The VO_2-on kinetics in constant load exercise sub-anaerobic threshold reflects endothelial function and dysfunction in muscle microcirculation

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Abstract

To propose a test to evaluate endothelial function, based on VO_2 on-transition kinetics in sub-anaerobic threshold (AT) constant load exercise, **w**e tested healthy subjects and patients with ischemic-hypertensive cardiopathy by two cardiopulmonary tests on a cycle ergometer endowed with an electric motor to overcome initial inertia: a pre-test and, after at least 24h, one 6 min constant load exercise at 90% AT. We measured net phase 3 VO_2-on kinetics and, by phase2 time constant (τ), valued endothelial dysfunction. We found shorter τ in repeated tests, shorter time between first and second test, by persisting endothelium-dependent arteriolar vasodilatation and/or several othermechanisms.Reducing load to 80% and 90% AT did not produce significant changes in τ of healthy volunteers, while in heart patients an AT load of 70%, compared to 80% AT, shortened τ (Δ=4.38±1.65s, p=0.013).In heart patients, no correlation was found between NYHA class, ejection fraction (EF), and the two variables derived from incremental cycle cardio-pulmonary exercise, as well as between EF and τ; while NYHA class groups were well correlated with τ duration (r=0.92, p=0.0001).Doxazosin and tadalafil also significantly reduced τ. In conclusion, the O_2 consumption kinetics during the on-transition of constant load exercise below the anaerobic threshold are highly sensitive to endothelial function in muscular microcirculation, and constitute a marker for the evaluation of endothelial dysfunction.

Key words: Anaerobic threshold, Constant load exercise, Endothelial function, O2 consumption

Introduction

At the onset of constant load, sub-anaerobic threshold (AT) cycle cardio-pulmonary exercise (CPX), a sudden and rapid increase in VO_2, usually lasting around 15-20s, is observed; this is called the 1st phase (φI). The appearance of φI is so immediate, and its duration is so short, that VO_2 during this phase is believed to reflect, in large part, the early increase in pulmonary blood flow caused by an increase in heart rate and myocardial contractility, which are mediated both neurologically and by an increased venous return (Casaburi et al. 1989). This phase frequently shows artifacts that prevent it from being analyzed systematically. A 2nd phase (φII) follows the first with a very variable delay, occasionally more than 30s from the imposition of the load, and is characterized by a slower increase in VO_2 according to an approximately exponential time course. This is followed, after around 3 minutes, by a 3rd phase (φIII), which coincides with the steady-state (Figure1) (Linnarsson et al. 1974, Whipp and Wasserman 1972, Whipp 1987).

The 2nd phase has been attributed to a combination of continuous increase of venous return from active muscles and a significant reduction of O_2 content in blood which has been subjected to gas exchange (Barstow and Molé 1987, Barstow et al. 1990, Hughson 1990, Sietsema et al. 1989). In this context, the φII time constant (τ) is the time taken during φII for VO_2 to reach 63% of the rise to its steady state net value (φIII). It has been demonstrated that VO_2 measured in φII and φIII closely reflects the intramuscular O_2 consumption (Barstow and Molé 1987, Barstow et al. 1990, Barstow et al. 1994, Berg et al. 1997, Grassi et al. 1996). The time constant τ can be reduced with training (Sietsema et al. 1989). ATP demand, net of rest quota, is initially met in part thanks to muscle endogenous energy sources, oxymyoglobin and oxyhaemoglobin, but chiefly by phosphocreatine splitting (PCr), whose fall mirrors inversely the rise of VO_2. This keeps the contribution of anaerobic glycolysis in ATP production at a negligible level (Mahler 1985, Marsh et al. 1993, Rostow et al. 1987). This phenomenon was described by Cerretelli and coll. as "early lactate" (Cerretelli et al. 1979), it is always present and has been linked both to flow misdistribution of muscle microcirculation (Delp and Laughlin 1998, Delp 1999, Hughson 1990,

Laughlin 1987, Schoemaker 1999, Remensnyder 1962, Tschakovsky and Hughson 1999), or to inertia in the mitochondrial oxidative metabolic machinery due to activation times for enzymes in the Krebs cycle and electron transport chain activity (Tschakovsky and Hughson 1999, Yoshida et al. 1995). In fact, during the first few seconds of a stress test two conditions that can cause flow misdistribution of muscle microcirculation coexist: on one hand, sympathetic hypertonia at restlimits the influx of blood to muscle microcirculation for the condition needs, and subsequently increases during stress (Whipp and Ward 1990); on the other hand, the initial hyperemia is misdistributed because it occurs without changes in vascular conductance so prefunding still resting as well as already activated fibers (Delp and Laughlin 1998, Delp 1999, Laughlin 1987, Rådegran and Saltin 1998, Remensnyder 1962, Tschakovsky and Hughson 1999). This misdistribution causes mitochondrial PO_2 to dropin active myocytes towards values that produce the “early lactate” phenomenon (Cerretelli et al. 1979, Tschakovsky and Hughson 1999) and moderates the rate of VO_2 increase during the very beginning of φII. Vasodilation is progressively reinforced both by local adenosine release and, more importantly, by endothelium-mediated substances, especially nitric oxide (NO) and prostacyclin, which diffuse to underlying vascular smooth muscle causing it to relax, and dilatenearby arterioles (Flammer and Lüscher 2010, Hughson 1990, Maiorana et al. 2003, Tschakovsky and Hughson 1999), thus creating rapid, widespread "functional sympatolysis" (Remensnyder et al. 1962). The resulting increase in conductance of vascular muscle microcirculation drives increased flow to dilated vessels, thus both improving the match between blood flow and the metabolic rate of active muscle cells and keeping the φII time constant short. The duration τcan therefore yielda purely indirect measure of endothelial function and, if lengthened, also can be considered as a marker of endothelial dysfunction. In cardiovascular disease, myocyte perfusion is often hindered due to a combination of factors: strong reinforcement of sympathetic tone, both centrally-mediated and secondary to renin-angiotensin system stimulation; endothelial dysfunction, resulting in reductions in both NO synthesis/activity and the signaling pathway from NO to cyclic guanosin monophosphate (cGMP)

via soluble guanylyl-cyclase (sGC); increased release of powerful vasoconstrictors such as endothelium-derived contracting factors including several prostanoids, as prostaglandins and thromboxane A2 (arachidonic acid metabolism-derived by cyclooxygenase), endothelin-1 and reactive oxygen species generated by several sources, which, along with other substances, can produce oxidative stress, destroying NO and increasing vascular tone (Vanhoutte 2011, Virdis et al. 2010, Virdis et al. 2013). For these reasons O_2 uptake is hampered in contracting fibers so that they are forced to increase the contribution of anaerobic lactacid metabolism to meet the increased energy requirements, and this prolongs τ duration (Barstow and Molé 1987, Hughson 1990, Maiorana et al. 2003). Therefore, τ lengthening can be considered a marker of endothelial dysfunction. In this paper, to evaluate endothelial function and dysfunction, we propose a test which uses VO_2 on-transition kinetics in sub-AT constant load exercise, always preceded by an incremental CPX, conducted on a different day to determine the load needed to reach AT.

