Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Medicine and Health Sciences
Department of Neuromedicine and Movement Science

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Comparison of muscle mitochondrial capacity between young and elderly subjects using Near-Infrared Spectroscopy

Master's thesis in Physical Activity and Health
Supervisor: Mireille Van Beekvelt
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Abstract

INTRODUCTION: Aging brings many changes in the body and a drop in skeletal muscle oxidative capacity is among them. Skeletal muscle oxidative capacity or mitochondrial capacity can be investigated non-invasively with the use of Near-Infrared Spectroscopy (NIRS), by calculating the time constant (Tc). Only few studies have used NIRS to investigate differences in mitochondrial capacity between young and old subjects. The aim of the current thesis was to investigate whether there is a difference in mitochondrial capacity between healthy young and old participants after performing isolated muscle exercise and a whole-body exercise.

METHODS: In this cross-sectional experimental study, 13 young (18-40 years) and 12 old (60-80 years) non-smoking, recreationally active participants with no previous history of cardiovascular- or pulmonary diseases with a BMI < 30 were recruited through posters and social media platforms around Trondheim municipality, Norway between December 2021 and February 2022. Subjects' flexor digitorum superficialis (FDS) muscle and vastus lateralis (VL) muscle from the right forearm and thigh were investigated with NIRS repeated occlusion (RO) method to determine mitochondrial capacity. On the first day, participants performed incremental handgrip test and an incremental cycling test to establish the individual's maximal handgrip strength and peak power. On the second day, sustained submaximal handgrip test and cycling test were performed at 25% (WR1) and 50% (WR2) of the maximal strength and power attained on the first day. During the 4 minutes recovery periods after the exercise bouts, RO were applied, and muscle oxygen consumption (mVO_2) was measured. mVO_2 values were fitted to a monoexponential function to calculate the Tc.

RESULTS: There was no statistically significant difference in Tc between groups ($F(1, 23.14) = 0.673$; $p = 0.420$) regardless of the muscle or the work rate tested. A statistically significant main interaction effect of muscle ($F(1, 66.53) = 15.41$, $p<0.001$) was found, FDS muscle showed a significantly larger Tc (69.5 ± SE 4.36) value than the VL muscle (50.06 ± SE 4.35). A main interaction effect of work rate was shown ($F(1, 64.89) = 31.36$, $p<0.001$); WR2 yielding significantly larger Tc values (Tc= 73.6 ± SE 4.29) compared to WR1 (Tc= 45.96 ± SE 4.42).

DISCUSSION and CONCLUSION: No difference in mitochondrial capacity between the young and old participants was found at the relative WRs of 25% and 50% measured in FDS muscle after sustained submaximal handgrip test and in VL muscle after cycling test. VL muscle showed a superior mitochondrial capacity compared to the FDS muscle. Work rate significantly influenced the Tc, after WR2, more time was necessary for the muscle mitochondria to recover to resting values than after WR1.

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1. Introduction

In the beginning of our life, we are not aware of what aging brings us, we only start noticing it when we can't do the things that we used to be able to do. We might not have the strength and endurance required to perform our daily routines. According to the World Health Organization (WHO 2022) 50 years ago the population over 60 years in Norway was 18.5%, and today it has reached 28.8%. In 50 years, it is expected to rise to 32.8%. In Europe the population above 60 is expected to increase from 25.85% today to 36.38% in 2072. From an economic standpoint, the increasing older generation can be perceived as a burden, especially if we add the health-costs of the different health-related conditions which emerge as people age. The increasing number of studies focusing on aging in the last decades, aim to understand the aging process, and to discover methods which can increase the quality of life, independence, and disease-free years in the elderly population.

Many studies have been focusing on the effect of aging on the human skeletal muscle and on the endurance capacity of the body. Studies show, that after the age of 25 years, cardiorespiratory fitness drops by approximately 10% per decade (Heath, Hagberg et al. 1981, Hawkins and Wiswell 2003, Pimentel, Gentile et al. 2003). Furthermore, starting from the age of 30 years, aging brings structural and functional modifications in the skeletal muscles (Lexell, Taylor et al. 1988, Nair 2005). Among the most important structural modifications are the reduction in muscle cross-sectional area due to loss of muscle mass and muscle fibers. Additionally, a shift of the remaining muscle fiber types, from type II, fast twitch fibers known to produce powerful forces for short duration supporting quick movements, towards type I muscle fibers, fatigue resistant fibers which support endurance activities. Furthermore, the muscles contractile properties drop, and together with the loss in muscle mass, contributes to the observed decrease in muscle strength with age (Roos, Rice et al. 1997, Nair 2005). All these changes are tied to muscle weakness and reduced endurance capacity seen in the elderly (Nair 2005).

Aging is known to bring along alterations in the human skeletal muscle mitochondria (Peterson, Johannsen et al. 2012). These can lead to the overall weakness and loss in function witnessed by the older people. The mitochondria are found in all cells of the human body, including muscles, and they are the energy production engines which generate energy in the form of adenosine triphosphate (ATP) with the use of oxygen, in a biochemical process called oxidative metabolism. These ATP molecules are then used by the muscle to perform work in form of muscle contractions. Aging was shown to negatively influence the

mitochondrial ATP production rate (Short, Bigelow et al. 2005), skeletal muscle oxidative capacity showing a decline with aging (Adami and Rossiter 2018).

Skeletal muscle oxidative capacity can be investigated locally, in a non-invasive way, by using the gold standard method of magnetic resonance spectroscopy (MRS) (Hamaoka and McCully 2019). Although MRS is a valuable technology in this field, it is difficult to use, it is expensive, has limited availability and requires much time for maintenance (Hamaoka, McCully et al. 2007, Hamaoka and McCully 2019).

Another cost and time-efficient method used in investigating muscle mitochondrial oxidative capacity in a non-invasive way is Near-Infrared Spectroscopy (NIRS)(Motobe, Murase et al. 2004, Ryan, Southern et al. 2013, Ryan, Brophy et al. 2014). NIRS has been widely utilized in the studying of local tissue oxygenation, blood flow and oxygen consumption (Hamaoka, McCully et al. 2007, Jones, Chiesa et al. 2016). NIRS can measure local oxygen delivery to the skeletal muscle and the utilization of the oxygen within the muscle, which are two important elements of determining the ability of a muscle to perform exercise (Jones, Chiesa et al. 2016). NIRS can be used as a tool for investigating and understanding the prime mechanisms of decline in exercise capacity in the context of, but not limited to, aging (Jones, Chiesa et al. 2016).

NIRS is an optical method which uses light in the near-infrared spectrum. The NIRS devices are portable, wireless, and easy to use. They allow continuous measurements in resting and movement conditions. The NIRS-emitted light passes through human tissues describing a banana shape trajectory while travelling from the transmitter towards the receiver (Cui, Kumar et al. 1991). On its journey towards the receiver, the light is absorbed by the body's chromophores, part of it is scattered in the tissue, and the rest is picked up by the receiver. In the human body the main chromophores investigated by NIRS are haemoglobin and myoglobin. They are known to absorb specific wavelengths within the near-infrared spectrum of light. Both haemoglobin and myoglobin have identical spectral characteristics, therefore NIRS can't distinguish them. NIRS can only differentiate their oxygenated and deoxygenated form, given that their spectral characteristic changes when oxygen is bound to them. Absorption changes of oxyhaemoglobin/myoglobin and deoxyhaemoglobin/myoglobin at multiple penetration depths are used to measure tissue saturation index (TSI) with spatially resolved spectroscopy (Patterson, Chance et al. 1989).

