

Non-invasive screening for peripheral oxygenation dysfunction in healthy and pathological populations

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PART I

GENERAL INTRODUCTION

1. PREFACE

Metabolic myopathies are diseases where muscle dysfunction acts as one of the major symptoms, usually caused by genetic defects or hormonal dysfunction. These myopathies comprise several subgroup diseases resulting from defects in biochemical energy metabolism. Mitochondrial Myopathy (MM) is one of these subgroups. Initial research to unravel underlying mechanisms started in the late 1980's with the discovery of large scale mitochondrial DNA (mtDNA) mutations. During the past decades, the primordial focus was of course the severe phenotype of this disease, whereby less attention was given to mild types of this myopathy. An important symptom in this MM population is a disturbed peripheral oxygenation pattern at muscle level, resulting from mitochondrial malfunctioning. Interestingly, the last two decades, several studies have investigated muscle abnormalities in another patient population as well, i.e. Chronic Fatigue Syndrome (CFS). This latter disease is based on exclusion criteria and requires four out of eight minor criteria for diagnosis. It is remarkable however, that muscle pain, post-exertional malaise, and muscle fatigue are included in these criteria. A number of papers have already focused on a putative disturbed peripheral oxygenation pattern in these CFS patients, however, with contrasting results. In this dissertation we aimed to focus on peripheral oxygenation in both patient and healthy populations. To investigate in particular how patients with a disturbed oxygenation pattern, probably due to mitochondrial malfunctioning, could be identified in a non-exhaustive and non-invasive way. Because both diseases are quite "young" and little is known about eventual mild phenotypes of mitochondrial myopathy, we wanted to make a contribution in this scientific area by developing a screening tool to identify such patients. In the first part of this dissertation, we aimed to present a short introduction about peripheral oxygenation in healthy subjects from a historical perspective. Consequently, in the second part of the introduction, a description of both MM and CFS diseases and their exercise tolerance spectrum is presented as well as peripheral oxygenation patterns in these patient populations.

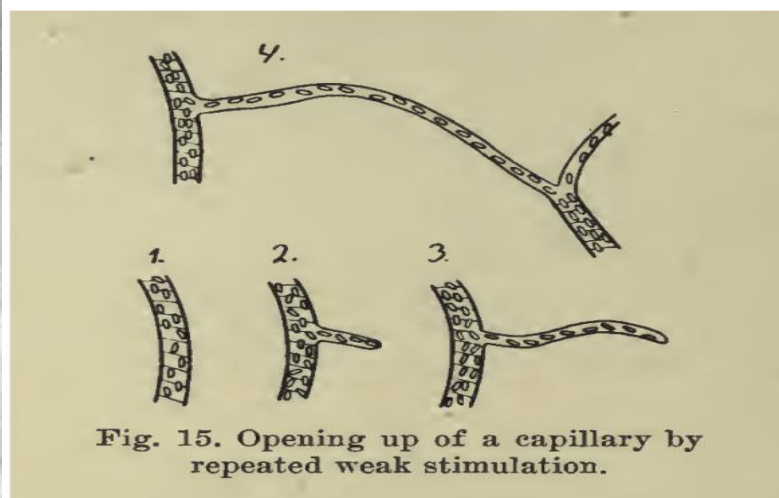
2. PERIPHERAL OXYGEN CONSUMPTION DURING EXERCISE: THE FICK PRINCIPLE

The history of studying oxidative metabolism at whole body level and in peripheral tissues has initially been dominated by two eminent physiologists named Adolf Eugen Fick (1829- 1901) and August Krogh (1874- 1949). More than one century ago, Adolf Eugen Fick died in Belgium, leaving behind a physiological heritage about oxygen (O_2) diffusion and measuring cardiac output (Q). As he was the first scientist capable of measuring Q , the formula to calculate this was called the Fick principle. Essentially, this Fick principle includes blood flow to an organ or tissue which can be calculated if the following information is known. (i) The amount (ml) of oxygen consumption (VO_2) per unit time, (ii) the oxygen concentration of arterial blood supplying the organ or tissue (representing oxygenated blood concentration) and (iii) the oxygen concentration of venous blood leaving the organ or tissue. As such, the following equation could be made: $VO_2 = Q \cdot D_{a-v}O_2$, where $D_{a-v}O_2$ represents arteriovenous O_2 difference. Interestingly, this method was not only applicable at whole body level, but could be used in multiple human body systems. At muscle level for example, peripheral oxygen consumption (VO_{2m}) can be calculated from muscle perfusion (Q_m) and muscle O_2 extraction ($D_{a-v}O_2$). Hence, when exercise is initiated, O_2 demand at muscle level will increase as a consequence of a higher skeletal muscle metabolic rate whereby the oxidative metabolism will predominantly be activated when rhythmic dynamic contractions are sustained. In this case, VO_{2m} will increase by increasing both $D_{a-v}O_2$ and/or Q_m , which is of crucial importance to have an expanded passage of O_2 , free fatty acids (FFA) and glucose at muscle level. The O_2 requirements of contracting skeletal muscle may increase up to a 100-fold compared to resting conditions.

