is more effective in improving the subjects' physical performance and eventually their health (Riebe et al. 2018; Bull et al. 2020). Exercise prescription is often done in terms of exercise domains (Riebe et al. 2018; Bull et al. 2020), which have distinct characteristics in terms metabolic responses and fatigue. Too often, however intensity prescription for aerobic exercise, both in healthy and diseased populations, is defined in terms of work rates corresponding to a fixed percentage of heart rate (HR) reserve or of peak HR (Gormley et al. 2008; Nybo et al. 2010; Macko et al. 2005; Ivey et al. 2007, Piepoli et al. 2016; Riebe et al. 2018), mainly for the facility of tracking HR by wearable HR meters or cell phones. Studies by our group, however, demonstrated that both in healthy young subjects (Zuccarelli et al. 2018; Baldassarre et al. 2022) and in obese patients (Zuccarelli et al. 2021) a disproportionate increase in HR ("slow component" of HR kinetics) is present during constant work rate exercises. The slow component of HR kinetics occurs at lower work rates (below the gas exchange threshold [GET]) compared to the slow component of pulmonary O_2 uptake ($\dot{V}O_2$) kinetics (Zuccarelli et al. 2018, Baldassarre et al. 2022). Furthermore, above GET the relative amplitude of the HR slow component is more pronounced than that of $\dot{V}O_2$ slow component (Zuccarelli et al. 2018, Baldassarre et al. 2022). As a consequence of this phenomenon, during exercise carried out for 15 minutes at a constant HR, slightly above that corresponding to GET, an intensity often recommended by guidelines for aerobic exercise (Garber et al. 2011; Riebe et al. 2018; Bull et al. 2020), work rate had to significantly decrease in order to maintain HR constant, both in healthy young subjects (Zuccarelli et al. 2018; Baldassarre et al. 2022) and in obese patients (Zuccarelli et al. 2021). At times the decreased work rate led to changes in exercise domain, from the heavy- to the moderate-intensity (Baldassarre et al. 2022). All this obviously makes exercise prescription and evaluation based on specific percentages of HR peak potentially inaccurate.

The phenomena described above have never been investigated in cardiac patients, a population in which exercise prescription is often performed in terms of fixed percentages of HR max (Piepoli et

al. 2016; Fletcher et al. 2001). Therefore, the main aim of the present study was to identify and quantify in coronary artery disease patients in stable conditions the decrease in work rate during exercise performed at a fixed HR, set at a value slightly higher than that corresponding to GET. We also intended to test the hypothesis that the decrease in work rate, being associated with a slow component of HR kinetics, would be significantly attenuated by the administration of β-blockers. These drugs significantly improve the prognosis of these patients (Ponikowski et al. 2016) by reducing the β-adrenergic drive, which has been proposed as a potential cause of the HR slow component (Orizio et al. 1988; Zuccarelli et al. 2021).

MATERIALS AND METHODS

Patients

Nineteen patients (17 men and 2 women) followed by the Center for Exercise Prescription and Administration, Department of Medicine, University of Udine, situated in the Hospital of Gemona del Friuli, were selected for this study. All the subjects had cardiovascular diseases (17 coronary artery disease, 1 heart failure and 1 hypertensive cardiac disease) and were in stable clinical conditions. Participants were divided into two groups according to the presence or absence of a therapy with β-blockers: in group BB (n = 10; age 69 ± 7 [mean ± SD], height 1.75 ± 0.11 m, body mass 86.0 ± 13.7 kg) patients were treated with β-blockers (atenolol 50 mg/day, or bisoprolol 2.5-7.5 mg/day, or metoprolol 2 x 50 mg/day), whereas in group no-BB (n = 9; age 71 ± 11 [mean ± SD], height 1.73 ± 0.08 m, body mass 79.5 ± 11.8 kg) patients were not treated with β-blockers.

One patient (group no-BB) was excluded from the study because of the occurrence of frequent ventricular ectopic beats during the incremental exercise, which led to the premature interruption of the test. Another patient of group BB complained of increasing exertional dyspnea and was excluded due to a moderate anemia ([Hb] 9.6 $g \cdot dL^{-1}$). Therefore, only seventeen patients (9 for group BB and 8 for group no-BB) completed the testing procedures and were included in the statistical analysis.

All subjects gave their written informed consent after they received a detailed explanation of the experimental procedures before the start of the study, whose protocol was approved (Prot. IRB: 84/2022, 6th of June 2022) by the Institutional Review Board of the Department of Medicine, University of Udine.

Experimental Protocol

Participants were required to come to the laboratory on three separate occasions. On their first visit subjects underwent a physical examination and anthropometric measurements were performed. During the second visit the participants completed an incremental exercise on an electronically braked cycle ergometer (Ergoselect 100, Ergoline GmbH) until voluntary exhaustion. Pedaling frequency was digitally displayed to the subjects, who were asked to keep a constant cadence throughout the tests between 60 and 65 revolutions·min^{-1}. Voluntary exhaustion was defined as the incapacity to maintain the imposed load and pedaling frequency despite vigorous encouragement by the researchers. The incremental exercise protocol consisted of 15-30 W/min ramp increases (preceded by a resting baseline and by 2 minutes at 10-20 W), depending on the characteristics and the predicted functional capacity of each patient; the aim was to reach voluntary exhaustion in 10-15 minutes.
During the third visit to the laboratory patients performed a "HR-controlled" exercise ($HR_{CLAMPED}$), in which work rate was continuously adjusted to maintain a constant HR, equivalent to GET + 15%. During the first 2 minutes of $HR_{CLAMPED}$ work rate was progressively increased in order to reach the work rate target value, thereafter it was kept constant for 3 minutes, and then it was adjusted by the operator by decreasing/increasing by 2 W every 5 s, in order to maintain HR constant for the remaining 15 minutes of the exercise.

Measurements

Pulmonary ventilation ($\dot{V}E$), O_2 uptake ($\dot{V}O_2$), and CO_2 output ($\dot{V}O_2$) were assessed 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 three different flow rates. 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 $\dot{V}O_2/\dot{V}CO_2$. GET was determined using the "V-slope" method and "secondary criteria" (Beaver et al. 1986); the respiratory compensation point (RCP) was determined by standard criteria (Wasserman et al. 1986). To identify the work rate and HR corresponding to $\dot{V}O_2$ 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 relationship to the left, by an amount corresponding to the mean response time of the $\dot{V}O_2$ kinetics (Whipp et al. 1981) of a similar patients' population (Koike et al. 1995; Karsten et al. 2011). HR was determined from a 12-lead electrocardiography (Quark C12x, Cosmed, Rome, Italy). Mean values of ventilatory, pulmonary gas exchange and cardiovascular variables were calculated during the last 20 s of each minute of exercise for both the incremental and the $HR_{CLAMPED}$ exercises;

values obtained during the exhausting work rate of the incremental exercise were considered peak values.