To interpret the changes in TSI signal, an intervention is needed. For instance, by applying an arterial occlusion (AO) to the investigated limb, we can calculate muscle oxygen consumption (mVO_2) under the NIRS probe (Hamaoka, Iwane et al. 1996, Van Beekvelt, Colier et al. 2001), by looking at the rate of decrease in TSI signal. By combining NIRS with multiple, short duration repeated arterial occlusions (RO) after a period of muscle activation, we get a series of mVO_2 measurements. If we plot these mVO_2 values obtained over the recovery period, we form an idea of how fast the mVO_2 recovers to resting values. The mVO_2 values are fitted to a monoexponential curve and the time constant (Tc) is calculated, which can be used as a marker for muscle mitochondrial capacity (Nagasawa, Hamaoka et al. 2003, Motobe, Murase et al. 2004, Buchheit, Ufland et al. 2011, Brizendine, Ryan et al. 2013). A higher Tc represents a slower recovery, a lower Tc shows a faster recovery to resting values, therefore a better mitochondrial capacity (Motobe, Murase et al. 2004). This approach was shown to be reproducible (Southern, Ryan et al. 2014) and was validated against MRS-derived phosphocreatine recovery time constants (Ryan, Southern et al. 2013) and against mitochondrial respiratory capacity derived from muscle biopsies (Ryan, Brophy et al. 2014).

Multiple studies have used the above-mentioned technique in the past decade. Most studies have investigated small muscle groups performing short (7s to 1 minute), isolated, dynamic muscle contractions (Ryan, Southern et al. 2013, Ryan, Southern et al. 2013, Southern, Ryan et al. 2014, Chung, Rosenberry et al. 2018, Beever, Tripp et al. 2020, Lagerwaard, Nieuwenhuizen et al. 2020) or the muscles were activated through electrical stimulation (Ryan, Erickson et al. 2012, Brizendine, Ryan et al. 2013). Other studies have taken the advantage of the ease of usability of NIRS and have investigated skeletal muscle oxidative capacity in locomotor muscles after running (Buchheit, Ufland et al. 2011) and cycling exercises (Zuccarelli, do Nascimento Salvador et al. 2020).

Studies that have used this NIRS approach to investigate the effect of aging on the mitochondrial function within human skeletal muscle found a decrease in mitochondrial capacity in both non-locomotor (Chung, Rosenberry et al. 2018) and locomotor muscles (Lagerwaard, Nieuwenhuizen et al. 2020), suggesting that aging negatively affects mitochondrial function. Furthermore, Lagerwaard, Nieuwenhuizen et al. (2020) highlighted a muscle-specific decline in mitochondrial capacity with aging, but there is still not much data available in this field using NIRS.

Given that this approach has been scarcely used in studies of muscle aging, and most of the studies investigated isolated muscles after a short activation period; in this thesis we lengthened the exercise period and included both isolated muscle work in the form of dynamic handgrip exercise and whole-body, cycling exercise bouts. NIRS was shown to be feasible determining muscle oxidative metabolism following standard cycle ergometer exercises at varying intensities (Zuccarelli, do Nascimento Salvador et al. 2020). The reason for lengthening the exercise period was to ensure a sufficient stimulus for the oxidative metabolism within the skeletal muscle mitochondria, underlined by Adami and Rossiter (2018) as being necessary for an adequate measurement of the oxidative capacity. The aim of the present thesis was to investigate whether there is difference in mitochondrial capacity after performing isolated muscle exercise and more general, whole-body exercise between healthy young and elderly participants.

2. Methods

2.1. Participants

25 healthy, recreationally active subjects participated in this study which were divided in two groups: young (5 women, 8 males) and old (6 women, 6 males). The inclusion criteria for age were 18-40 years for the young group, and 60-80 years for the elderly group. Participants were recruited through posters and social media platforms in Trondheim municipality, Norway between December 2021 and February 2022. Inclusion criteria: healthy, non-smoking, recreationally active individuals with no previous history of cardiovascular-pulmonary diseases or any current musculoskeletal or neurological conditions which could impede performing the exercise tests, BMI < 30, no diabetes or uncontrolled high blood pressure. The protocol was conducted with the approval of the Regional Committee for Medical and Health Research Ethics, Norway.

The two test days took place at NTNU laboratory in the St. Olavs Hospital, Nevro-Øst, Trondheim, Norway. The body composition analysis took place on a separate day, at AHL center at St. Olavs Hospital. All subjects signed a written informed consent before testing.

2.2. Experimental design and protocol

This was a cross-sectional experimental study, where participants were invited to the laboratory on 3 different occasions on 3 separate, nonconsecutive days. On the first day participants filled a questionnaire regarding their training habits, with questions about training frequency, duration of the sessions, intensity and type of exercises performed.

Afterwards, resting muscle oxygen consumption (mVO_2) and maximal voluntary contraction force (MVC) were measured, followed by the incremental handgrip test (IHT) used to measure the maximal handgrip strength (HG_{max}). After testing the arm, the participant was moved to a cycle ergometer, where lactate profile test (LPT) was performed, followed by an incremental cycling test (ICT). On the second day subjects performed a sustained submaximal handgrip test (SHT) and submaximal cycling test (SCT) at two different work rates (WR), representing 25% (WR1) and 50% (WR2) of their maximal force and power measured on the first day during the IHT and ICT. Furthermore, the second test day ended with measuring skinfold thickness at the site of NIRS-device placement. On the third day, the subjects body composition was measured.

Subjects were instructed to refrain from caffeine on the day of the tests, alcohol intake and from performing exercise training at least 48 hours before the test days.

2.3. Measurements

2.3.1. Near-infrared spectroscopy (NIRS)

Muscle oxygen consumption was measured in a non-invasive manner using two, continuous-wave near-infrared portable NIRS devices (Portamon, Artinis Medical Systems, The Netherlands). NIRS optodes were placed on the bulk of the flexor digitorum superficialis muscle (FDS) on the right forearm, parallel to the major axis of the forearm; and on the lower third of vastus lateralis muscle (VL) of the right leg, parallel to the major axis of the thigh (Figure 2.3.1). Before optode placement, the skin areas were shaved and cleaned with alcoholic wipes. The exact position of the NIRS optodes was marked on the skin, using skin markers, for accurate repositioning of the devices on the second test day. NIRS optodes were fastened on the muscles, a black cloth was used to cover them, to avoid infiltration of ambient light.

The NIRS devices emitted light at 845 and 761 nm wavelength through the LED transmitters at source-detector distances of 30-, 35- and 40-mm. With these specific wavelengths, it was possible to detect absorption changes of oxyhaemoglobin and deoxyhaemoglobin under the NIRS device. TSI was calculated using spatially resolved spectroscopy approach (Patterson, Chance et al. 1989). NIRS measurements were made on the FDS and VL muscles, as being among the most active muscles in handgrip and cycling exercises, respectively (Ryan and Gregor 1992, Van Beekvelt, Colier et al. 2001). Data was

collected using data acquisitions software (Oxysoft, Artinis Medical Systems, BV, The Netherlands). Data was displayed in real-time and stored for later analysis.

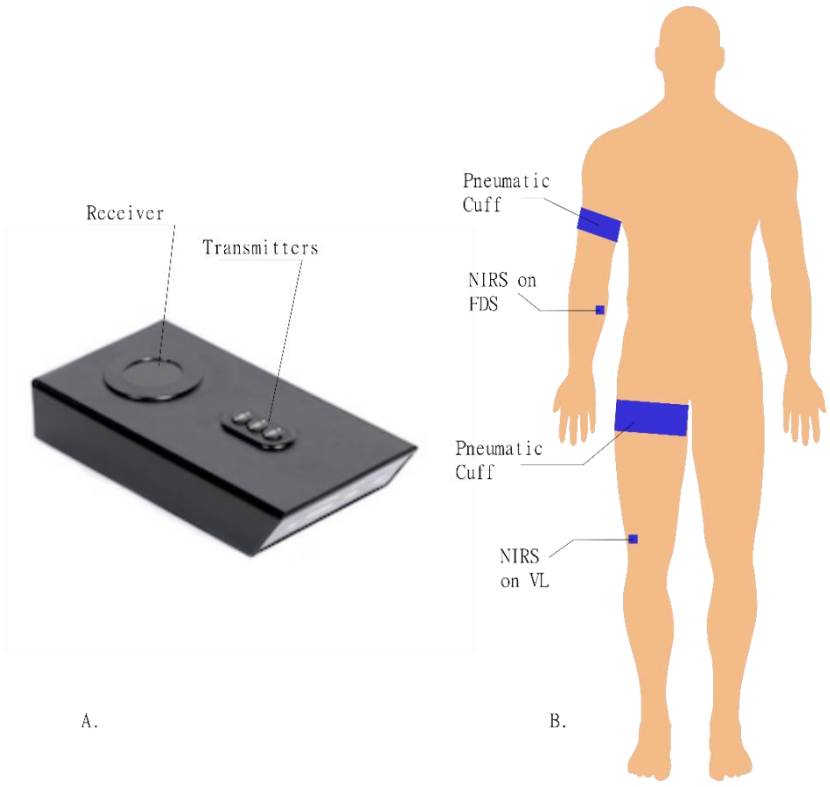

Figure 2.3.1

A: NIRS PortaMon device with the three transmitters and the receiver (Artinis 2022).