Methods

Population. We screened more than 360 normal subjects and patients with heart disease (NYHA class II and III) (overall called heart patients), in stabilized treatment according to ESH-ESC guidelines (McMurray et al. 2012), and 62 (M. 52, F. 10) agreed to participate in our researches. These represent examples of the method's applicability.

Exercise protocol. All tests were performed in the morning, after 2 hours of resting in a room adjacent to the our laboratory, for reasons described in discussion, using the Medifit1000 (Holland) cycloergometer, calibrated before each session; an ULTIMA-CPX (Medical Graphics Corporation, USA) ergospirometer, calibrated before each test, interfaced with a Case16 (Marquette, USA) electrocardiograph. The subjects first performed a maximal ramping CPX, preceded by a 3 min. rest, starting from 10-15 W, as first load, following by load increments: 15-20 W/min, depending on the status of the patient: healthy and moderately active, sedentary, or heart failure (NYHA functional class II or III). Details of load increments are shown in Table 1. For the purposes of this

study, sedentary subjects are those not participating in activities with a training effect. Then, in a second session, at least 24 hours later, subjects performed a single 6 min. cycle CPX at constant intensity, preceded by a 3 min. rest. For each subject the load was calculated at 90% of the AT (except in the second trial as noted in Table 1),identified in the incremental test by the V slope method (Beaver et al. 1986). No warm-up phase was included in any tests.

During both the incremental and the constant load test, we used a Rudolph mask (7930-7940 series, USA) and sealed the space between mask and face with a special gel modeled on the internal geometry of the mask (Ultimate Seal, Rudolph, USA). To further improve adhesion between face and mask, we replaced the cap and fastening straps of the Rudolph mask with PVC ones. To overcome the flywheel inertia at the onset of exercise, the cycle ergometer was equipped with an electric motor that accelerated the flywheel to its planned rotational velocity, namely 60 rpm for both incremental and constant load tests, while the pedals remained at rest, and this was turned off when pedaling began. Pedaling frequency during exercises was maintained constant with the help of a digital metronome.

All studies were conducted on volunteers in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all study participants. Protocols were approved by the local ethical committee.

Data analysis. A monoexponential model was fitted to the raw unaveraged breath-by-breath VO_2 data of the constant load tests, interpolated every 0.01s, by an our software package: Cardio Pulmonary Exercise Parameters Estimator (C.P.E.P.E.), written in the Matlab language. This iteratively optimizes the model parameters to fit to the VO_2 experimental data (Fig.1), but φI is excluded from the fitting process to reduce artifacts. To further improve the fit and make the test suitable for clinical use, our software started curve fitting from a baseline point, hidden by φI, that was identified as the start of the 2nd phase, using trends in O_2 end-expiration partial pressure ($PETO_2$), the corresponding CO_2 value ($PETCO_2$), and respiratory exchange ratio to mouth (RER). In fact, according to Whipp et al. 1982 “these three variables throughout φI typically were

maintained reasonably stable at their prior control values, on occasion hyperventilation" a very frequent condition" was evidenced in φI, causing $PETO_2$ and RER to increase and $PETCO_2$ to decrease. Thereafter, $PETCO_2$ rose to a new steady level during φII, which was maintained in φIII. $PETO_2$ and R decreased in φII, exhibiting transient undershoots".

A vertical line passing through the point where these latest changes occur, and that therefore corresponds to the start of φII, reaches the baseline in a point from which fitting curve starts. The fitted curve must pass through the point where φI meets φII, which then becomes visible (Fig.1,2). Net VO_2 at steady-state (φIII) was measured and theφII time constant was calculated on the fitting curve. If curve fitting were forced to detach from the baseline at the point where the load is applied, as has always been done in past, φI would be incorporated into the calculation of τ, whose duration would then be lengthened (Whipp et al. 1982).

Statistical analysis. In all cases the parameter of interest was τ, the exponential rise time in φII. The normality of the distribution of the tested parameters was evaluated by the Kolmogorov-Smirnov test. Differences in τ between first and repeated runs in the first trial were tested by the ANOVA test for repeated measures with Bonferroni correction; while in the other trials differences of τ before and after treatment were computed by the t-test for paired data. In the third trial the relationships betweenτ and the selected variables were obtained by linear correlation. In all statistical analyses the significance level was set at $p<0.05$. Summary difference statistics are presented as mean ± standard deviation (SD), and percentage differences and effect sizes, expressed as Cohen's d, are also given.

The characteristics of the trials population, noted below, are summarized in Tab 2.

- First trial. The effect of retest onτ.

The effect of repeat testing on τ variation was examined in a set of28 males: 3 healthy subjects and 25 treated heart patients NYHA class II (Table 2),, separated in 3 equal number groups, based on the time separating two successive tests, approximately 30, 60 and 120 min. The tests, rigorously with a load corresponding to 90% of the AT, were performed in different days with same constant

load. 30 tests were performed because one of the patients was inserted in two groups, and another one was in all three groups; the normal subjects were distributed between all 3 groups.

- Second trial. The dependence of τ on the load intensity.

The study of the dependence of τ on the load intensity was conducted on five healthy subjects and four male treated heart patients NYHA class II (Table 2). Two exercises at constant intensity were performed on different days, with a load, corresponding to 90% and 80% of the AT in healthy subjects and 90% and 70% in heart patients.

- Third trial. Relationship between τ and some heart efficiency ratios.

In 17 treated heart patients (Table 2)12in NYHA class II and 5 in NYHA III linear correlations were calculated between τ duration, and variables obtained from cycle CPX: peak VO_2 (pVO_2), measured as the % of its theoretical maximal value; VO_2 at AT, measured as the % on the theoretical minimum (40% of theoretical pVO_2); echocardiographic ejection fraction (EF); and NYHA classes II and III, assigning a score to these of 2 or 4, respectively.

- Fourth trial. Effect of α1-blocker administration.

2 mg/day doxazosin was administered to four NYHA class II heart patients, in standard stabilized treatment (Table 2). The constant τ was assessed immediately before treatment, and following one week of administration.

- Fifth trial. Effect of tadalafil administration

20 mg tadalafil was administered to four NYHA class II heart patients (Table 2), in standard stabilized treatment. The constant τ was assessed immediately before treatment, and 28 hours following administration.

Results

We found that τ values of healthy individuals do not exceed 13s, regardless of age, as long as physically active, while values above 16s are clearly pathologic; values within this range could be considered in a grey zone which may occur both in healthy people with a sedentary lifestyle and in

patients with mild pathology.