Statistical analysis

Results are expressed as mean ± SD values. Statistical significance of the differences between the two groups (BB vs. no-BB) was checked by two-tailed Student's unpaired t-tests. Respiratory and cardiovascular 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 Turkey *post hoc* test. Single and two linear regressions were carried out by the least-squared residuals method. Comparisons between the two fitting models were carried out using the F-test. The level of significance was set at 0.05. Statistical analyses were carried out by a commercially available software package (Prism 8.0; GraphPad).

RESULTS

Peak values of the main respiratory and cardiovascular variables obtained during the incremental exercises are reported in **Table 1.** No significant differences were found in BB *vs.* no-BB for $\dot{V}O_{2peak}$, peak work rate and other variables ($\dot{V}CO_2$, R, $\dot{V}E$, V_T, fR, $PetO_2$, $PetCO_2$) determined at peak exercise. As expected, HR_{peak} was significantly lower in BB than in no-BB, both when the variable was expressed in $b \cdot min^{-1}$ or in percentage of the age-predicted maximum values (calculated as 208 - 0.7 · age [Tanaka et al. 2001]). More specifically, expressed as a % of the age-predicted maximum, HR_{peak} values were about 90% in no-BB and about 70% in BB, and the extent of the HR_{peak} decrease after β-blockers administration (about -20%) was similar to that reported in literature (Petersen et al. 1983). $\dot{V}O_2$, work rate and HR at GET, as well as $\dot{V}O_2$ at RCP were not different between the two groups. Both in BB and in no-BB $\dot{V}O_2$ at GET and at RCP corresponded to about 65% and to about 90% of $\dot{V}O_{2peak}$, respectively.

Table 1. Peak values of the main respiratory, cardiovascular and metabolic variables determined during the incremental exercise in coronary artery disease patients in stable conditions treated (BB) or not treated (no-BB) with β-blockers. Data related to the gas exchange threshold (GET) and to the respiratory compensation point (RCP) are also presented.

	BB	**no-BB**	**P-value**
Peak work rate (W)	155 ± 50	165 ± 55	0.71
$\dot{V}O_{2peak}$ ($L \cdot min^{-1}$)	1.814 ± 0.501	1.753 ± 0.510	0.81
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	21.2 ± 4.8	22.9 ± 8.5	0.61
$\dot{V}CO_{2peak}$ ($L \cdot min^{-1}$)	2.050 ± 0.587	2.180 ± 0.652	0.67
R_{peak}	1.13 ± 0.10	1.24 ± 0.13	0.06
$\dot{V}E_{peak}$ ($L \cdot min^{-1}$)	80.5 ± 20.1	77.3 ± 21.7	0.76
V_{Tpeak} (L)	2.22 ± 0.51	2.06 ± 0.46	0.50
fR_{peak} ($breaths \cdot min^{-1}$)	37 ± 6	37 ± 6	0.73
$PetO_{2peak}$ (mmHg)	118.1 ± 3.6	116.9 ± 5.5	0.60
$PetCO_{2peak}$ (mmHg)	31.4 ± 4.0	33.9 ± 4.7	0.26
HR_{peak} ($b \cdot min^{-1}$)	114 ± 19*	139 ± 23	0.03
HR_{peak} ($\%HR_{MAX\ pred}$)	71 ± 12*	88 ± 11	0.01
$\dot{V}O_{2GET}$ ($L \cdot min^{-1}$)	1.198 ± 0.211	1.150 ± 0.285	0.70
$\dot{V}O_{2GET}$ ($\%\dot{V}O_{2peak}$)	67 ± 12	66 ± 6	0.82
Work $rate_{GET}$ (W)	62 ± 18	62 ± 28	0.93
HR_{GET} ($b \cdot min^{-1}$)	81 ± 9	90 ± 17	0.18
$\dot{V}O_{2RCP}$ ($L \cdot min^{-1}$)	1.603 ± 0.405	1.566 ± 0.418	0.86
$\dot{V}O_{2RCP}$ ($\%\dot{V}O_{2peak}$)	89 ± 6	90 ± 5	0.83

Data are means ± SD. Work rate; $\dot{V}O_2$, 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; $HR_{max\ pred,}$ age-predicted maximal heart rate; $\dot{V}O_{2GET}$, pulmonary oxygen uptake at GET; work $rate_{GET}$, work rate at GET; HR_{GET}, heart rate at GET; $\dot{V}O_{2RCP}$, pulmonary oxygen uptake at RCP. P values relate to differences between groups by means of Student's unpaired t-test.

In **Figure 1** mean (± SD) values of HR and work rate obtained during the $HR_{CLAMPED}$ exercise are shown. In both groups, HR mean target value (set at 90 ± 8 and 107 ± 16 $b \cdot min^{-1}$ in BB and no-BB, respectively, corresponding to 115% of GET) was reached within the first 5 minutes of exercise and remained constant throughout the test, indicating that the aim of the protocol (keeping HR constant) was successfully reached in both groups. HR values during exercise were significantly lower in BB than in no-BB. In both groups work rate had to decrease in order to keep HR constant, and the decrease was more pronounced in no-BB *vs.* BB.

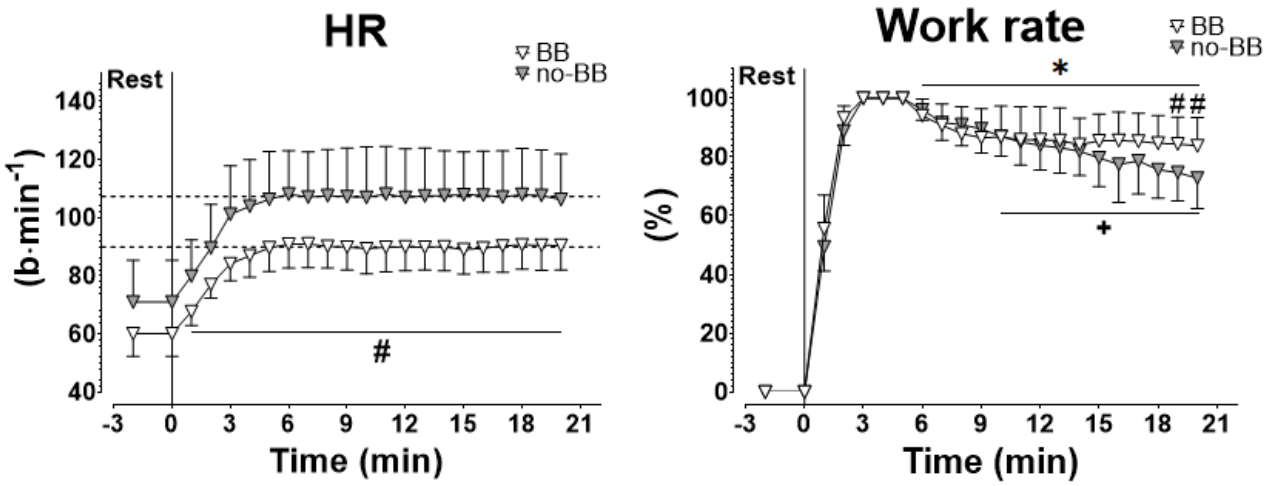

Figure 1. Mean (± SD) values of heart rate (HR) and work rate during $HR_{CLAMPED}$ exercises, in patients treated with β-blockers (BB) and in patients not treated with β-blockers (no-BB). The horizontal dashed line indicates the mean HR target value. $^{*,+}$Statistically different from the highest value of the variable ($p < 0.05$). $^{\#}$Statistically different from the value obtained in the no-BB group ($p < 0.05$). See text for further details.