B: Placement of the NIRS devices and the pneumatic cuffs on the right forearm and thigh of the body (OpenClipart 2020).

In the present study, we derived mVO_2 values from the rate of decrease in TSI signal during AO. During AO it is assumed that no blood flows in or out from the investigated limb, total hemoglobin is constant (De Blasi, Almenrader et al. 1997), therefore all the disappearing oxygen is assumed to be consumed by the muscle mitochondria.

Resting mVO_2 was calculated during the 10 minutes vascular occlusion test (VOT) (fig. 2.4.1). A simple linear regression was fitted over the unfiltered NIRS TSI signal. The regression analysis was performed over a period of 3 minutes with a fixed delay period of 30 seconds after the start of the AO. Resting mVO_2 was expressed in *percent/second (% / s)*.

On the second day, the submaximal handgrip and cycling exercise bouts were followed by RO after both WR1 and WR2. During these RO, multiple mVO_2 values were derived using simple linear regression over varying delay and regression periods. The linear regressions were done over the unfiltered NIRS TSI signal. The mVO_2 values were fitted to the following monoexponential function:

$$y = End - \Delta \times e^{-1/Tc}$$

In this equation, y represents relative mVO_2 during the arterial occlusion, *End* is the mVO_2 immediately at the end of exercise, Δ is the change in mVO_2 from rest to end exercise, Tc is the fitting time constant (Ryan, Erickson et al. 2012).

2.3.2. Occlusions/Repeated occlusions

Two pneumatic cuffs (Hokanson SC12L; Marcom Medical ApS, Denmark) were placed as proximal as possible on the right leg and arm (figure 2.3.1) to enable application of AO using a rapid automatic inflation system (Hokanson E20 Rapid Cuff Inflator + Hokanson AG-101 Air Source, Marcom Medical ApS, Denmark), which inflated the cuffs in 0.3s to 300mmHg, held the pressure and instantaneously deflated the cuff at the press of a button.

These cuffs were used to apply the AO during rest and the RO applied after the exercise periods on the second test day. For the RO, the device was set to inflate the cuff to 300mmHg and deflate in a manner that the cuff was repeatedly on-and-off for 5 seconds (Figure 2.4.1).

2.3.3. Maximal voluntary contraction force and maximal handgrip strength

Subject's maximal voluntary contraction force (MVC) of the right arm was measured with a dynamometer (Lafayette Professional Hand Dynamometer; Lafayette Instruments, Model No. 5030L1. Indiana, USA).

A custom-made handgrip dynamometer was used to perform the IHT and the SHT.. HG_{max} was determined by the highest load lifted by the participant at the last complete 15 second stage where all the test requirements were met.

2.3.4. Cycle ergometer

Excalibur cycle ergometer (Excalibur, Lode B.V. Medical Technology Groningen, The Netherlands) was used on both test days when cycling exercises were performed. This was coupled with a data acquisition software (Lode Ergometry Manager 10.12.0, Lode BV., The Netherlands) which enabled the definition of different protocols, and recorded cadence, wattage, and time during the different stages of the cycling protocols. Warm-up and active recovery periods were set and recorded manually.

2.3.5. Spirometry

Measurements of ventilatory parameters and pulmonary gas exchange were obtained by using open circuit indirect calorimetry (Oxycon Pro, Jaeger GmbH, Hoechberg, Germany). Calibration was done at the start of each test day. The calibration of the volume transducer was done by continuous, constant pumps of a 3L calibration syringe (Hans Rudolph Inc,

Kansas City, MO, USA). The gas concentration sensor was calibrated automatically by the system switching for several times from ambient air to a known, 15% CO_2 and 5% O_2 gas concentration composition from a gas tube. While testing, the exhaled air was collected into a tube through a mouthpiece and delivered to a mixing chamber where the gas concentrations and volumes of the exhaled air were analyzed, with sampling frequency of 10 seconds.

Spirometry was performed continuously during the cycling protocol on both test days.

2.3.6. Heart rate monitor

HR was measured using a HR monitor belt placed on the chest at the height of the sternum (Polar RS800, Polar Electro OY, Kempele, Finland).

HR measurements were continuous, from the start to the end of both test days.

2.3.7. Lactate sampling

To measure blood lactate concentration ($[La]_b^+$), a lactate analyzer (Lactate Pro LT-1730, Arkray, Kyoto, Japan) was used. After cleaning with alcoholic wipe one of the participants fingers, the finger was pinched with a special needle. The first blood drop was removed, and sampling took place from the second drop with the lactate strip which was attached to the lactate analyzer. The result was shown on the screen of the analyzer in a few seconds and recorded manually.

The exact sampling time-points can be seen in figure 2.4.1.

2.3.8. Skinfold thickness

Skinfold thickness at the sites of application of the NIRS probes was measured with a skinfold caliper (Holtain, Crymmych U.K.), and it was divided by 2 to determine adipose tissue thickness (ATT).

Sampling took place at the end of the second test day.

2.3.9. Body composition analysis

Body composition analysis was done by bio impedance analysis (Inbody 770, Biospace, Seoul, Korea). Participants were invited to the laboratory during the morning, and the measurement was performed on an empty stomach. Subjects had to stand still on the device, with the feet on the electrodes while holding handles in their hands with extended arms for about 1 minute.

2.4. Experimental procedures and protocol

The protocol of the testing days can be visualized in figure 2.4.1.