Trial 1 – Representative time courses are shown in Figure 3.

The time intervals separating two successive tests were: in the 1st group 31.40±4.74 min, in the 2nd group 66.60±10.92 min, in the 3rd group 113.30±10.41 min; in short 30 min, ~1 hour, ~ and 2 hours. In the 1st group τ average duration in initial test was 28.19±7.56s and 20.21±9.37s in repeated tests, with a difference (Δ) of -7.98±2.00 s, (= -28.31%; d=0.94) ($p<0.0001$); in the 2nd group τ was respectively 23.52±10.91s and 19.87±8.26s, with a Δ of -3.65±3.39 s, (= -15.52%; d=0.23) ($p= 0.008$); in the 3rd group 19.64±7.24s and 18.44±7.39s with a Δ of -1.20±1.00s, ($p= 0.004$), which, expressed as a percentage is -6.11% (d=0.16). A positive correlation was also found between τ value in 1st test, independent variable, and the difference between τ values of the two successive tests of each subject: in 1st group, r=0.739 ($p=0.015$) as in 2nd group, r=0.843 ($p=0.002$). However, this effect did not occur in 3rd group, r=0.072 ($p=0.842$), likely due to the sample size, that was too limited compared to the small difference in τ duration between the two tests. Thus, τ duration is significantly decreased in repeated exercises and the τ differences between first and repeated tests were reduced significantly from 1st to 3rd group, while remaining still statistically significant in the 3rd. Furthermore, in the first two groups a greater τ duration in the first tests was associated with a greater reduction of τ in the repeated tests.

Trial 2 –

The reduction of load from 90% of AT (τ=11.90s) to 80% (τ=11.10s) in healthy subjects did not produces significant changes in τ (Δ=-0.80±1.20 s or -6.72%, d=0.16,$p=0.212$), while in heart patients the reduction from90% AT (τ=24.87s) to 70% AT (τ=20.50s) has significant shortenedτ (Δ=- 4.38±1.65 s or = -17.57%,d=0.40, $p= 0.013$).

Trial 3 –

No direct relation, by linear correlation, was found between NYHA class, EF, and the two variables derived from incremental cycle CPX, or between EF and τ: r was 0.34, $p= 0.78$; while

NYHA II and III classes groups were well correlated with τ duration (r =0.92, p= 0.0001).

Trial 4 – Representative time courses are shown in Figure 4.

The effect in treated heart patients of the α1-blocker, 2 mg/day doxazosin, was to reduce τ from 34.10±6.26sbefore treatment to 21.27±3.89s one week after treatment, with a mean Δ between before and after treatment of -12.83±3.36s, or-37.5±5.03%, d=2.46, p=0.005.

Trial 5 – Representative time courses are shown in Figure 5.

The effect of 20 mg tadalafil 28 hours after administration was to decrease τ, from 31.15±16.51s to 22.90±15.26s with a mean Δ between before and after treatment of -8.25±4.06s, or -26±12%, d=0.52, p=0.027.

Discussion

Trial 1 – Effect of the retest onτ.

Development of protocol (Fig.3).

Initially, we subjected each subject to at least four tests at constant load separated by 30-40 min, following the example of previous researchers, who performed up to eight or nine repetitions (Casaburi et al. 1989, Sietsema et al. 1989, Whipp et al. 1982). The tests were then interpolated once per second, time aligned to exercise start, superimposed and averaged, for subsequent curve fitting. For later tests, including those presented here, only a single exercise at constant load was performed, for two reasons: to prevent the inevitable time lag, increased with the number of repetitions requested, between the verbal command to start a test and the actual onset of test, with repercussions on the data; but, more particularly, because in the repetitions of the same load we found that the τ duration was much shorter, the lower was the interval between the two tests. Besides, the greater was the τ duration in the first tests the greater was the reduction in repeated tests. It is well-known that in healthy subjects endothelial NO release is significant even at rest, but increasingly compromised with greater degrees of endothelial dysfunction (Afanas'ev 2009, Förstermann 2010, Halcox et al. 2009, Hughson 1990, Li and Förstermann 2009, Münzel et al.

2008). Thus, both arteriolar vasodilation at the beginning of dynamic effort, which represents a powerful physiological stimulus to increase NO bioavailability, and inertia in the mitochondrial oxidative metabolic machinery, can be improved during the repeated exercise of subjects with increased endothelial dysfunction compared to normal, physically active, subjects, in which these effects are more evident and already present at rest (Green et al. 2004): i.e. it is more difficult for an already normal endothelial function to improve. Several mechanisms to explain the persistence of vasodilation in repeated tests have been proposed (Camley et al. 2007, Francis et al. 2008, Smith et al. 1996), but as yet there is no consensus on the matter. Consequently, in our protocol, subjects performed the tests after 2 hours of rest to avoid the influence of walking, prior to their arrival at our laboratory, on the results of the incremental and constant load tests and remained at rest between tests and subsequent. For the same reason, all tests were performed without warm-up. In the third group of the first trial, the difference in τ average duration between initial and repeated tests was of -6.11%; the small difference can be taken as an indication of the repeatability of the test.

A shorter φII τ was also found by Rossiter et al. in seven well-trained males in the second of two consecutive 6 min. bouts of high-intensity square-wave knee-extensor exercise in prone position with 6 min rest interval (Rossiter et al. 2001). Besides, several authors invoked the NO-Cox interaction to explain the O_2 cost reduction in moderate-intensity exercise, 80% AT, in well-trained healthy subjects after consumption of nitrate supplementation or of nitrate-rich vegetables (Bailey et al. 2009,Lansley et al. 2011, Larsen et al. 2007, Larsen et al. 2010), and of a beverage containing L-arginine (Bailey et al. 2010), an effect that is generally observed in high-intensity, supra-AT exercise (Maione et al. 2013). If this effect were present in our tests it could have affected the τ duration, but even in our patients treated with a PDE5 inhibitor (trial 5, Figure 5), and hence with increased NO bio-availability, the O_2 cost was unchanged. Literature data (Brown and Cooper 1994, Erusalimsky and Moncada 2007, Flammer and Lüscher 2010, Palacios-Callender et al. 2007, Sarti et al. 2000) support the belief that this mechanism, which reduces O_2