The different work rate decreases in the two groups are more clearly evident in **Figure 2**, in which individual and mean (± SD) values of the decreases of the variable from the 3rd to the 20th minute of exercise are presented. Work rate decreases in no-BB were significantly greater than in BB, both when expressed in W (-12 ± 7 vs. -20 ± 6 W, $p = 0.02$) (left panel) and as a % of the 3rd minute value (-16 ± 10 vs. -27 ± 10%, $p = 0.04$) (right panel).

Interestingly, the work rate decreases determined a shift in the exercise intensity domains (**Figure 3**). Both in BB and in no-BB the mean values of work rate were above GET (that is, in the heavy intensity domain) at the 3rd minute of exercise, but were substantially identical to GET (that is, at the boundary between moderate-intensity and heavy-intensity domains) at the end of the exercise. Individual values in no-BB (left panels) show that at the end of the exercise 5 patients out of 8 were exercising below GET, that is in the moderate-intensity domain.

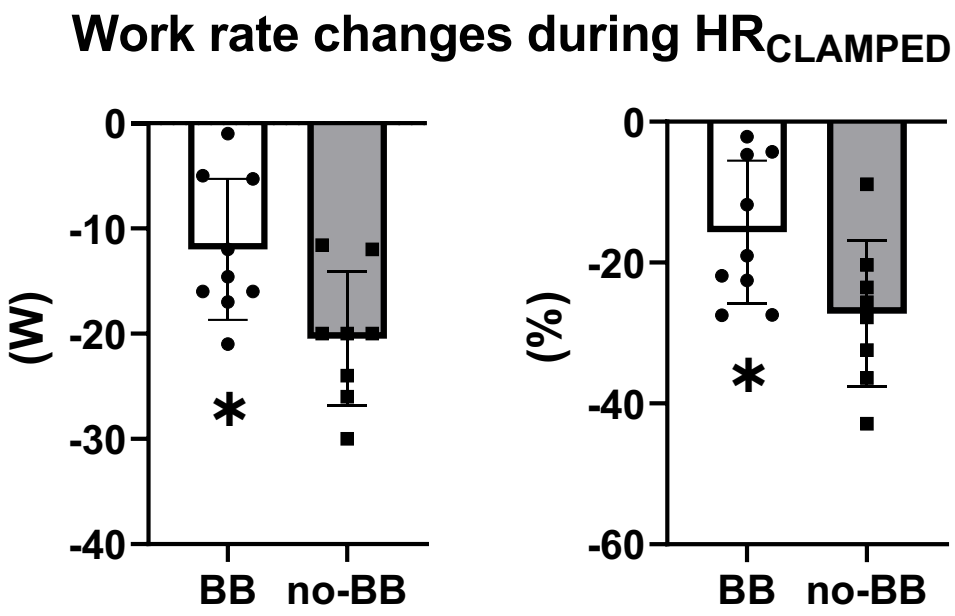

Figure 2. Individual and mean (± SD) values of the absolute and percentage changes in work rate during the $HR_{CLAMPED}$ exercise in cardiac patients treated with β-blockers (BB) or not treated with β-blockers (no-BB). *Statistically different from the value obtained in the no-BB group ($p < 0.05$).

Also the time courses of the work rate decreases in the two groups appear of interest. In **Figure 4** the work rate data presented in the left panel of Figure 1 are shown with expanded x (from minute 3 to minute 20) and y axes, in order to better appreciate the time courses of the variables. Whereas in no-BB the work rate decrease *vs.* time followed a linear function throughout the period, in BB, after a linear decrease from minute 3 to about minute 9 (this linear decrease was substantially superimposed on that observed in no-BB), the variable kept substantially constant until the end of the exercise.
The equation that best fitted the experimental data (single *vs.* two linear regressions) was determined using the F-test (see Statistical analysis). Implications of these different time courses will be analyzed in the Discussion.
Mean (± SD) values of $\dot{V}O_2$, $\dot{V}CO_2$ and R obtained during the $HR_{CLAMPED}$ exercise are shown in **Figure 5**. Individual and mean (± SD) values of the of the variable at the 3rd and the 20st minute of exercise are presented in **Figure 6**. $\dot{V}O_2$ did not change in BB, whereas it significantly decreased in no-BB. $\dot{V}CO_2$ and R decreased in both groups.

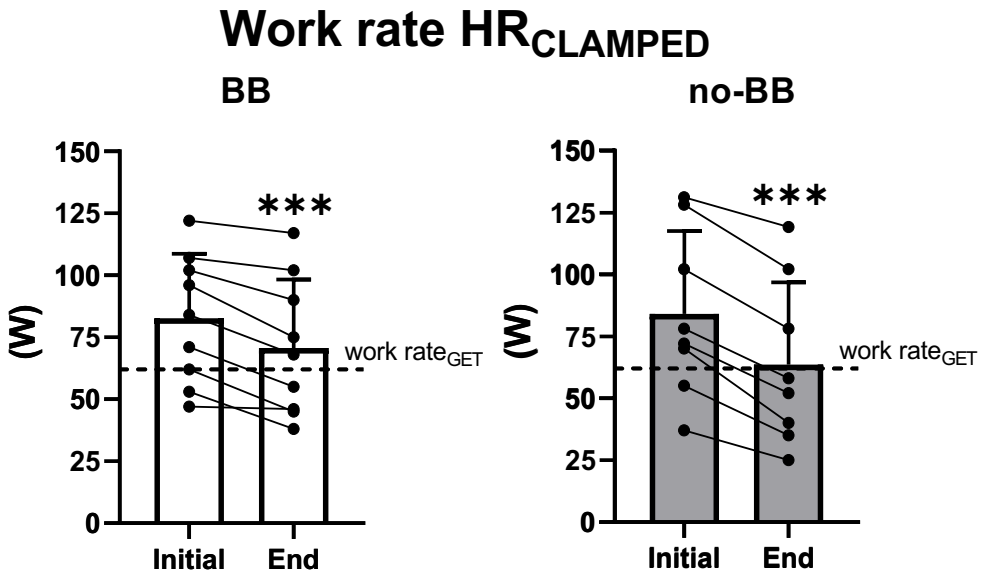

Figure 3. Individual and mean (± SD) values of work rate at the 5th (Initial) and 20th minute (End) of the $HR_{CLAMPED}$ exercise, in cardiac patients treated with β-blockers (BB) or not treated with β-blockers (no-BB). The horizontal dashed line indicates the mean value of the work rate at the gas exchange threshold (GET). ***$p < 0.001$. See text for further details.