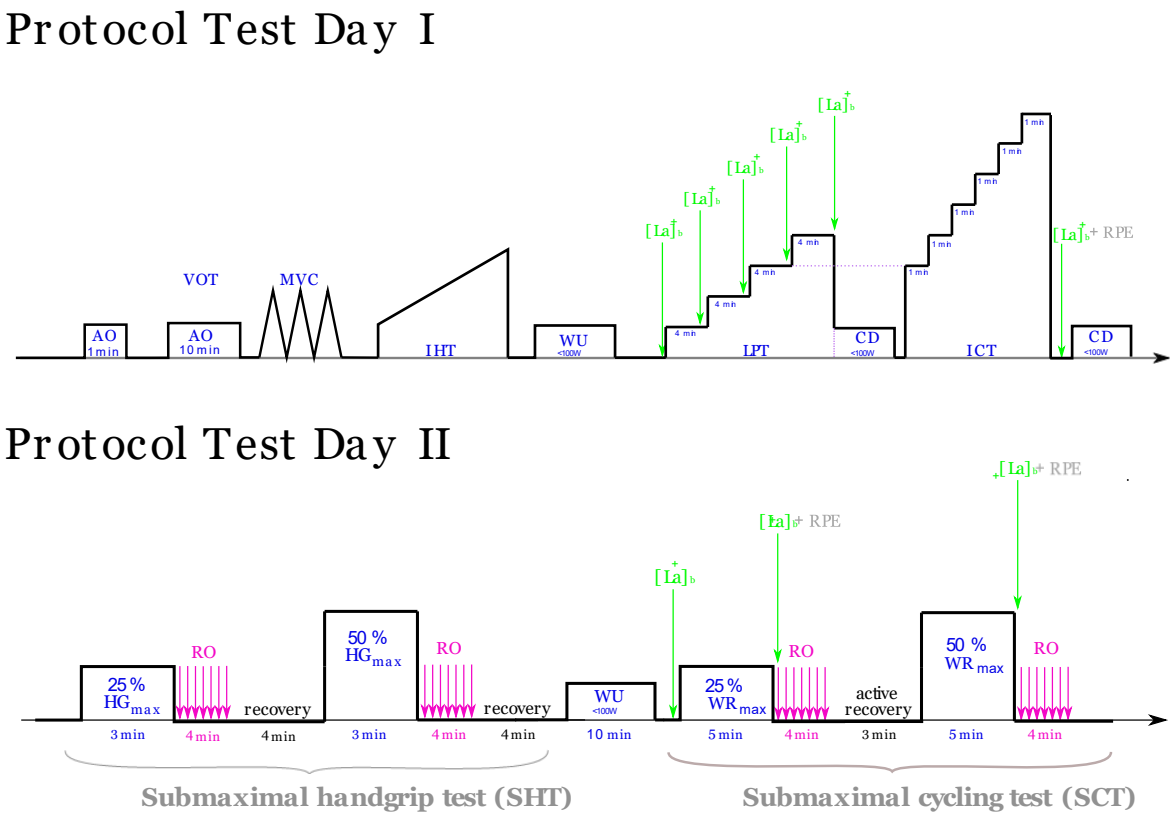

Figure 2.4.1.: Chronological order of test procedures during the two test days. Arterial Occlusion (AO), Vascular Occlusion Test (VOT), Maximal Voluntary Contraction force test (MVC), Incremental Handgrip Test (IHT), Warm Up (WU), Lactate Profile Test (LPT), Cool Down (CD), Incremental Cycling Test (ICT), Maximal Handgrip strength (HG_{max}), Repeated Occlusions (RO), Peak Power (WR_{peak}), Blood Lactate concentration ($[La]_b^+$), Rate of Perceived Exertion (RPE)

2.4.1. Vascular occlusion test

The subject lay in a comfortable semi-supine position on a bench, with the right arm rested in an extended position along the body, sustained by pillows at the level of heart. The legs were parallel and in a relaxed position.

The test started with 5 minutes of baseline measurement of muscle oxygenation, followed by a short, 1-minute arterial occlusion (AO) to familiarize the subject with the sensation. This was followed by 3 minutes of recovery, and then the vascular occlusion test (VOT) started. During the VOT, the AO was held for 10 minutes.

2.4.2. Maximal voluntary contraction test and incremental handgrip test

The subject was seated in an upright position, with the elbow flexed at 90˚ and by the side of the body. The handle of the dynamometer was adjusted, so it rested on middle of the four

fingers, in a vertical position. MVC force was determined as the highest value attained out of 3 trials of isometric grip force performed, where the subject was instructed to squeeze the dynamometer as much as possible without moving any other body part. 1 minute rest was held between trials.

Afterwards, subjects lay with the back supported in a comfortable position, with the right arm rested on the handgrip dynamometer at the level of the heart. The forearm was supported at the wrist and elbow, leaving the rest of the arm free, so the NIRS device did not compress on the tissues and the forearm was maintained in a slight upward position. After approximately 5 minutes of rest, the dynamic IHT started, used to measure HG_{max}. Continuous, rhythmic handgrip exercise was performed with a contraction rate of 1 s on / 1 s off, guided by a metronome. The WR increased constantly with 250 g every 15 sec until reaching voluntary exhaustion. The test was considered terminated when the contraction amplitude or rate could not be maintained, or the participants expressed desire to stop the test.

2.4.3. Lactate profile test and Incremental cycling test

At the start of the test participants performed a warm-up at low intensity (50-100 W) which lasted for 10 minutes. $[La]_b^+$ was measured after warm-up and before starting the Lactate Profile Test (LPT).

LPT consisted of 4 minutes cycle periods per WR, maintaining cadence above 60 rpm. Three predefined protocols were used with different starting wattage and increments: for young men 100W start with 25W increments; for young women and old men 95W/ 20W and for old women 75W/15W. Lactate measurements were performed at the end of each 4-minute stage. When $[La]_b^+$ values exceeded 4 mmol/L, the LPT test was finished, considering that the participant reached the onset of blood lactate accumulation (OBLA).

After an active recovery period of 5-10 minutes, an ICT until voluntary exhaustion was performed, aiming to measure peak oxygen consumption (VO_{2peak}). Starting WR was defined as one stage before the WR where OBLA was reached. The increments used were the same as the ones used at the LPT, only that the time spent per each WR was reduced to 1 minute per stage. The test was finished if pedaling frequency dropped lower than 60 RPM or the inability to continue despite the energetic encouragements of the researchers. $[La]_b^+$ and the Rate of Perceived Exhaustion (Borg scale 6-20) at the end of test were taken as secondary

markers of exhaustion (fig. 2.4.1.). In addition, as another marker of exhaustion, the peak Respiratory Exchange Ratio (RER_{peak}) was derived from the spirometry measurements.

2.4.4. Submaximal handgrip and cycling test

On the second day, isolated handgrip exercise was performed at 25% (WR1) and 50% (WR2) of the HG_{max} attained on the first day. The exercise setting was identical with the one where IHT was performed. The participants were instructed to perform the handgrip exercise at a rate of 60 contractions per minute. To ensure proper pace, a metronome was used. The exercise periods lasted 3 minutes at both WR. WR1 was performed first, followed by WR2. Both exercise periods were followed by RO which lasted 4 minutes. Participants were instructed to avoid any movements during the RO. The two WRs were separated by a recovery period of 4 minutes when movement was allowed (fig. 2.4.1.).

The cycling protocol followed the same pattern. After a warm-up of 10 minutes, participants cycled first at 25% (WR1) and then at 50% (WR2) of the peak power (WR_{peak}) attained on the ICT. The load at warm-up was set lower than the wattage used at WR1. The duration of each exercise period was 5 minutes, followed by 4 minutes of RO. Between the two cycling periods, an active recovery of 4 minutes was performed, where participants cycled at the warm-up intensity.

During the SCT, participants were instructed to maintain the pedaling frequency above 60 rpm, at a comfortable pace. At the end of each cycling period, participants were suddenly stopped from pedaling with a wooden block placed under the pedal by one of the researchers. This happened at the exact moment when the RO were applied. During RO participants were instructed to refrain from any movements and relax the entire leg.

Two WRs were chosen, to analyze if potential differences between the age groups in Tc occurred at both WRs or only at a higher or a lower WR.

At the end of test day 2, ATT was determined at the sites of application of the NIRS probes.

2.5. Data processing and statistical analysis

NIRS data was processed using proprietary code in Matlab (Matlab version R2021b). Statistical analysis was conducted using SPSS (IBM SPSS Statistics Version 27).

Data are presented as means ± SD or as means ± SE. The level of statistical significance was set at $p \leq 0.05$. All physical and physiological parameters were tested for

normality within the two groups using Shapiro-Wilk test and compared by independent sample t-test if the data was normally distributed. The non-normally distributed data was analyzed using Mann-Whitney U test.

The effects of group, muscle, WR and their interaction on the Tc were analysed using linear mixed models approach. Only the Tc values derived from a curve-fit with an R^2 value ≥ 0.8 were included in the analysis. The model was buildt-up using a step-by-step approach, starting with the analysis of the main effects of group, muscle and WR, and the interaction effects of all these factors. The effects which yielded a non-significant result were removed one-by-one from the model, starting with the complex interactions first, and then with the ones with the highest p-values. The quality check of the model was done after every step by inspection of the Hurvich and Tsai's criterion (AICC), to see the actual improvemens of the model .