This leads us to another eminent scientist who has done revolutionary physiological research with respect to muscle oxygenation. Auguste Krogh and his wife Marie Krogh studied the O_2 cascade presuming that the capillaries were the primary site for O_2 exchange between blood and tissue. This belief was based on the fact that capillaries have a high surface to volume ratio and that O_2 transit time is relatively long. In 1920, Auguste Krogh even won a Nobel Prize in Physiology or Medicine for the mechanisms of oxygen regulation of the capillaries in skeletal muscle tissue. In Figure 1, a picture of Professor Krogh is presented as well as one of his figures

of capillary recruitment. According to this theory, capillaries regulate blood-muscle O_2 flux and muscle energetics, thereby assigning a largely supportive role for the residual vascular system (Krogh 1919a,b, 1920b). This laid the foundation for Krogh and Eshlag's very elegant mathematical model concerning muscle oxygenation which is physiologically still widely accepted today. Basically, Krogh and Eshlag's model has led to two important axioms regarding peripheral oxygenation. (i) Intramuscular partial O_2 pressure (PO_2) will decrease when the distance of red blood cells (RBCs), located in the capillaries, is increased. (ii) There are more 'open' capillaries during and after aerobic exercise compared to resting conditions, thereby increasing RBC flux to the activated muscle and decreasing O_2 diffusion distances. Hence, in contracting muscles, more capillaries will be recruited in order to increase the blood-muscle O_2 , glucose and free fatty acids (FFA) flux and to reduce intramuscular PO_2 by shortening capillary to mitochondrial diffusion distances. A key concept regarding this 'capillary recruitment' is the contractile capability of these capillaries to be able to close-off during resting conditions. Krogh himself recognized two major problems using his surgical coloring technique inside the capillaries (Krogh 1919a,b), i.e. the extreme surgical interventions which possibly influence the perfusion pressures and the clumping together of the India ink particles, used as an observable marker in the capillaries.

Fig. 1: A picture of Auguste Krogh (left) and one of his first figures concerning the capillary recruitment theory (right) (Krogh 1919a,b).