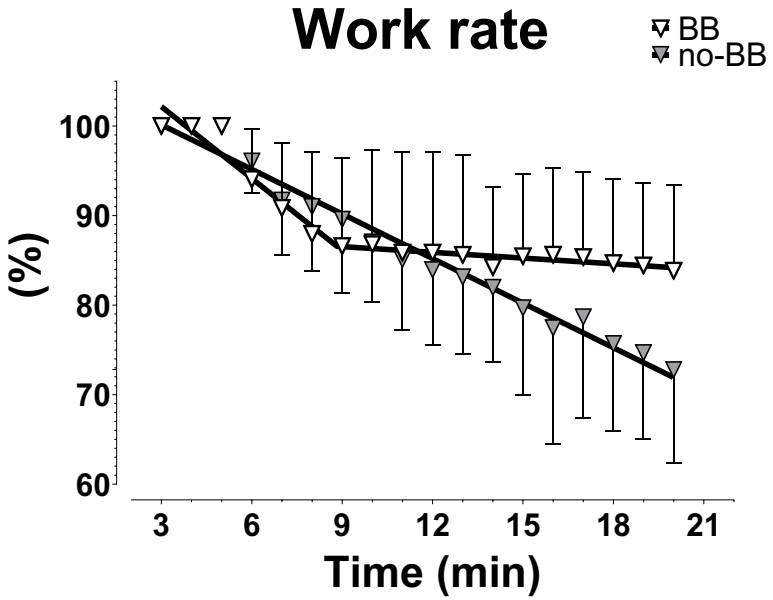

Figure 4. Mean (± SD) values of work rate from the 3rd to the 20th minute of $HR_{CLAMPED}$ exercises, in patients treated with β-blockers (BB) and in patients not treated with β-blockers (no-BB). The fitted single and two linear regression lines are also shown. See text for further details.

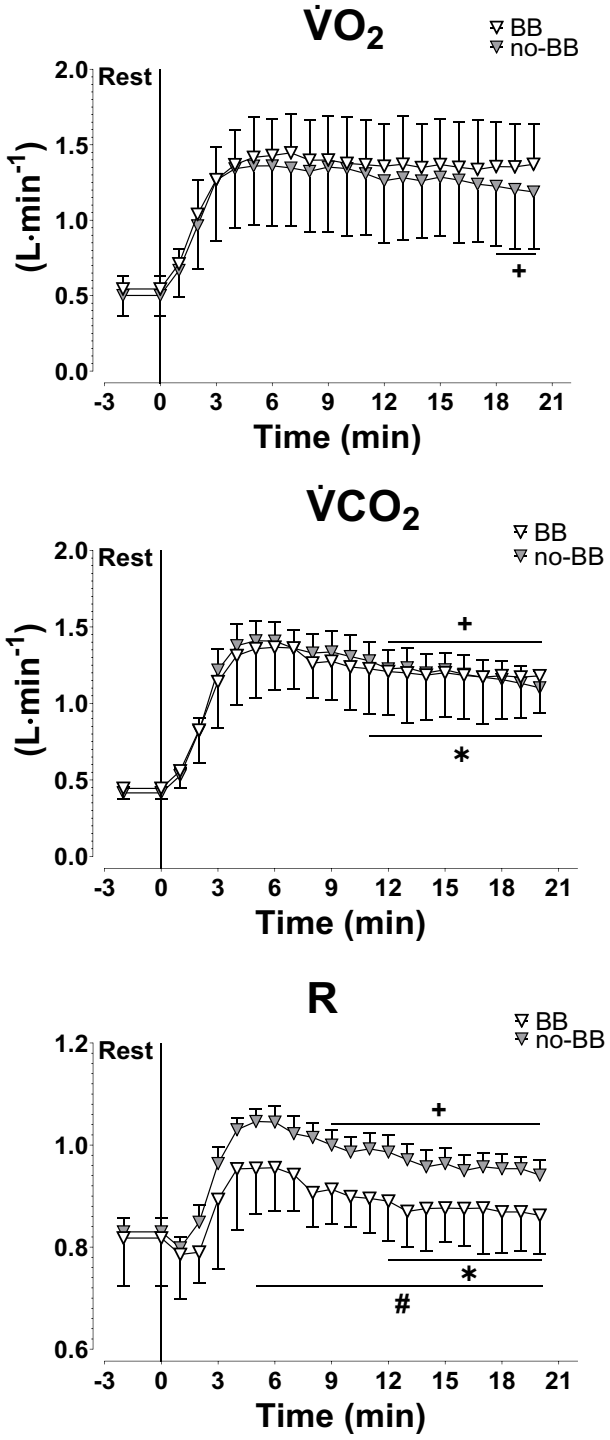

Figure 5. Oxygen uptake ($\dot{V}O_2$), CO_2 output ($\dot{V}O_2$) and gas exchange ratio (R) during $HR_{CLAMPED}$ exercises, in patients treated with β-blockers (BB) and in patients not treated with β-blockers (no-BB). *,+Statistically different from the highest value of the variable ($p < 0.05$). #Statistically different from the value obtained in the no-BB group ($p < 0.05$). See text for further details.