3. Results

3.1. Subject description

All subjects completed the entire protocol, no adverse events were encountered. Main physical characteristics of the subject are included in table 3.1.

All participants were included in the data analysis (13 old and 12 young). In comparison to the elderly, the young participants were significantly taller (p=0.002), had a significantly higher fat free mass (FFM p=0.015) and thigh circumference at the level of NIRS-device placement on the VL muscle (p=0.006). There were no statistical differences between groups in weight (p=0.117), BMI (p=0.510), and skinfold thickness at the sites of optode placement on FDS (p=0.403) and VL (p=0.446) muscles, whereas body fat percentage was significantly higher in the older group (p=0.046).

Table 3.1 Physical characteristics of the participants

	Group		
	Young	Old	p-value (Independent sample t-test)
	Mean (± SD)	Mean (± SD)	
Total number of participants	13	12	-
Male	8	6	-
Female	5	6	-
Age (years)	27.5 (± 7.22)	64.4 (± 3.94)	-
Height (cm)	177.7 (± 7.64)	168.7 (± 5.21)	**0.002***
Weight (kg)	73.3 (± 7.72)	67.7 (± 9.51)	**0.117**
BMI (kg/m^2)	23.2 (± 1.49)	23.7 (± 2.41)	**0.510**
FFM (kg)	59.5 (± 8.70)	50.9 (± 7.58)	**0.015***
Body Fat (%)	18.5 (± 5.36)	23.4 (± 6.38)	**0.046***
Skinfold thickness FDS (mm)	4.39 (± 1.32)	3.88 (± 1.68)	**0.403**
Circumference FDS (cm)	26.4 (± 1.77)	25.0 (± 2.59)	**0.128**
Skinfold thickness VL (mm)	9.72 (± 3.66)	8.42 (± 4.65)	**0.446**
Circumference VL (cm)	46.8 (± 1.95)	43.8 (± 2.89)	**0.006***

'*' Statistically significant difference between the groups, p< 0.05.

3.2. Training-status and activity level

The two groups were similar in terms of training status. In both young and elderly group, the declared average training intensity was medium to high. Both groups reported training sessions lasting either between 30-60 minutes or above 60 minutes. 46.2% of the young group trained between 30-60 minutes, and 53.8% above 60 minutes. The training session duration was equally distributed in the elderly group, half of the participants having training session lasting between 30-60 minutes, and the other half training above 60 minutes per session. Training frequency differed among young and elderly groups, 53.8% of the younger participants trained almost every day, opposed to only 25% of the old participants. The remaining participants in both groups trained on average 2-3 times/week.

The peak respiratory, cardiovascular, and metabolic end-exercise values determined during IHT, ICT, SHT, and SCT are shown in table 3.2.1. Neither MVC (Young(Y): 46.38 ± 8 kg vs. Old(O): 39.83 ± 10.86 kg) nor HG_{max} (Y: 13.13 ± 1.48 kg vs. O: 12.33 ± 1.8 kg) tests showed a statistically significant difference between young and elderly participants. The results of the ICT show, that the young group achieved a significantly higher VO_{2peak} (Y:

51.27 ± 9.81 ml/kg/min vs. O: 41.84 ±7.3 ml/kg/min), WR_{peak} (Y: 274.23 ± 64.12 W vs. O: 212.5 ± 53.53 W), HR_{peak} (Y: 188.82 ± 6.32 bpm vs. O: 172.17 ± 15.49 bpm), RER_{peak} (Y: 1.01 ± 0.07 vs. O: 0.93 ± 0.07), peak Rate of Perceived Exertion (RPE_{peak}) (RPE: scale 6-20, Y: 18.42 ± 0.89 vs. O: 17.67 ± 0.91) and peak blood lactate concentration ($[La]^{+}_{peak}$) values (Y: 15.54 ± 3.22 mmol/L vs. O: 11.83 ± 4.56 mmol/L) than the elderly group (Table 3.2.1.).

Table 3.2.1. Peak respiratory, cardiovascular, and metabolic end-exercise values

		Group		
		Young	Old	p-values
		Mean (± SD)	Mean (± SD)	(Independent sample t-test / Mann-Whitney U-test*)
MVC (kg)		46.38 (± 8.00)	39.83 (± 10.86)	**0.098**
IHT	Start HG (kg)	2.50 (± .00)	2.50 (± .00)	**-**
	HG_{max} (kg)	13.13 (± 1.48)	12.33 (± 1.80)	**0.235**
SHT	WR1 (kg)	3.28 (± 0.37)	2.96 (± 0.67)	**0.151**
	WR2 (kg)	6.58 (± 0.74)	6.17 (± 0.90)	**0.220**
ICT	WR_{peak} (W)	274.23 (± 64.12)	212.50 (± 53.53)	**0.027 #***
	VO_{2peak} (ml/kg/min)	51.27 (± 9.81)	41.84 (± 7.30)	**0.013 ***
	RER_{peak}	1.01 (± .07)	.93 (± .07)	**0.006 ***
	$[La]^{+}_{peak}$ (mmol/L)	15.54 (± 3.22)	11.83 (± 4.56)	**0.030 ***
	HR_{peak} (beats/min)	188.82 (± 6.32)	172.17 (± 15.49)	**0.004 ***
	RPE_{peak} (6-20)	18.42 (± 0.89)	17.67 (± 0.91)	**0.47 ***
SCT	WR1 (W)	68.77 (± 16.15)	53.75 (± 14.02)	**0.027 #**
	$[La]^{+}$ at WR1 (mmol/L)	1.65 (± 0.36)	1.96 (± 0.78)	**0.219**
	RPE at WR1 (6-20)	8.89 (± 1.23)	8.33 (± 1.61)	**0.344**
	WR2 (W)	137.38 (± 31.90)	107.42 (± 28.07)	**0.027 #**
	$[La]^{+}$ at WR2 (mmol/L)	3.25 (± 0.90)	2.44 (± 0.90)	**0.034 ***
	RPE at WR2 (6-20)	12.96 (± 1.25)	12.36 (± 1.29)	**0.298 #**
WR at OBLA (W)		160.38 (± 48.58)	133.33 (± 36.58)	**0.132**

p-values marked with '#' are derived from Mann-Whitney U-test, '*' Statistically significant difference between the groups, $p<0.05$.

3.3. NIRS-derived resting mVO_2

An independent sample t-test showed a statistically significant difference between the two groups in resting mVO_2 (p=0.003) measured in FDS muscle, the younger group having a higher rate of desaturation (-0.13 % / s ± SD 0.04) during rest compared to the old participants (-0.08 % / s ± SD 0.04). When the resting mVO_2 in the VL muscle was

investigated, both groups showed similar response (Y: -0.08 % / s ± SD 0.03 vs. O: -0.07 % / s ± SD 0.04; p=0.860).

3.4. NIRS-derived mitochondrial capacity

Representative NIRS raw TSI signal from an individual subject measured during SHT at WR1, followed by the resting period with the RO is shown in figure 3.4. In addition to this, the secondary illustration shows how the curve fit of the mVO_2 values measured during the RO was done. Tc values of the curve-fit together with the goodness of fit (R^2) are presented in the upper left corner of the curve fit illustration.