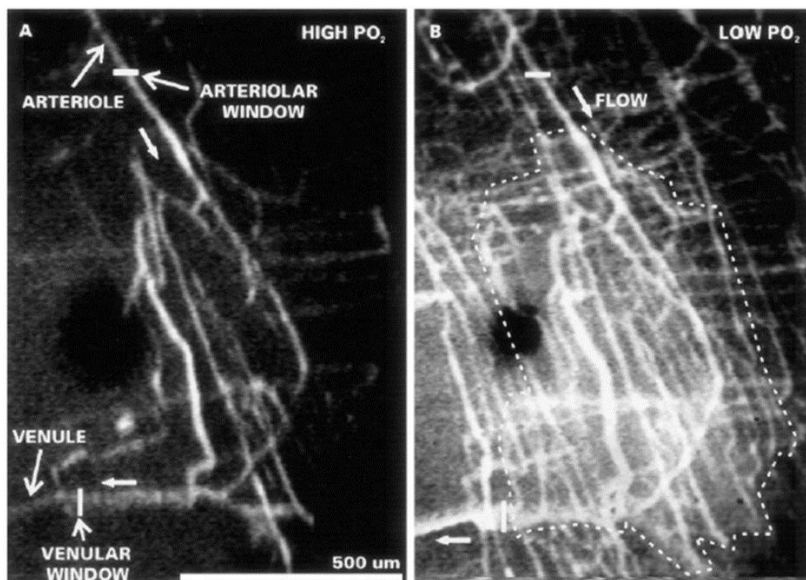


With great respect to Auguste Krogh's early observations, it is very interesting to question what we know now, one century later. His notion "capillary recruitment" during exercise is still widely accepted and described in physiological textbooks (Astrand and Rodahl, 1986; Mcardle et al., 2007) and contemporary studies (Parthasarathi and Lipowsky, 1999; Baron et al., 2000; Wheatley et al., 2004; Clark et al., 2008) to explain peripheral oxygenation. Despite impressive technological advances to study microcirculation and muscle haemodynamics, this existent dogma is very difficult to abandon (Poole and Musch, 2010). Compelling evidence from the last two decades requires consideration of new models for blood-myocyte O₂ exchange in exercising muscle. Poole et al. (2011) summarized most of this evidence in a very clear review concerning peripheral muscle oxygenation.

Firstly, there is overwhelming evidence demonstrating muscle RBC flux at rest in more than 80% of the capillaries in rat extensor digitorum longus (Anderson et al., 1997), diaphragm (Kindig and Poole, 1998) and spinotrapezius (Hudlicka et al., 1982; Kindig et al., 2002) muscles as well as in hamster sartorius and cremaster (Damon and Duling, 1984), rabbit tenuissimus (Vrieling et al., 1987) and cat sartorius (Burton and Johnson, 1972) muscles. Also, it has been shown that microvascular hematocrit in resting muscle capillaries is far below systemic (Klitzman and Duling, 1979; Sarelius and Duling, 1982; Desjardins and Duling, 1987; 1990; Poole et al., 1997; Frisbee and Barclay, 1998; Kindig and Poole, 1998; 2001; Kindig et al., 2002; Copp et al., 2009). Hence, the perfusion in most of the capillaries during resting conditions with a low microvascular hematocrit is an indication for a flux at rest of predominantly blood plasma. When exercise is initiated, haemoglobin will be increased up to two- or threefold at muscle capillary level indicating a differential blood flow (i.e. more RBC flux- less plasma) during exercise (Kindig et al., 2002; Copp et al., 2009). The "classical" view of hypoxic initiated capillary recruitment was never more clearly shown as in the images of Parthasarathi and Lipowsky (1999), presented here in Figure 2. According to the classical view, the drop in PO₂ in Figure 2b will stimulate capillaries to open, thereby increasing capillary RBC flux and decreasing blood- myocyte O₂ diffusion distances and increasing intramuscular PO₂. However, some issues can be raised towards this classical view, which assumes capillaries to close off at rest. Firstly, muscle capillaries are enormously fragile vessels and do not contain any contractile capability (Poole et al., 2011). Secondly, PO₂ values (in Figure 2b) of 35 mmHg are

supposed to be hypoxic while resting muscle cytosolic normoxia values are around 17 mmHg (Whalen et al., 1974, 1976). Despite these early findings of an intracellular cytosolic PO_2 of 17 mmHg, there is a general consensus that cytosolic intracellular PO_2 values are even lower (3-7 mmHg) (Gayeski et al., 1991; Gnaiger et al., 1998; Wittenberg et al., 1989). Consequently, Figure 2b represents actually a hyperoxic condition at capillary muscle level. Instead of the hypoxic induced capillary recruitment from Figure 2a to Figure 2b as an indication for recruitment of non-flowing capillaries at rest, this may rather show a hyperoxic induced vasoconstriction from right (2b) to left (2a).

Fig. 2: The images of Parthasarathi and Lipowsky (1999) of the resting cremaster muscle using fluorescently labelled plasma at PO_2 conditions of 130 mmHg (a) and 35 mmHg (b).