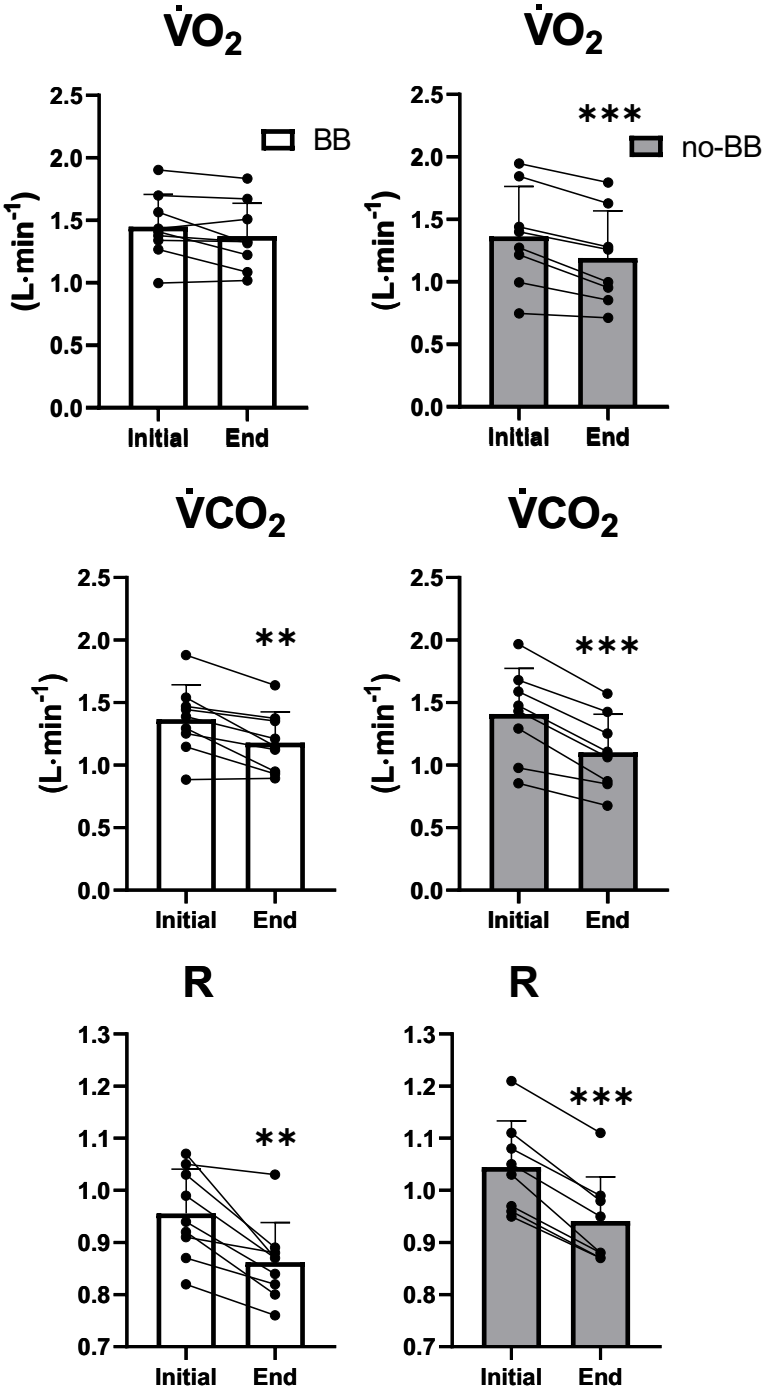

Figure 6. Individual and mean (± SD) highest (Initial) and last minute (End) values of oxygen uptake ($\dot{V}O_2$), CO_2 output ($\dot{V}CO_2$) and gas exchange ratio (R) recorded during $HR_{CLAMPED}$ exercise, in cardiac patients treated with β-blockers (BB) and in patients not treated with β-blockers (no-BB). **p < 0.01; ***p < 0.001.

DISCUSSION

During a 15-min exercise set at a work rate corresponding to a HR slightly above GET, patients with coronary artery disease in stable conditions have to significantly decrease work rate in order to keep HR constant. Confirming our hypothesis, the work rate decrease at a fixed HR was attenuated by β-blockers administration, suggesting a causative role for the phenomenon attributable to β-adrenergic stimulation. The decreased work rate at a fixed HR slightly above GET, observed in the present study in cardiac patients, confirms previous observations by our group in young subjects (Zuccarelli et al. 2018), obese adolescents (Zuccarelli et al. 2021) and young subjects undergoing bed rest (Baldassarre et al. 2022).

Textbook of physiology says that a higher HR for the same work rate indicates a decreased exercise tolerance (Astrand 1986; McArdle 1986; Wasserman 1999). In the present study, as well as in our previous ones (Zuccarelli et al. 2018; 2021; Baldassarre et al. 2022) we observed a "mirror image" of the phenomenon, that is a lower work rate for the same HR. We postulate that the observation represents a sign, or a biomarker, of impaired exercise tolerance as well. In support of this concept are the observations that the work rate decrease at a fixed HR was smaller following exercise training (Zuccarelli et al. 2021) and was greater following bed-rest deconditioning (Baldassarre et al. 2022). In the present study we demonstrate that this biomarker of impaired exercise tolerance can be identified also in patients with cardiovascular diseases. The approach should be of interest also from a practical point of view, since it is based upon variables (HR and work rate) which can be easily determined with great precision during exercise, carried out with different ergometers or even in field studies. Moreover, the method does not need gas exchange measurements, or the need for the subject/patient to perform several repetitions of submaximal, maximal and supramaximal exercises, as imposed by other approaches frequently utilized to evaluate exercise tolerance, and based on the determination of the power-duration curve and critical power (Jones et al. 2010). If adequately standardized the approach described in the present study may be valuable also in diseased populations. The observed phenomena (decreases in work rate and [in no-BB] in $\dot{V}O_2$ at a fixed HR) represent a sort of "mirror image" of the progressive increases in HR and $\dot{V}O_2$ as a function of time (traditionally termed "slow component") observed during heavy-intensity (above GET) constant work rate exercise (Zuccarelli 2018, 2021; Baldassarre et al. 2022; Teso et al. 2022; Engelen et al. 1996). Whereas the $\dot{V}O_2$ slow component is associated with decreased efficiency of contractions and fatigue (Grassi et al. 2015), the physiological significance of the HR slow component is less clear, and mostly anecdotal observations are present in the literature (Linnarsson 1974, Engelen et al. 1996; Orizio et al. 1988; Hebestreit et al. 1998; Wasserman et al. 1967; Grassi et al. 1997; Bearden &