cost in supra-AT exercises, is primed (and it can be supported by a possible and not necessary reduction of O_2 concentration) by an increase of NO concentration which exceeds some definite but unpredictable level (Cooper and Giulivi 2007), but also by AMP/ATP-dependent AMPK activation, when metabolism becomes primarily aerobic (Hardie and Sakamoto 2006). Our subjects were either heart patients or healthy, sedentary or moderately active but otherwise untrained, volunteers; and their exercise load, namely 90% AT, was considerably less than that employed by Rossiter et al. to challenge the well-trained subjects, at 80% AT because these subjects reached AT at a significant higher load. Thus we conclude that the O_2 cost reduction, always present in supra-AT exercise, occurs in sub-AT only if the load is sufficiently high to both inhibit mitochondrial respiration by NO over-expression, and to activate AMPK, consequences naturally more apparent in trained individuals. Besides, it is unlikely, with the loads used in the our protocol, that NOinhibitedmitochondrial cytochrome c oxidase (CcO),during φII and φIII, as is observed at high concentrations of NO, with consequent O_2 savings (Brown and Cooper 1994, Erusalimsky and Moncada 2007, Flammer and Lüscher 2010, Sarti et al. 2000), a mechanism that characteristically depends just on the NO/O_2 ratio (Palacios-Callender et al. 2007). Also, sGC, the enzymatic target of NO, is approximately 50 times more sensitive to NO than to CcO (Bellamy et al. 2002) so it is unlikely that NO would be able both to maximally activate sGC and to inhibit cellular respiration (Bellamy et al. 2002, Rodríguez-Juárez et al. 2007), not least because CcO can also be partially inhibited by NO without an effect on cellular respiration (O_2 consumption) (Palacios-Callender et al. 2007).

Trial 2 – Dependence of τ on load intensity.

In this trial, the τ of healthy subjects was not significantly changed by varying the load, provided that the exercise was always performed below the AT; in contrast, in cardiac diseases a lower load was associated with a significantly shorter τ. Consequently, the patients' responses to effort depend not only on the clinical conditions but also on the load. A pathologic response is observed at a higher load, while normal responses are maintained with a lower load. Therefore, at least in

subjects without severe impairment, it is not possible to talk of myopathy caused by heart failure, but of non-permanent metabolic adaptations to stress in conditions of impaired local perfusion.

Trial 3 –Correlation between τ, NYHA class, and some heart efficiency ratios.

In heart failure patients, by linear correlation, a direct relation was demonstrated between the τ duration and the NYHA class while no correlation was found with echocardiographically obtained EF. These results showed that τ duration enables the severity of heart failure to be classified in a manners parallel to the NYHA classification.

Trial 4 – Effect of the addition of doxazosin onτ (Fig.4).

In treated heart patients, standard pharmaceutical treatment β-blockers inclusive lengthens the τ duration by bradycardia and increase in peripheral resistance induced, as a result of the α tone escape. This β-block effect is also present with the specific β-blocker bisoprolol, while does not occur with the latestβ-blockers with intrinsic vasodilating action. Treatment with the α1-blocker doxazosin has speeded upτin our study compared to before values observed prior to administration due to its vasodilator activity. Therefore, the association between β-blockers and doxazosin is favorable because it may partially limit the not always useful effects of β-blockade.

Trial 5 – Effect of addition of tadalafil onτ(Fig.5).

Tadalafil, a long lasting phosphodiesterase 5 inhibitor (PDE-5) that prolongs NO and cGMP bioavailability, used in men with erectile dysfunction, was administered to four patients, inducing, even 28 hours after its administration, a significant reduction of τ.The persistence after so many hours of arterial (and venous) vasodilation by tadalafil is still not entirely clear (Camley et al. 2007, Francis et al. 2008, Smith et al. 1996).

The results of the trials suggest that the pharmacological and non-pharmacological treatment of heart disease, which reduces both shear-stress and oxidative stress (Nediani et al. 2011, Versari et al. 2009),quickly improves endothelial function and can also normalize the τ duration even without changing, initially, the severity of myocardial damage, which is persistently detected by echocardiography. In treated heart patients, in which the τ duration is not yet completely in the

normal range, we speculate that the test could be used to optimize the treatment, by add ingnew drugs and/or increasing the dosage of those already administered, up to achieve the normalization of τ and probably the recovery of a proper endothelial function.

An alternative, and currently more popular, technique to indirectly assess endothelial function, "flow mediated dilation", uses the shear stress induced in the endothelium of peripheral conductance arteries (normally the brachial artery) by a non-physiological stimulus, namely post ischemic hyperemia, which involves the application of a blood pressure cuff for 5 minutes, causing limb ischemia, and its abrupt deflation to restart a hyperemic limb blood flow. Ischemia of such duration can cause the formation of reactive O_2 species (ROS), such as superoxide anion (O_2^{-*}) and H_2O_2, which, apart from increasing vascular tone, readily react with vascular NO to form peroxynitrite (ONOO-), a powerful oxidant. Tetrahydrobiopterin (BH4), an eNOS-dependent cofactor to NO synthesis, is highly sensitive to oxidation by ONOO-. Excessive oxidation and BH4 depletion promote eNOS uncoupling, the production of O_2^{-*} by eNOS rather than NO (31), and interfere with the test sensibility. Even organic nitrate administration is not infallible as a means to show a possible ROS effect. Hemoprotein sGC, the first enzyme in the NO signal cascade, is sensitive to the gas only if the iron in its prosthetic heme group is in a reduced or bivalent form. In pathological conditions, the iron may be in an oxidized or trivalent form due to oxidative stress, thus rendering sGC insensitive to NO. The prosthetic group may even be removed, thereby blocking the downstream signal (Miller et al. 2009, Stash et al. 2006). Thus unchanged test results in patients after nitrate administration may signify an NO insensitive catalytic sGC center, rather than ROS production.

On the other hand, the physiological stimulus outlined in the current work, namely dynamic cycloergometer exercise at mild to moderate intensity, altering VO_2 kinetics sub-AT, allows the sensitive evaluation of endothelium-dependent vasodilation at onset exercise within muscular microcirculation where gas exchange occurs. Obviously the method has limitations, as well as advantages: the procedure is relatively complex and unable to provide correct results in cases of

major reduction of lower limb blood flow due to arterial stenosis, in severe bronchopulmonary disease with serious obstacle to air flow and/or pulmonary gas exchange or significant pulmonary hypertension. Additionally, in athletes the φII time constant is very often less than 7 sec, too short to be properly estimated by exponential fitting of breath-by-breath data. However, with the method described we obtained a fitting curve which was closer to the actual breath-by-breath data collected during this type of exercise. In this operation we followed the path suggested by Prof. Whipp (Whipp et al. 1982).

Conclusion

In conclusion the kinetic of O_2 consumption during the on-transition of constant load exercise below the anaerobic threshold appears to be highly sensitive to endothelial function in muscular microcirculation, and may represent a marker for the evaluation of endothelial dysfunction.

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Conflict of interest

No author has direct or indirect conflict of interest in the publication of this paper.