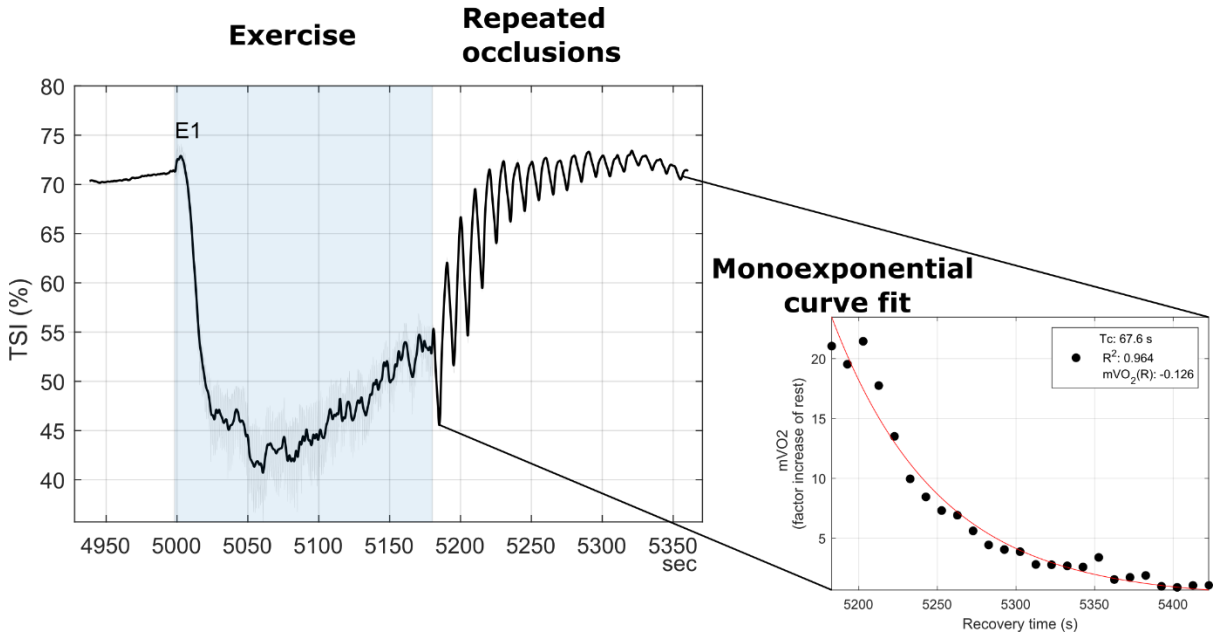

Figure 3.4 : Representative NIRS TSI signal from an individual subject. *NIRS TSI signals measured in the FDS muscle during the 3 minutes SHT at WR1, followed by the 4 minutes recovery period, where the RO were applied. During every occlusion from the RO series, mVO_2 was calculated from the slope of TSI signal. On the added figure the monoexponential curve fit of the individual mVO_2 can be seen. The independent mVO_2 values were plotted against time and fitted to a monoexponential function to compute the Tc. The TSI signals look similar during handgrip and cycling exercises at both WR.*

8 Tc values were excluded from the analysis due to R^2 values of the monoexponential curve fit being lower than 0.8.

The linear mixed models analysis revealed no statistically significant difference in Tc between groups, regardless of the muscle or the WR tested (F (1, 23.14) = 0.673; p= 0.420).

Furthermore, all the interaction effects between muscle, work rate and group resulted in non-significant results, $p>0.05$.

There was a statistically significant main interaction effect of muscle ($F(1, 66.53) = 15.41$, $p<0.001$), FDS muscle showed a significantly larger Tc (69.5 ± SE 4.36) value than the VL muscle (50.06 ± SE 4.35). Moreover, there was a main interaction effect of WR ($F(1, 64.89) = 31.36$, $p<0.001$); WR2 causing a significantly larger Tc values (Tc= 73.6 ± SE 4.29) compared to WR1 (Tc= 45.96 ± SE 4.42). The average Tc for the recovery of mVO_2 after exercise for both exercise modes and WRs, divided by group are presented in table 3.4.

Table 3.4 : Average Tc (s) values attained after handgrip and cycling exercises at both work rates and in both groups.

Muscle	Work rate	Group	Tc (s) Mean (± SE)	95% Confidence Interval
FDS	WR1	Young	58.62 (± 6.20)	46.155 - 71.077
		Old	52.73 (± 6.17)	40.292 - 65.164
	WR2	Young	86.27 (± 6.01)	74.153 - 98.376
		Old	80.38 (± 6.27)	67.747 - 93.006
VL	WR1	Young	39.18 (± 6.29)	26.559 - 51.806
		Old	33.29 (± 6.17)	20.854 - 45.735
	WR2	Young	66.83 (± 6.00)	54.759 - 78.903
		Old	60.94 (± 6.17)	48.507 - 73.378

4. Discussion

The main finding of the present thesis was that there was no significant difference in mitochondrial capacity between young and elderly participants after sustained exercise at relative WRs of 25% and 50% measured in a non-locomotor muscle after isolated muscle work and in a locomotor muscle after whole-body exercise. Another important finding was that there was a significant main interaction effect of muscle, where FDS muscle showed larger Tc values than VL muscle. This interaction effect reveals a lower mitochondrial capacity in the FDS muscle than in the VL muscle at both tested WRs. Furthermore, there was a main interaction effect of WR. After WR2, Tc was significantly larger than after WR1 in both muscles and both groups. This translates into a longer time needed for the muscle mitochondria to recover to resting levels after a higher WR.

Since the declared activity levels of the participants were similar among the groups, it was possible to assess solely the effect of aging on muscle mitochondrial capacity. For future studies, it would be advisable to better control for the participants physical activity habits. One solution could be to combine activity diary with accelerometers, pedometers, or other activity monitors, to determine the exact duration and intensity level spent engaged in physical activity.

Our statistical analysis revealed no significant difference in Tc between young and elderly, regardless of the muscle or the WR tested. This result was not in alignment with previous studies in the field. To the best of our knowledge, there has been only two previous studies conducted which have used NIRS to analyze the effects of aging on mitochondrial function by comparing young with old participants (Chung, Rosenberry et al. 2018, Lagerwaard, Nieuwenhuizen et al. 2020). Chung, Rosenberry et al. (2018) investigated the flexor digitorum profundus muscle and identified significant ($p=0.04$) increase of the postexercise muscle oxygen consumption recovery kinetics in elderly ($Tau = 51.8 \pm 5.4$ sec) as opposed to the young participants ($Tau = 37.1 \pm 2.1$ sec). Similarly, Lagerwaard, Nieuwenhuizen et al. (2020) exposed data showing significantly lower mitochondrial capacity in older males when the leg muscles were tested. They found no difference in mitochondrial capacity between young and old subjects in the tibialis anterior muscle ($p=0.64$), whilst there was a significantly lower mitochondrial capacity in the gastrocnemius ($p=0.048$) and VL ($p= 0.036$) muscles of the older participants as opposed to the younger ones.

Even though the methodological approach of determining the Tc was similar between the present thesis and the aforementioned studies, the duration of muscle activation, applied resistance and exercise mode differed. For example, in our study we prolonged the exercise duration for both FDS and VL muscles. Whilst in the study conducted by Chung, Rosenberry et al. (2018), the isometric handgrip exercise lasted for ~10-30 sec, similar to the study of Lagerwaard, Nieuwenhuizen et al. (2020), where 30 seconds of twitch electrical stimulation was applied on the VL and 30 seconds rubber resistance band exercise for the other investigated muscles. In our study we dynamically contracted the FDS with the rate of 60 contractions/minute for 3 minutes and activated the VL through cycling for 5 minutes at both WR. The main reason why these time periods were chosen was to ensure sufficient stimulus on the muscles. A secondary reason was to analyze a possible interchangeability between determining mitochondrial capacity on an isolated muscle and a whole-body exercise, so that

tests in the future for elderly participants could be narrowed to an easier isolated muscle. Both Chung, Rosenberry et al. (2018) and Lagerwaard, Nieuwenhuizen et al. (2020) controlled the level of the oxygen saturation/oxygenation signal during the exercise, so all the participants started the RO from the same relative level. We did not do this, instead, we were interested in how the mitochondria recovers after fixed period of sustained dynamic contractions of the muscle at the same relative intensity across the groups.