Moffatt, 2001). A previous study from our group (Zuccarelli et al. 2018) demonstrated that the HR slow component: i) occurs also during moderate-intensity exercise; ii) during heavy-intensity exercise is more pronounced, percentage-wise, than the $\dot{V}O_2$ slow component. Potential mechanisms responsible for the HR slow component include an increased body temperature (González-Alonso et al. 1995) and an increased β-adrenergic drive (Orizio et al. 1988). Independently from the cause, however, an increasing HR for the same work rate (HR slow component) would inevitably be associated with a decreasing work rate for the same HR, which was the phenomenon observed in the present as well as in our previous studies (Zuccarelli et al. 2018, 2021; Baldassarre et al. 2022). In the present study, by observing a less pronounced work rate decrease for a constant HR in the patients of the BB-group, we confirmed a causative role in the phenomenon attributable to β-adrenergic stimulation. The interplay between the $\dot{V}O_2$ and HR slow components is an issue that needs to be better elucidated. In the present study the work rate decrement was, in the no-BB group, more pronounced than that needed to prevent the $\dot{V}O_2$ slow component. Indeed, during $HR_{CLAMPED}$ exercise in the no-BB group $\dot{V}O_2$ actually decreased (by about 13%), confirming previous observations in healthy young subjects (Zuccarelli et al. 2018, Baldassarre et al. 2022). Significant decreases were observed also for $\dot{V}CO_2$ and R, variables whose progressive increase would be indirectly associated with fatigue. Which mechanism(s) could be held responsible for the more pronounced decrease of work rate, compared with that necessary to keep $\dot{V}O_2$ and R constant (that is to say, to prevent "slow components" or continuous increases of these variables)? No answer to this question can derive from the present results. Another mechanism that might play a role in the determination of the HR slow component is the so called "cardiovascular drift", a phenomenon characterized by a progressive decline in stroke volume associated with a parallel increase in HR, occurring after ~10 min of moderate-intensity exercise (Coyle & González-Alonso, 2000). It has been hypothesized that this phenomenon is linked to hyperthermia and dehydration-induced hypovolemia caused by prolonged exercise (González-Alonso et al. 1995). Unfortunately, body temperature or indices of dehydration were not measured in the present study. However, the mirror image of the HR slow component occurred well before the 10^{th} min of exercise. Furthermore, Zuccarelli et al. (2018) reported during both moderate and heavy-intensity constant work rate exercises no changes in stroke volume despite a progressive increase in HR. It appears unlikely that hyperthermia or dehydration occurred during the relatively short and very submaximal exercise bouts adopted in the present study. Therefore, different factors other than the cardiovascular drift should explain the observed phenomena.

The results of the present study should be relevant also in terms of aerobic exercise prescription. Training intensity is often prescribed at fixed HR value (Gormley et al. 2008; Nybo et al. 2010; Macko

et al. 2005; Ivey et al. 2007, Piepoli et al. 2016; Riebe et al. 2018), mainly for its ease of use, and this common practice is based on the concept of a linear relationship between HR, $\dot{V}O_2$ and work rate (Riebe et al. 2018). However, previous studies by our group (Zuccarelli et al. 2018, 2021; Baldassarre et al. 2022), as well as the present one clearly show that this notion does not hold true. Exercise prescription at fixed HR values, slightly higher than that corresponding to GET (as it is often done when aerobic training is involved) is inevitably associated, even within a relatively short time period (about 15 minutes), with progressive and significant work rate and $\dot{V}O_2$ decreases. This phenomenon occurred in young healthy subjects (Zuccarelli et al. 2018, Baldassarre et al. 2022), in obese adolescents (Zuccarelli et al. 2021) as well as in cardiac patients (no-BB patients of the present study). Exercise prescription at a fixed HR value, therefore, may not allow to adequately control the metabolic stimulus and, presumably, the adaptations to training.

Exercise intensity prescription based on fixed HR values is widely utilized also among patients with cardiovascular diseases (see *e.g.* Piepoli et al. 2016; Fletcher et al. 2001). In the present study we demonstrate that, also in this population, even a short duration task (15-min), which corresponds to half of the minimum duration of aerobic exercise recommended by guidelines (Piepoli et al. 2016), leads to a substantial reduction in work rate (-27%) and in $\dot{V}O_2$ (-13%). In the no-BB group the work rate progressively decreased from a value slightly above GET (high-intensity domain [Rossiter 2011]) at the beginning of the $HR_{CLAMPED}$ exercise to a value corresponding to GET (boundary between high-intensity and moderate-intensity domains [Rossiter 2011]) at the end of 15-min task (see Figure 3). During exercise training sessions of longer duration (e.g. 30-60 min, which is the typical duration prescribed by guidelines [Riebe et al. 2018; Piepoli et al. 2016]) a shift to the moderate-intensity domain appears likely, possibly altering the exercise training stimulus. In more general terms, the effects on training efficacy deriving from keeping HR constant or work rate constant, during training sessions, remain to be specifically evaluated in future studies. The above-mentioned issue seems to be attenuated in BB, in whom the work rate decrease during $HR_{CLAMPED}$ was less pronounced than in no-BB, and presented a plateau after about 9 minutes of exercise. In BB patients, moreover, no $\dot{V}O_2$ decrease was observed during $HR_{CLAMPED}$.

In conclusion, in coronary artery disease patients in stable conditions, during a 15-min exercise on a cycle ergometer initially set at a work rate corresponding to a HR slightly above GET (as frequently done for aerobic exercise prescription), in order to keep HR constant work rate substantially decreased. Confirming our hypothesis, and suggesting a causative role by β-adrenergic stimulation, the work rate decrease was less pronounced (about 16%) in the patients treated with β-blockers (BB) *vs.* that observed (about 27%) in patients not treated with β-blockers (no-BB). The decrease in work rate at a fixed HR may represent, also in cardiac patients, a sign of impaired exercise tolerance (lower

work rate for the same HR), and makes aerobic exercise prescription based on fixed submaximal HR values rather problematic. The issue is less relevant in patients treated with β-blockers. Future studies will have to define if training intensity should be prescribed at a fixed HR or at a fixed work rate.

GRANTS

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4 CONCLUSIONS

The present research project dealt, in general terms, with the evaluation of biomarkers of oxidative function, potentially useful for assessing exercise (in)tolerance as well as evaluating effective preventive and/or therapeutic interventions in diseased and/or inactive population (e.g., patients with injuries, chronic diseases, or astronauts during spaceflight missions). Particular attention was paid to a new non-invasive and simple approach, that is the quantification of the decrease in work rate necessary to keep heart rate (HR) constant at a value slightly above that corresponding to gas exchange threshold (GET), a level frequently utilized in exercise prescription.

Confirming previous studies by our group (Zuccarelli et al. 2018, 2021), we demonstrated that cycling exercise at a fixed HR (slightly higher than that corresponding to GET) is inevitably associated with a progressive and significant reduction in work rate over a 15-minute period, both in healthy untrained subjects (*Study 1*) and in cardiac patients in stable conditions (*Study 2*). Textbooks of physiology say that a higher HR for the same work rate indicates a reduced exercise tolerance (Astrand et al. 1986; McArdle et al. 1986; Wasserman et al. 1999). In *Study 1* and *2*, as well as in our previous ones (Zuccarelli et al. 2018, 2021), we observed a "mirror image" of the above-mentioned phenomenon, that is a lower work rate for the same HR. We postulate that this finding represents a sign of exercise intolerance as well. In support of this concept, we observed that the work rate decrease at a fixed HR was significantly aggravated after 10 days of bed rest (*Study 1*), whereas it was greatly attenuated in obese patients following a 3-week exercise training program (Zuccarelli et al. 2021). Furthermore, work rate decreases needed to keep HR constant following bed rest were significantly correlated with the individual decreases in $\dot{V}O_{2peak}$ (*Study 1*), which is considered one of the most important parameters of functional evaluation of oxidative metabolism. Other "systemic" biomarkers of exercise intolerance were investigated in the same study. Peak work rate, $\dot{Q}_{peak}$, GET and RCP were about 10-20% lower after *vs.* before bed rest, whereas $\dot{V}O_2$ kinetics during transitions from rest to moderate-intensity exercise were about 20% slower after bed rest. These data further demonstrate a significant impairment of oxidative function following 10 days of exposure to microgravity.