References

- AFANAS'EV I:*Superoxide and nitric oxide in senescence and aging.* Front Biosc. **14:**3899-912, 2009
- BAILEY SJ, WINYARD P, VANHATALO A, BLACKWELL JR, DIMENNA FJ, WILKERSON DP, TARR J, BENJAMIN N, JONES AM:*Dietary nitrate supplementation reduces the O_2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans.* J Appl Physiol. **107:**1144-55, 2009.
- BAILEY SJ, WINYARD PG, VANHATALO A, BLACKWELL JR, DIMENNA FJ, WILKERSON DP, JONES AM:*Acute L-arginine supplementation reduces the O_2 cost of moderate intensity exercise and enhances high-intensity exercise tolerance.* J Appl Physiol. **109:**1394-403, 2010.
- BARSTOW TJ AND MOLÉ PA:*Simulation of pulmonary O_2 uptake during exercise transients in humans.* J Appl Physiol. **63:**2253-61, 1987.
- BARSTOW TJ, LAMARRA N, WHIPP BJ:*Modulation of muscle and pulmonary O_2 uptakes by circulatory dynamics during exercise.* J Appl Physiol. **68:**979-89, 1990.
- BARSTOWTJ, BUCHTHAL S, ZANCONATO S, COOPER DM:*Muscle energetics and pulmonary oxygen uptake kinetics during moderate execise.* J Appl Physiol. **77:**1742-9, 1994.
- BEAVER WL, WASSERMAN K, WHIPP BJ:*A new method for detecting the anaerobic threshold by gas exchange.* J Appl Physiol. **60:**2020-7, 1986.
- BELLAMY TC, GRIFFITHS C, GARTHWAITE J:*Differential sensitivity of guanylyl cyclase and mitochondrial respiration to nitric oxide measured using clamped concentrations.* J Biol Chem. **277:**31801-7, 2002.

- BERG BR, COHEN KD, SARELIUS ICH:*Direct coupling between blood flow and metabolism at the capillary level in striated muscle.* Am J Physiol. **272:**H2693-700, 1997.
- BROWN GC, COOPER CE:*Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal cytochrome oxidase respiration by competing with oxygen at cytochrome oxidase.* FEBS Lett. **356:**295-298, 1994.
- CAMLEY SM, SAWYER CL, BRUNELLE KF, VAN DER VLIET A, DOSTMANN WR:*Nitric oxide-evoked transient kinetics of cyclic GMP in vascular smooth muscle cells.* J Cell Sign **19:**1023-33, 2007.
- CASABURI R, DALY JA, HANSEN J, EFFROS R:*Abrupt changes in mixed venous blood gas composition after the onset of exercise.* J Appl. Physiol. **67:**1106-12, 1989.
- CERRETELLI P, PENDERGAST D, PAGANELLI WC, RENNIE W:*Effect of specific muscle training on VO_2 on-response and early blood lactate.* J Appl Physiol Respir Environ Exerc Physiol. **47:**761-9, 1979.
- COOPER CE, AND GIULIVI C:*Nitric oxide regulation of mitochondrial oxygen consumption II: molecular mechanism and tissue physiology.* Am J Physiol Cell Physiol. **292:**C1993-2003, 2007.
- DELP MD, LAUGHLIN MH:*Regulation of skeletal muscle perfusion during exercise.* Acta Physiol Scand. **162:**411-9, 1998.
- DELP MD:*Control of skeletal muscle perfusion at the onset of dynamic exercise.* Med Sci Sports Exerc. **31:**1011-8, 1999.
- ERUSALIMSKY JD, MONCADA S:*Nitric oxide and mitochondrial signaling: from Physiology to pathophysiology.* Arterioscler Thromb Vasc Biol. **27:**2524-31, 2007.
- FLAMMER AJ, LÜSCHER TF:*Three decades of endothelium research: from the detection of nitric oxide to the everyday implementation of endothelial function measurements in cardiovascular diseases.* Swiss Med Wkly.**140:**w13122, 2010.

- FÖRSTERMANN U:*Nitric oxide and oxidative stress in vascular disease.* Pflugers Arch. **459:**923-39, 2010.
- FRANCIS SH, MORRIS CZ, CORBIN JD:*Molecular mechanisms that could contribute to prolonged effectiveness of PDE5 inhibitors to improve erectile function.* Int J Impot Res. **20:**333-42, 2008.
- GRASSI B, POOL DC, RICHARDSON RS, KNIGHT DR, ERICKSON BK, WAGNER PD:*Muscle O_2 uptake in humans: implications for metabolic control.* J Appl Physiol. **80:**988-98, 1996.
- GREEN DJ, MAIORANA A, O'DRISCOLL G, TAYLOR R:*Effect of exercise training on endothelium-derived nitric oxide function in humans.* J Physiol. **561:**1-25, 2004.
- HALCOX JPJ, DONALD AE, ELLINS E, WITTE DR, SHIPLEY MJ, BRUNNER EJ, MARMOT G, DEANFIELD JE:*Endothelial function predicts progression of carotid intima-media thickness.* Circulation. **119:**1005-12, 2009.
- HARDIE DG, SAKAMOTO K: *AMPK: a key sensor of fuel and energy status in skeletal muscle.* Physiology. **21:**48-60, 2006.
- HUGHSON RL:*Exploring cardiorespiratory control mechanisms through gas exchange dynamics.* Med Sci Sports Exerc. **22:**72-9, 1990.
- LANSLEY KE, WINYARD PG, FULFORD J, VANHATALO A, BALLEY SJ, BLACKWELL JR, DI MENNA FJ, GILCHRIST M, BENJAMIN N, JONES AM:*Dietary nitrate supplementation reduces the O_2 cost of walking and running: a placebo-controlled study.* J Appl Physiol. **110:**591-600, 2011.
- LARSEN FJ, WEITZBERG E, LUNDBERG JO, EKBLOM B:*Effect of dietary nitrate on oxygen cost during exercise.* Acta Physiol. **191:**59-66, 2007.
- LARSEN FJ, WEITZBERG E, LUNDBERG JO, EKBLOM B:*Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise.*

Free Radical Biol Medicine. **48:**342-7, 2010.