Another important methodological difference which might have influenced our results is the R^2 threshold used for the curve fitting of the mVO_2 values measured during the RO. The R^2 represents the goodness of fit of the mVO_2 values to the monoexponential decay and have large influence on the Tc values. To the best of our knowledge there is no crisp threshold for ideal R^2 values, therefore in our study we included all Tc derived from a curve fit with $R^2 \geq 0.8$. Zuccarelli, do Nascimento Salvador et al. (2020) chose R^2 values between 0.93 and 0.99, meantime Chung, Rosenberry et al. (2018) and Lagerwaard, Nieuwenhuizen et al. (2020) have included into their analysis only the curve fits with R^2 values > 0.91 and > 0.95, respectively. For the VL muscle, due to lower muscle activation Lagerwaard, Nieuwenhuizen et al. (2020) has lowered the inclusion threshold to $R^2 > 0.9$. This high threshold used by Chung, Rosenberry et al. (2018) and Lagerwaard, Nieuwenhuizen et al. (2020) may have led them to define more accurate Tc values of the muscles tested and therefore show a difference between the age groups. From our observations, the R^2 values have a high impact on Tc. In case of a participant having R^2 values increased from 0.976 to 0.986 and the Tc changed from 58s to 50.8s. Both R^2 values are high, but the Tc is 12.4 % smaller in the second case (Figure 4.1). If a small difference in the R^2 values introduce such a large difference in Tc, then the threshold value of 0.8 chosen by us might introduce such a large variability in the Tc that we could not show any difference between young and elderly, even though this might have existed.

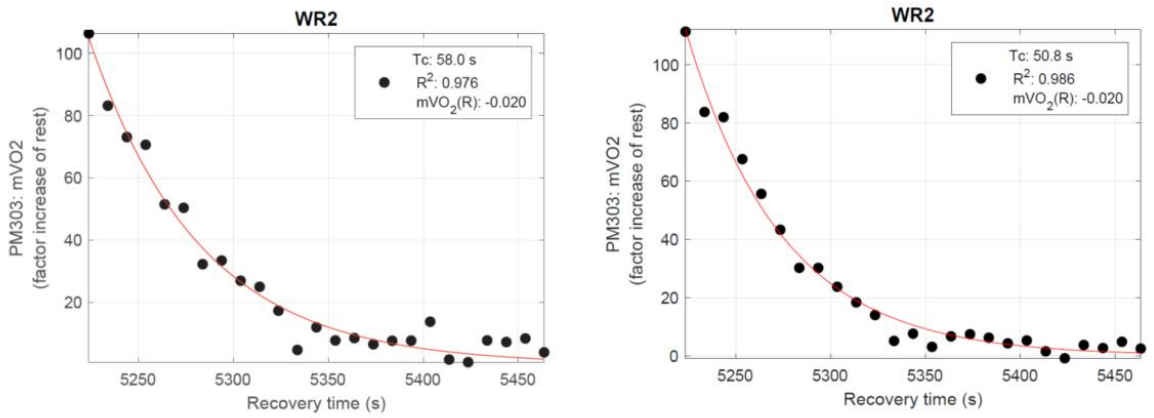

Figure 4.1: Two monoexponential curve-fits of the same individual, showing the recovery curve after performing SHT at WR2. The delay and regression periods to derive individual mVO_2 during the repeated occlusions used differed. Both present high R^2 values, but the slight increase in R^2 value on the right image results in 12.4% decrease in Tc.

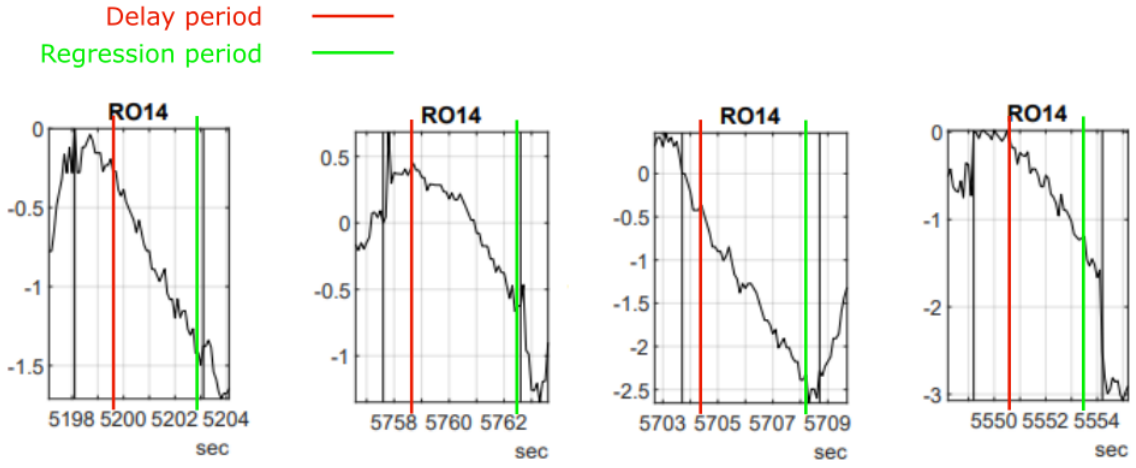

Figure 4.2 The 14th occlusion from the RO series from different participants. Variable delay and occlusion periods are shown. Delay period starts with the first vertical grey line and stops at the red vertical line. The regression period used to derive mVO_2 started from the red line and ended at the green line. The second grey line represents the end of the 5s AO.

Determining the start point and the end point for sampling of the mVO_2 during the RO, directly influences the R^2 and therefore the Tc values. Figure 4.2 shows the 14th occlusion from the RO series from different participants. We focused on maximizing the R^2 by selecting the best combination of delay and regression period within a participant for

determining mVO_2 from a limited number of possibilities (figure 4.2). Our method of using a variable start point and endpoint for determining mVO_2 might be a limiting factor in the selection of ideal slope of the TSI signal for determining the mVO_2 within the RO.

Lagerwaard, Nieuwenhuizen et al. (2020) reported 25% lower mitochondrial capacity in the elderly compared to younger participants in the VL muscle. On one hand, Lagerwaard, Nieuwenhuizen et al. (2020) used local, 30s of twitch electrical stimulation of the VL muscle, whilst we applied 5 minutes whole-body cycling exercise. Even though, Ryan, Brizendine et al. (2013) highlighted the possibility to compare the NIRS-derived measurements of mitochondrial function after voluntary muscle activation and electrical stimulation exercises, we don't know if there is an influence on the Tc if muscles are activated in a different mode, as if Tc derived after local activation through electrical stimulation can be compared with Tc derived after global, whole body exercise. On the other hand, Lagerwaard, Nieuwenhuizen et al. (2020) included low to moderately physically active participants who trained 1-2 h per week. Our recruited participants had higher declared training status. People with higher training status are expected to show superior fitness levels. Superior fitness levels are associated with lower Tc values, therefore better mitochondrial capacity (Brizendine, Ryan et al. 2013, Ryan, Southern et al. 2013, Jones, Chiesa et al. 2016). There is a possibility that our elderly participants physical activity habits maintained their mitochondrial capacity to a better extent than the moderately trained participants tested by Lagerwaard, Nieuwenhuizen et al. (2020), therefore we could not show a difference in Tc values between the young and elderly participants tested. It is also crucial to underline the fact that Lagerwaard, Nieuwenhuizen et al. (2020) included a low number of measurements of the VL muscle, and this might have influenced their final results.

As opposed to Ryan, Brizendine et al. (2013) , who found NIRS recovery measurements of mVO_2 not to be influenced by the intensity of the exercises, we found a statistically significant impact of the increase in exercise intensity on the Tc values. Tc after WR2, was significantly lengthened compared to Tc attained after WR1. Ryan, Brizendine et al. (2013) controlled the increase in intensity by maintaining the resistance and increasing the frequency of contractions, whilst we did the opposite: we increased the resistance and maintained the contraction frequency. Our results are consistent with the results of Zuccarelli, do Nascimento Salvador et al. (2020) and Buchheit, Ufland et al. (2011), who did find an exercise intensity dependency of mVO_2 recovery kinetic even though, Buchheit, Ufland et al. (2011) used another, but comparable method. Zuccarelli, do Nascimento Salvador et al.