Overall, these data should confirm the validity of the proposed approach (work rate decrease at a fixed HR) as a new tool for evaluating exercise (in)tolerance both in healthy and diseased population. However, our data do not specifically identify a threshold (work rate decrease) for a functionally relevant impairment.

The proposed new method should also be of interest from a practical point of view, since it is based on variables (i.e., HR and work rate) which can be easily and reliably measured utilizing different

types of ergometers also in particular experimental conditions (e.g., spaceflights, or in patients with chronic diseases) or even on the field. The new proposed approach offers also other several advantages compared to some traditional tools of exercise (in)tolerance evaluation. For instance, it would obviate the need to perform multiple repetitions of submaximal, or maximal and supramaximal tests to task failure, respectively necessary for an accurate evaluation of the $\dot{V}O_2$ kinetics (Whipp et al. 1982; Lamarra et al. 1987), or for the determination of the power-duration relationship (Poole et al. 1988; Jones et al. 2010), often considered the "gold standard" in terms of exercise tolerance.

Furthermore, the quantification of the decrease in work rate during exercise at a fixed HR does not need complex mathematical analysis, and would also elude the methodological uncertainties associated with GET or RCP determination, requiring the intervention of multiple expert observers and the need for the often invoked "ancillary criteria" (Beaver et al. 1986).

The results of *Study 1* and *2* should also be relevant in terms of aerobic exercise prescription. Both in healthy and disease population, training intensity is usually prescribed at fixed HR value (see e.g., Fletcher et al. 2001; Piepoli et al. 2016; Riebe et al. 2018; Powers & Howley, 2004), mainly for reasons of practicality. This common practice is based on the concept of a linear relationship between HR, $\dot{V}O_2$ and work rate which is however an oversimplification. Confirming previous observations by our group (Zuccarelli et al. 2018, 2021), we demonstrated that exercise prescription at specific HR values, slightly higher than that corresponding to GET (as it is often done when aerobic training is involved) is indeed accompanied by progressive and significant work rate and $\dot{V}O_2$ decreases, which may result in a fall of intensity in different domains (*Study 1* and *2*), possibly altering the training stimulus and resulting adaptations. This aspect remains be specifically evaluated by future studies, which will have to define the best modality for prescribing training intensity, whether for example on the basis of fixed HR or work rate values.

The mechanism(s) responsible for the decrease in work rate during exercise at a fixed HR have not been fully elucidated yet. It appears reasonable to claim that this phenomenon mirrors a "slow component" of HR kinetics during constant work rate exercise, as reported also in previous studies by our group (Zuccarelli et al. 2018; 2021). In support of this statement, we observed greater amplitudes of HR slow component during moderate-intensity exercise after bed rest, and moreover a significant correlation between the individual decreases in work rate at a constant HR and the corresponding values of the amplitude of the HR slow component (*Study 1*). Thus, investigating what determines the work rate decrease during exercise at a clamped HR should be equivalent to investigating the mechanism(s) responsible for the HR slow component during a constant work rate exercise. We observed that the decrease in work rate during exercise at a constant HR was greater than that needed to prevent "slow components" of $\dot{V}O_2$, as well as other variables indirectly associated

with a loss of efficiency and fatigue (i.e., R, blood lactate, muscle deoxygenation), which indeed decreased during exercise (*Study 1* and *2*). Another potential mechanism responsible for the HR slow component may be an augmented sympathetic activity, indeed a close correlation between the increments in HR (slow component) and the increases in blood catecholamines levels was reported by Orizio et al. (1988) during short dynamic exercise at ≥45% of $\dot{V}O_{2peak}$. This observation was substantially confirmed by the results of *Study 2*, namely by the less pronounced work rate decrease for a constant HR in cardiac patients treated with β-blockers. Therefore, a causative role in determining the HR slow component could be attributed, at least in part, to β-adrenergic stimulation. An additional phenomenon which could be hypothesized is the so called "cardiovascular drift", that is a progressive decline in stroke volume associated with a parallel increase in HR occurring after ~10 min of moderate-intensity exercise (Coyle & González-Alonso, 2000). This phenomenon is usually associated with hyperthermia and dehydration caused by prolonged exercise (González-Alonso et al. 1995). Unfortunately, body temperature or indices of dehydration were not measured in our studies. However, the decrease in work rate, namely the mirror image of the HR slow component, occurred early during exercise, well before the 10th min. Furthermore, Zuccarelli et al. (2018) reported no changes in stroke volume despite a progressive increase in HR during moderate and heavy intensity constant work rate exercises. Therefore, different factors other than hyperthermia or dehydration should explain the observed phenomena, and more studies are clearly needed to better address this issue.

The results of study 1, mainly dealing with "systemic" biomarkers of impairment of oxidative metabolism, must be interpreted in conjunction with those obtained by another study of our group on the same subjects (Zuccarelli et al. 2021). In that study we focused the attention on the identification of more "peripheral" biomarkers of impairment of oxidative metabolism following microgravity in order to provide a more complete framework of the site(s) of limitation along the entire O_2 pathway from ambient air to the mitochondria of skeletal muscles. Regarding this study, I mainly dealt with the non-invasive evaluation of the microvascular function and of the skeletal muscle oxidative metabolism by near-infrared spectroscopy (NIRS). In the early phase of a low intensity constant work rate exercise, we detected indirect signs of inadequate matching between O_2 delivery and O_2 uptake as demonstrated by a transient and sharp increase ("overshoot") of muscle O_2 fractional extraction by NIRS. This phenomenon mirrors the "undershoot" of microvascular O_2 partial pressure (PO_{2mv}) determined by phosphorescence quenching, which in turn represents the ultimate force driving blood-myocyte O_2 flux. Interestingly, after 10 days of horizontal bed rest we observed a greater transient overshoot of muscle deoxygenation by NIRS compared to before exposure to microgravity, suggesting an impaired microvascular function, which substantially confirms previous data by our

group (Salvadego et al. 2016, 2018; Porcelli et al. 2010). This microvascular impairment appears to be also in agreement with that identified by the passive leg movement (PLM) approach, that actually revealed a blunted blood flow response after 10 days of bed rest.