- LAUGHLIN MH:*Skeletal muscle blood flow capacity. Role of muscle pump in exercise hyperemia.* Am J Physiol. **253:**H993-1004, 1987.
- LI H, FÖRSTERMANN U:*Prevention of atherosclerosis by interference with the vascular nitric oxide system.* Curr Pharm Des. **15:**3133-45, 2009.
- LINNARSSON D:*Dynamics of pulmonary gas exchange and hearth rate changes at start and end of exercise.* Acta Physiol Scand Suppl. **415:** 1-68, 1974.
- MAHLER M:*First-order kinetics of muscle oxygen consumption and an equivalent proportionality between QO_2 and phosphoryl-creatinine level.* J Gen Physiol. **86:**135-65, 1985.
- MAIONE D, CICERO AFG, BACCHELLI S, COSENTINO E, MANNERS DN, D'ADDATO S, DEGLI ESPOSTI D,SENALDI R, STROCCHI E, AMBROSIONI E, BORGHI C: *VO2 Kinetics in Supra-Anaerobic Threshold Constant Tests Allow the Visualisation and Quantification of the O2 Saving after Cytochrome C Oxidase Inhibition by Aerobic Training or Nitrate Administration.* Physiol.Res.**62**: 671-679, 2013.
- MAIORANA A, O'DRISCOLL G, TAYLOR R, GREEN D:*Exercise and the nitric oxide vasoditatorsystem.* Sports Med. **33:**1013-35, 2003.
- MARSH GD, PETERSON DH, POTWARKA JJ, THOMPSON RT:*Transient changes in muscle high-energy phosphates during moderate exercise.* J Appl Physiol. **75:**648-56, 1993.
- MCMURRAY JJ, ADAMOPOULOS S, ANKER SD, AURICCHIO A, BÖHM M, DICKSTEIN K, FALK V, FILIPPATOS G, FONSECA C, GOMEZ-SANCHEZ MA, JAARSMA T, KØBER L, LIP GY, MAGGIONI AP, PARKHOMENKO A, PIESKE BM, POPESCU BA, RØNNEVIK PK, RUTTEN FH, SCHWITTER J, SEFEROVIC P, STEPINSKA J, TRINDADE PT, VOORS AA, ZANNAD F, ZEIHER A; ESC Committee for Practice Guidelines:*ESC Guidelines for the diagnosis and treatment of acute and*

chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. Eur Heart J. **33:**1787-847, 2012.

- MILLER TW, CHERNEY MM, LEE AJ, FRANCOLEON NE, FARMER PJ, KING SB, HOBBS AJ, MIRANDA KM, BURSTYN JN, FUKUTO JM:*The effects of nitroxyl (HNO) on soluble guanylate cyclase activity: interactions at ferrous heme and cysteine thiols.* J Biol Chem. **284:**21788-96, 2009.
- MÜNZEL T, SINNING C, POST F, WARNHOLTZ A, SCHULZ E:*Pathophysiology, diagnosis, and prognostic implications of endothelial dysfunction.* Ann Med. **40:**180-96, 2008.
- NEDIANI C, RAIMONDI L, BORCHI E, CERBAI E: *Nitric oxide, reactive oxygen species generation and nitroso redox imbalance in heartfailure: from molecular mechanisms to therapeutic implications.*Antioxid Redox Signal.**14:** 289-331, 2011.
- PALACIOS-CALLENDER M, HOLLIS V, FRAKICH N, MATEO J, MONCADA S:*Cytochrome c oxidase maintains mitochondrial respiration during partial inhibition by nitric oxide.* J Cell Sci. **120:**160-5, 2007.
- RÅDEGRAN G, SALTIN B:*Muscle blood flow at onset of dynamic exercise in humans.* Am J Physiol. **274:**H314-22, 1998.
- REMENSNYDER JP, MITCHELL JH, SARNOFF SJ. *Functional sympatholysis during muscular activity.* Circ Res 1962; 11: 370-80.
- RODRÍGUEZ-JUÁREZ F, AGUIRRE E, CADENAS S:*Relative sensitivity of guanylate cyclase and mitochondrial respiration to endogenous nitric oxide at physiological oxygen concentration.* Biochem J. **405:**223-31, 2007.
- ROSSITER HB, WARD SA, KOWALCHUK JM, HOWE FA, GRIFFITHS JR, WHIPP

BJ:*Effects of prior exercise on oxygen uptake and phosphocreatine kinetics during high-intensity knee-extension exercise in humans.* J Physiol. **537:**291-303, 2001.

- ROSTOW WI, WHIPP BJ, DAVIS JA, EFFROS RM, WASSERMAN K:*Oxygen uptake kinetics and lactate concentration during exercise in man.* Am Rev Respir Dis. **135:**1080-4, 1987.
- SARTI P, GIUFFRÈ A, FORTE E, MASTRONICOLA D, BARONE MC, BRUNORI M:*Nitric oxide and cytochrome c oxidase: mechanisms of inhibition and NO degradation.* Biochem Biophys Res Commun. **274:**183-7, 2000.
- SCHOEMAKER JK, HUGHSON RL:*Adaptation of blood flow during the rest to work transition in humans.* Med Sci Sports Exerc. **31:**1019-26, 1999.
- SIETSEMA KE, DALY JA, WASSERMAN K:*Early dynamics of O_2 uptake and heart rate as affected by exercise work rate.* J Appl Physiol. **67:**2535-41, 1989.
- SMITH JA, FRANCIS SH, WALSH KA, KUMAR S, CORBIN JD:*Autophosphorylation of type Ibeta cGMP- dependent protein kinase increases basal catalytic activity and enhances allosteric activation by cGMP or cAMP.* J Biol Chem. **271:**20756-62, 1996.
- STASH JP, SCHMIDT PM, NEDVETSKY PI, KUMAR A, MEURER H, TURGAY Y, ROTHKEGEL C, TERSTEEGEN A, KEMP-HARPER B, MÜLLER-ESTERL W, SCHMIDT HH:*Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels.* J Clin Invest. **116:**2552-61, 2006.
- TSCHAKOVSKY ME, HUGHSON RL:*Interaction of factors determining oxygen uptake at the onset of exercise.* J Appl Physiol.**86:** 1101-13, 1999.
- VANHOUTTE MP:*Endothelium-dependent contractions in hypertension when prostacyclin becomes ugly.* Hypertension. **57:**526-31, 2011.
- VERSARI D, DAGHINI E, VIRDIS A, GHIADONI L, TADDEI S: *Endothelial Dysfunction as a Target forPrevention of Cardiovascular Disease.* Diabetes Care. **32:** S314-

S31, 2009.

- VIRDIS A, GHIADONI L, TADDEI S:*Human endothelial dysfunction: EDCFs.* Pflugers Arch. **459:**1015-23, 2010.
- VIRDIS A, BACCA A, COLUCCI R, DURANTI E, FORNAI M, MATERAZZI G, IPPOLITO C, BERNARDINI N, BLANDIZZI C, BERNINI GP, TADDEI S:*Endothelial dysfunction in small arteries of essential hypertensive patients: role of cyclooxygenase-2 in oxidative stress generation.* Hypertension. **62:**337-44, 2013.
- WHIPP BJ, WASSERMAN K:*Oxygen uptake kinetics for various intensities of constant load work.* J Appl Physiol. **33:**351-6, 1972.
- WHIPP BJ, WARD SA, LAMARRA N, DAVIS JA, WASSERMAN K: Parameters of ventilator and gas exchange dynamics during exercise. J Appl Physiol Respir Environ Exerc Physiol. **52:**1506-13, 1982.
- WHIPP BJ:*Dynamics of pulmonary gas exchange.* Circulation. **76:**VI18-28, 1987.
- WHIPP BJ, WARD SA:*Physiological determinants of pulmonary gas exchange kinetics during exercise.* Med Sci Sports Exerc. **22:**62-71, 1990.
- YOSHIDA T, KAMIYA J, HISHIMOTO K:*Are oxygen uptake kinetics at the onset of exercise speeded up local metabolic status in active muscles?* Eur J Appl Physiol Occup Physiol. **70:**482-6, 1995.