(2020) used similar approach, as we did, following cycle ergometer exercises in young males. WRs for moderate and heavy exercises defined by Zuccarelli, do Nascimento Salvador et al. (2020) were 37 ± 6 % and 64 ± 5 % of WR_{peak}. They highlighted significantly lower Tc values in the VL muscle after moderate (Tc 29.1 ± 6.8 sec) compared with heavy (Tc 40.8 ± 10.9 sec) intensity cycling exercises. The Tc in VL muscle for the young group reported in the present thesis were 39.18 ± 6.29 sec and 66.83 ± 6 sec, for WR1 and WR2, respectively. Tc values measured by us are higher than the ones reported by Zuccarelli, do Nascimento Salvador et al. (2020) even though the relative intensities applied were lower; demonstrating a lower oxidative capacity in the VL muscle of the subjects tested by us. This is further highlighted by the fact that the VO_{2peak} values of the included participants in this thesis were similar with the ones included by Zuccarelli, do Nascimento Salvador et al. (2020) (51.27 ± 9.81 vs. 47.5 ± 6.7 ml/kg/min). Besides the higher R^2 threshold used for the monoexponential curve fit, another important methodological difference might be behind this finding. When defining Tc, they started the RO only when the muscle reached a desaturation target of 50% of the physiological calibration. As previously mentioned, we did not control for this in our study.

When inspecting the data from ICT performed on day 1, we can see that the younger participants might have pushed themselves more than the older counterparts. If we were to define attainment of VO_{2max} by achieving three of the following criteria: RER≥ 1, achievement of 96% of the predicted HR_{peak} according to the formula *208 – 0.7 * age* (Tanaka, Monahan et al. 2001), RPE> 18 (Borg scale 6-20) and $[La]^{+}_{max} \geq 8.0$ mmol/L (Howley, Bassett et al. 1995), 76,92% of the younger participants reached their VO_{2max}, opposed to only 50% of the older group. The remaining 50% from the older group could have reached a higher WR_{peak}, hence higher loads for WR1 and WR2. Our finding of similar Tc between young and elderly participants in the VL muscle after both WR1 and WR2 might hide a poorer mitochondrial function in the elderly. Our hypothesis arises from the fact that the Tc of the older participants reflected the recovery of mVO_2 succeeding a lower relative WR than the younger participants performed at. If the elderly would have been working at the same relative intensities as the younger ones, they might have shown an increased Tc, providing proof of poorer mitochondrial capacity.

5. Conclusion

In conclusion, in the present thesis, a difference could not be shown between young and old participants mitochondrial capacity measured after performing isolated muscle exercise and more general, whole-body exercise at the relative WRs of 25% and 50%.

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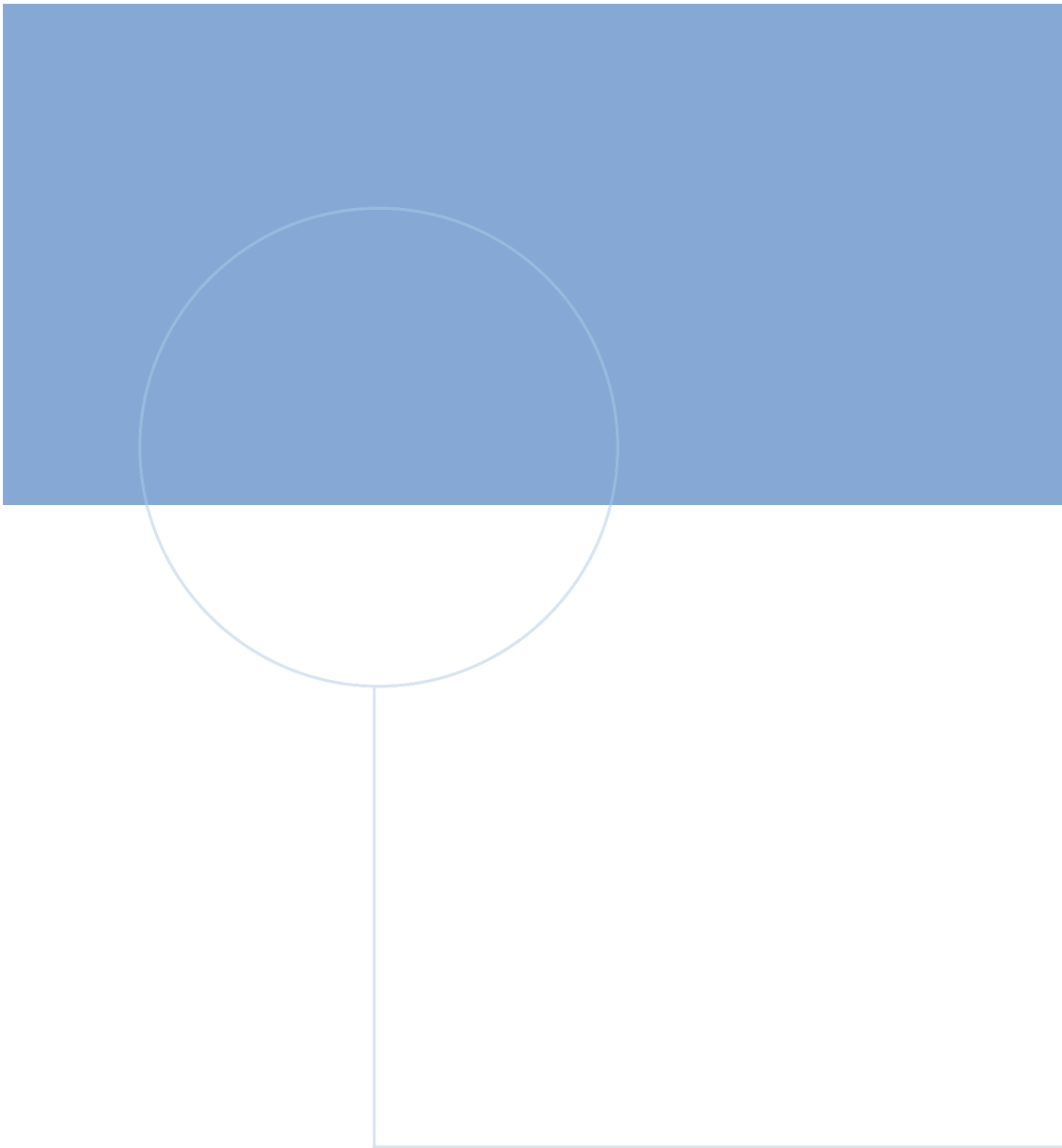
7. Appendix 1- Glossary

Adenosine Triphosphate	**ATP**
Arterial Occlusion	**AO**
Blood Lactate Concentration	$\mathbf{[La]_b^+}$
Body Mass Index	**BMI**
Fat Free Mass	**FFM**
Flexor Digitorum Superficialis	**FDS**
Incremental Cycling Test	**ICT**
Incremental Handgrip Test	**IHT**
Lactate Profile Test	**LPT**
Maximal Handgrip Strength	$\boldsymbol{HG_{max}}$
Maximal Voluntary Contraction	**MVC**
Muscle Oxygen Consumption	$\boldsymbol{mVO_2}$
Near-Infrared Spectroscopy	**NIRS**
Onset of Blood Lactate Accumulation	**OBLA**
Peak Blood Lactate Concentration	$\mathbf{[La]_{peak}^+}$
Peak Heart Rate	$\boldsymbol{HR_{peak}}$
Peak Oxygen Consumption	$\boldsymbol{VO_{2peak}}$
Peak Power	$\boldsymbol{WR_{peak}}$
Rate of Perceived Exhaustion	**RPE**
Repeated Occlusions	**RO**
Respiratory Exchange Ratio	**RER**
Submaximal Cycling Test	**SCT**
Submaximal Handgrip Test	**SHT**
Time constant	**Tc**
Tissue Saturation Index	**TSI**
Vascular Occlusion Test	**VOT**
Vastus Lateralis	**VL**
Work Rate	**WR**



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