For the first time after bed rest, we also evaluated *in-vivo* skeletal muscle oxidative function (mitochondrial respiration) by calculating the skeletal muscle $\dot{V}O_2$ ($\dot{V}O_{2m}$) recovery kinetics following moderate intensity exercises. This non-invasive approach is based on the concept that the linear increase in muscle deoxygenation by NIRS during a transient arterial occlusion represents an index of $\dot{V}O_{2m}$ (Hamaoka et al. 1996; Grassi & Quaresima 2016). Therefore, by performing a series of brief repeated occlusions in the recovery phase of exercise is possible to determine the $\dot{V}O_{2m}$ kinetics, which represents a classic tool of functional evaluation of oxidative phosphorylation (Ryan et al. 2012; Zuccarelli et al. 2020). After bed rest both time constant (τ) and velocity constant (K), which are two important descriptive parameters of $\dot{V}O_{2m}$ kinetics, were unchanged, suggesting that skeletal muscle mitochondrial function was not affected by the intervention. These results were in agreement with those *ex-vivo* obtained by high-resolution respirometry (HRR) on isolated and permeabilized skeletal muscle fibers, which confirmed previous observation by our group (Salvadego et al. 2016). Therefore, it seems that impairments of oxidative metabolism are "upstream" of muscle mitochondria, at the level of central and peripheral O_2 delivery, at least during short-term bed rest (10-day). Longer periods of bed rest exposure, in association or not with countermeasures, need to be investigated by future studies.

Finally, another interesting and novel finding reported by our group (Zuccarelli et al. 2021) during the same 10-d bed rest campaign was related to the resting skeletal muscle $\dot{V}O_2$, evaluated non-invasively by NIRS during a transient limb ischemia in the resting period before exercise onset. We observed a ~15% decrease in skeletal muscle basal metabolic rate after bed rest, which could represent an adaptive process following microgravity/disuse, probably due to the fact that catabolic processes induced by bed rest/inactivity are less energy-consuming than anabolic (Zuccarelli et al. 2021).

The studies presented in this thesis demonstrated that the quantification of the work rate decrease during exercise at a fixed HR (slightly above GET) can be considered a new simple and reliable tool for evaluating exercise (in)tolerance both in healthy (*Study 1*) and diseased population (*Study 2*). However, reproducibility studies are obviously needed, even with different ergometers, before the implementation of the proposed approach in practical terms, also to determine a threshold for the identification of a functionally relevant decrease.

Both in *Study l* and *2* the duration of the protocol was set at 15 minutes, which corresponds to half of the minimum duration of aerobic exercise recommended by guidelines (Riebe et al. 2018; Piepoli et

al. 2016). Nevertheless, a significant decrease in work rate was observed both in healthy young subjects and in patients with cardiovascular disease. Therefore, utilization of fixed percentages of HR during aerobic training session, which is a widely spread approach, may cause a fall of work rate (and metabolic rate) into different intensity domains, potentially leading to major errors in exercise intensity prescription. The problem could be aggravated during training sessions of longer duration (e.g., 30-60), as those prescribed by guidelines (Riebe et al. 2018; Piepoli et al. 2016). However, the effects on training efficacy deriving from keeping HR constant or work rate constant, during training sessions, remain to be specifically evaluated by future studies. On this regard, also the efficacy of training protocols alternating relatively short period of moderate- heavy-intensity exercise with brief active or passive recovery period, could be investigated.

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6 LIST OF PUBBLICATIONS INCLUDED IN THIS THESIS

STUDY 1

Baldassarre G, Zuccarelli L, Manferdelli G, Manfredini V, Marzorati M, Pilotto A, Porcelli S, Rasica L, Šimunič B, Pišot R, Narici M, Grassi B. Decrease in work rate in order to keep a constant heart rate: biomarker of exercise intolerance following a 10-day bed rest. *Journal of Applied Physiology* 132(6): 1569-1579, 2022.

STUDY 2 (submitted)

Baldassarre G, Azzini V, Zuccarelli L, Degano C, Graniero F, Plett G, Floreani M, Lazzer S, Mos L, Grassi B. In cardiac patients beta-blockers attenuate the decrease in work rate during exercise at a constant submaximal heart rate.

7 OTHER PUBBLICATIONS

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 a 10-day bed rest are upstream of mitochondrial respiration. Journal of Physiology 599(21): 4813-4829, 2021.

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Zuccarelli L, De Martino M, **Baldassarre G**, Monti E, Narici M, Isola M, Lippe G, Grassi B. Mitochondrial sensitivity to submaximal ADP concentrations following bed rest: a new approach aimed at identifying different mitochondrial populations. Journal of Physiology (*submitted*).

8 COMMUNICATIONS TO CONGRESSES

Baldassarre G, Azzini V, Zuccarelli L, Degano C, Graniero F, Plett G, Floreani M, Lazzer S, Mos L, Grassi B. In cardiac patients beta-blockers attenuate the decrease in work rate at a fixed heart rate. Poster at the American College of Sports Medicine's 69th Annual Meeting (ACSM), San Diego 31/05-04/06/2022.

Grassi B, **Baldassarre G**, Zuccarelli L. Peripheral impairments of oxidative metabolism during exercise following inactivity. Symposium: The curse of inactivity. 2022 Padua Days on Muscle & Mobility Medicine, Padua (I) 30/03-02/04/2022.

Zuccarelli L, **Baldassarre G**, Grassi B. Peripheral impairments of oxidative metabolism after 10 days of complete inactivity are upstream of mitochondrial respiration. Oral presentation at the XII National Congress of The Italian Society of Exercise and Sport Science (SISMES), Padova 08-10/10/2021.

Zuccarelli L, **Baldassarre G**, Grassi B. Comparison between slow components of heart rate and $\dot{V}O_2$ kinetics: functional significance and practical implications for exercise prescription. Oral presentation at the 71st National Congress of The Italian Society of Physiology (SIF), Milano (online) 07-09/09/2021.

Baldassarre G, Zuccarelli L, Manferdelli G, Manfredini V, Rasica L, Pilotto A, Marzorati M, Porcelli S, Šimunic B, Pišot R, Narici M, Grassi B. Work rate decrease at a fixed heart rate to evaluate exercise tolerance in microgravity. Poster at the American College of Sports Medicine's 68th Annual Meeting (ACSM), Washington 1-5/06/2021.

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. Short-term bed rest exposure impairs peripheral vascular and endothelial functions whereas mitochondrial respiration is unaffected. Poster at the American College of Sports Medicine's 68th Annual Meeting (ACSM), Washington 1-5/06/2021.

Baldassarre G, Zuccarelli L, Manferdelli G, Manfredini V, Rasica L, Pilotto A, Marzorati M, Porcelli S, Šimunic B, Pišot R, Narici M, Grassi B. Work rate decrease during exercise at a fixed

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