Table 1 Load protocol for different groups of subjects referred to in text

Group	**Incremental test load (W)** **Subgroup**			**Constant test load (%AT)**	
		Step 1	**Later steps**	**Trial 2**	**Trials 1,3-5**
Without heart failure	Active	15	20	Day 1: 90% Day 2: 80%	90%
	Sedentary	15	15		
NYHA class II	Active	15	15	Day 1: 90% Day 2: 70%	
	Sedentary	10	15		
NYHA class III	-	10	10		

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Tab. 2_ Characteristics of study populations

	gen	years	cm	kg
First trial				
N.S. 3	M. 3 —	66.41±9.19	168.78±8.07	82.04±14.64
Pts. 25	M. 22 F.3			
Second trial				
N.S. 5	M. 3 F.2	56.80±3.42		
Pts. 4	M. 4 —	72.50±1.73		
Third trial				
N.S. —				
Pts. 17	M. 12 F. 5	69.4, range 50-80	160.73±5.10	74.80±7.98
Fourth trial				
N.S. —				
Pts. 4	M. 4 —	73.8±5.12	164.8	75.5
Fifth trial				
N.S. —				
Pts. 4	M. 4 —	65±4.40	170±3.74	84±5.56

N.S.: normal subjects Pts.: patients

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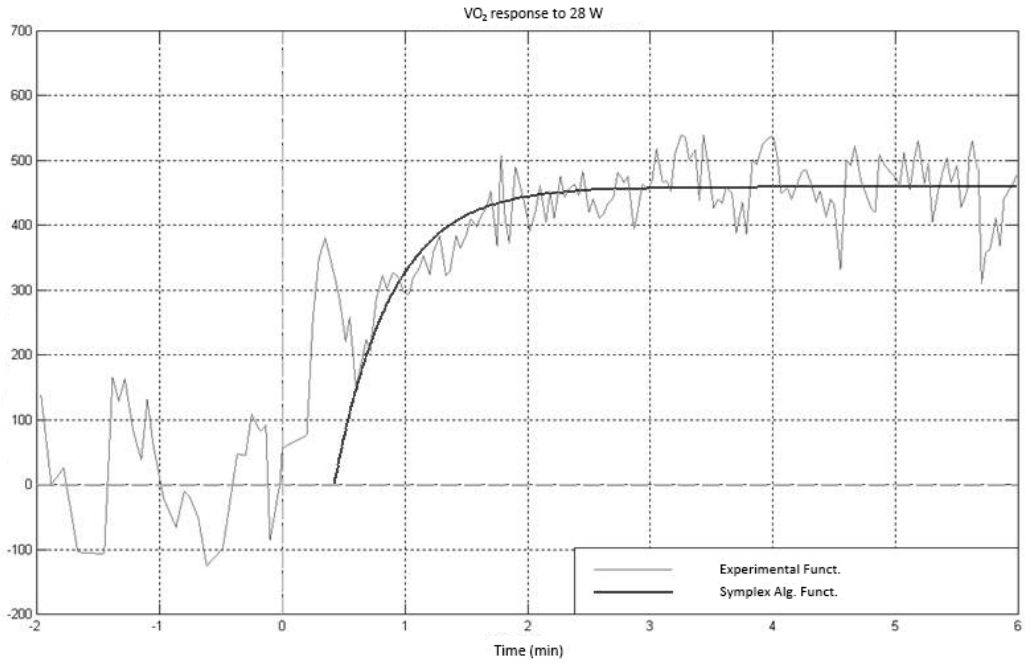
Fig. 1 - In grey the on-transition VO_2 kinetics in ml min^{-1} of rectangular exercise sub-AT; in black the fitting curve superimposed on second and third phase.Raw breath-by breath data.

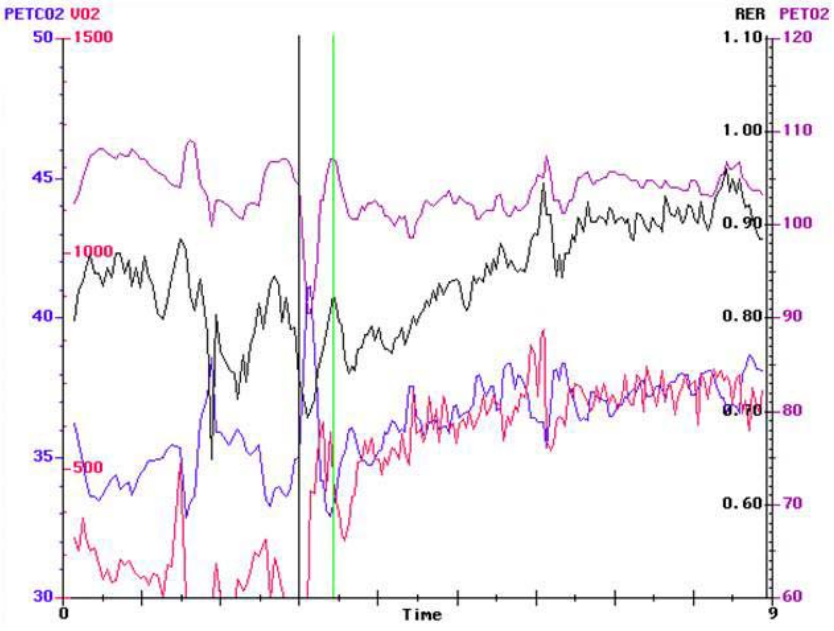
.Fig. 2 - Method of highlighting point from which fitting curve leaves basic line. In blue $PETCO_2$= end-tidal PCO_2 mmHg; in red VO_2- on kinetics sub-SA ml min^{-1}; in black RER= respiratory exchange ratio; in violet $PETO_2$= end tidal PO_2 mmHg.Raw breath-by breath data.

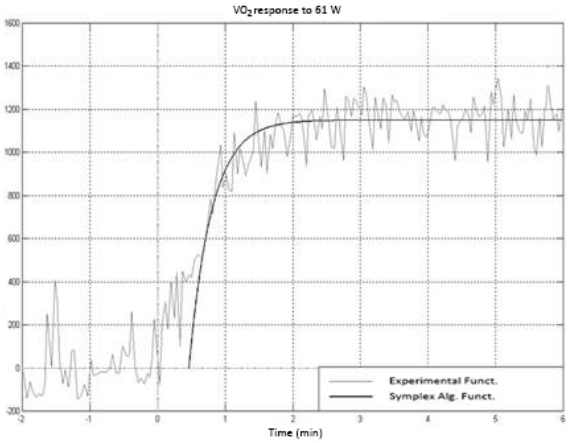
Fig. 3 –VO_2 compared to baseline (mi m^{-1}). Above: the first test in a patient (τ= 20.08 s).; below bottom: repeated test after ~ 30 min in same patient (τ = 13.06 s); difference of 7.02 s. Raw breath-by breath data..

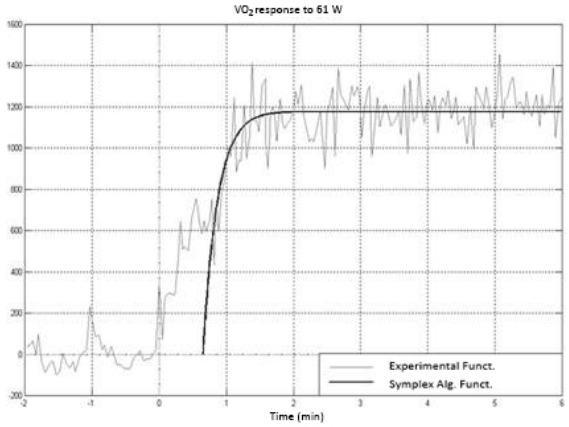
Fig. 4 VO_2 compared to baseline (mi m^{-1}). τ duration in a patient: at left,pre treatment (τ=34.10 s) and,at right, after taking 2 mg/day for 1 weekdoxazosin (τ=21.27 s.). Raw breath-by breath data.

Fig. 5 -τ duration in a patient: at left, pre treatment (τ = 38.52 s.) and, at right, after taking 20 mg tadalafil(τ = 24.71 s.); averaged data over 5 of 7 breaths.

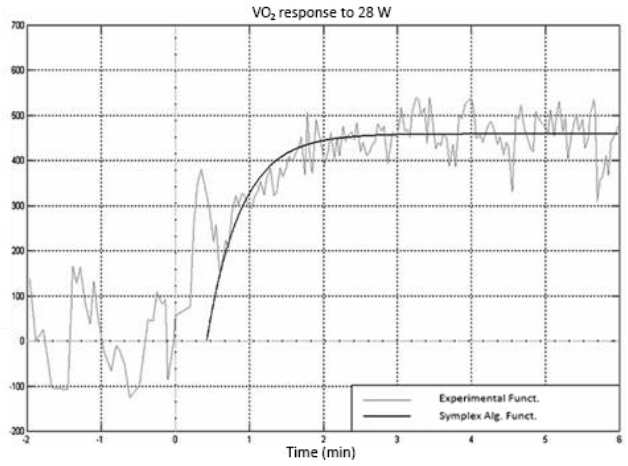

VO_2 response to 28 W
700
600
500
400
300
200
100
0
-100
-200
-2
-1
0
1
2
3
4
5
6
Time (min)
Experimental Funct.
Symplex Alg. Funct.

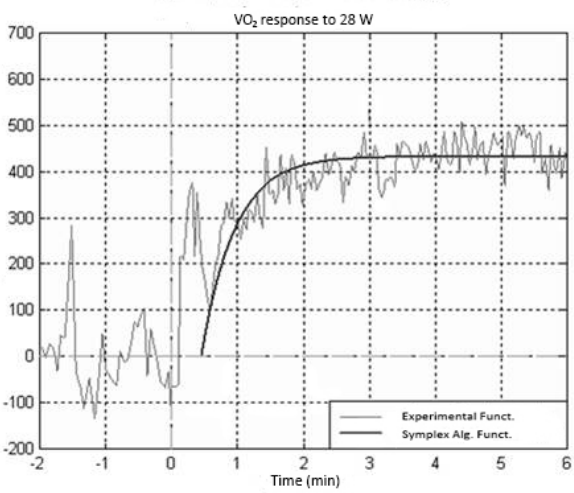

PETC02 V02
RER PET02
50
1500
45
1000
40
35
500
30
1.10
120
1.00
110
0.90
100
0.80
90
0.70
80
0.60
70
60
0
Time
9


VO_2 response to 61 W
Experimental Funct.
Symplex Alg. Funct.
Time (min)


VO_2 response to 61 W
Experimental Funct.
Symplex Alg. Funct.
Time (min)

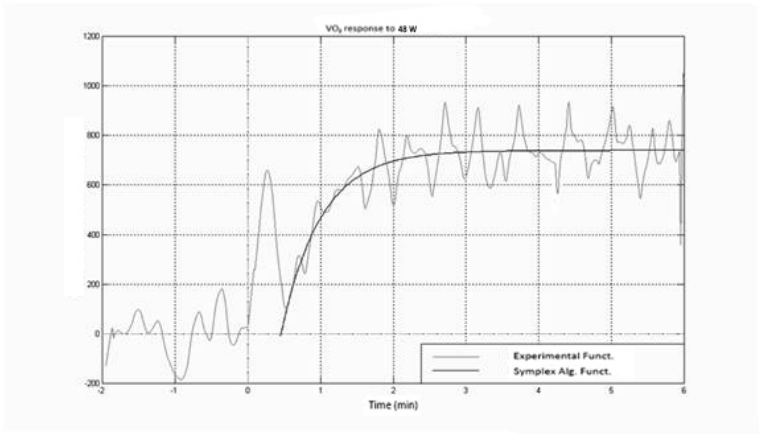
4

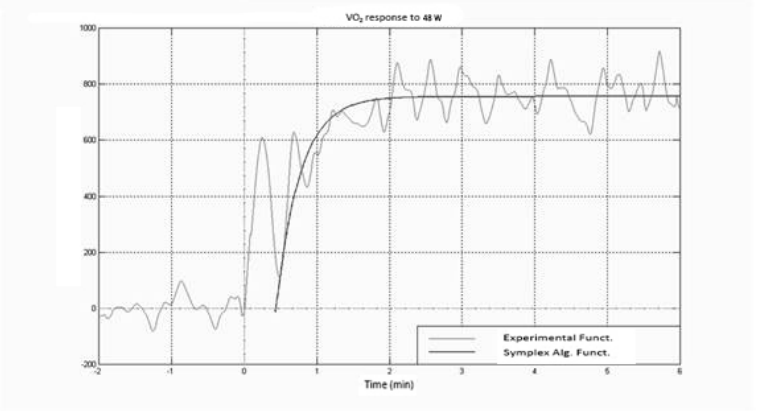

VO2 response to 28 W
Experimental Funct.
Symplex Alg. Funct.
Time (min)


VO2 response to 28 W
Experimental Funct.
Symplex Alg. Funct.
Time (min)

5

29


VO_2 response to 48 W
Experimental Funct.
Symplex Alg. Funct.
Time (min)


VO_2 response to 48 W
Experimental Funct.
Symplex Alg. Funct.
Time (min)

30