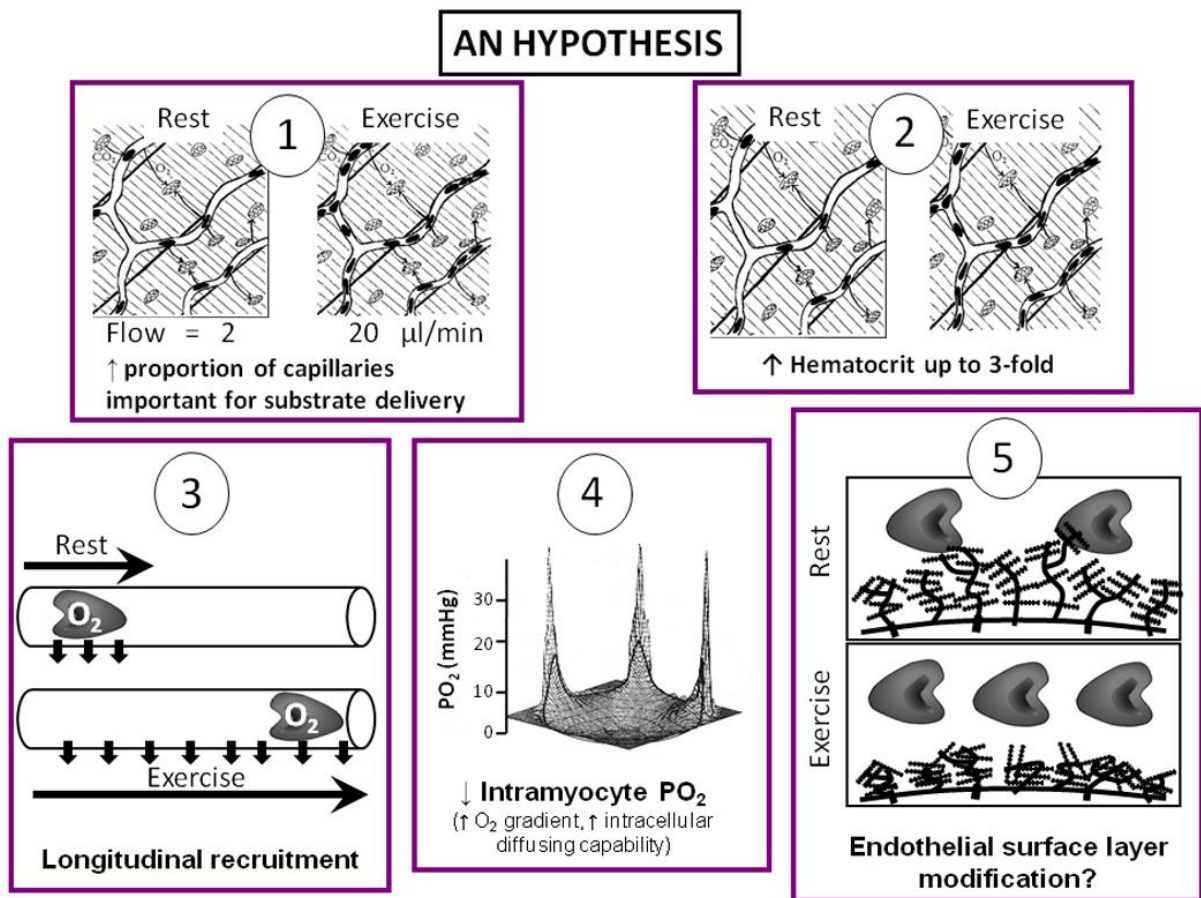


A second point that challenges the “classical view” is the fact that low intramuscular PO_2 is a trigger for increased capillary RBC flux, thereby shortening capillary-to-mitochondria diffusion distance (Poole et al., 2011). Surprisingly, intramuscular PO_2 during exercise was constantly very low, indicating a functional O_2 depleted region during exercise between capillaries and the mitochondrial reticulum (Gayeski and Honig, 1985, 1987; Honig et al., 1997; Voter and Gayeski, 1995; Richardsson et al., 1995a,b, 1998). Moreover, it has been shown that a decrease in diffusion distance between capillaries and mitochondria has absolutely no influence on capillary-to-mitochondrial PO_2 gradient, demonstrating that muscle diffusion capacity is independent from capillary density (Bebout et al., 1993; Hepple et al., 2000).

According to some modelling studies, it is more likely that the amount of RBCs flowing nearby the muscle fibers, rather than capillary density, are determining intramuscular O₂ diffusion (Federspiel and Popel, 1986; Groebe and Thews, 1990). However, if capillary density is higher, there will be a higher capacity for RBC flux surrounding the muscles. Hence, the first physiological axiom of Krogh and Ehrlang's modelling study is contradicted with convincing evidence.

If capillary recruitment does not take place during exercise, the question of how blood-myocyte O₂ flux increases during exercise must be answered distinctively. Five hypothetical mechanisms which are consistent with present scientific evidence are presented in Figure 3 (Poole et al., 2011). (1) When skeletal muscle contractions are initiated during exercise, RBC flux may abundantly increase in the capillaries. (2) Consequently, capillary hematocrit increases from 15% in resting condition to approximately 45% during exercise, indicating a differential blood flow in both conditions (blood plasma vs RBC's) (Klitzman and Duling, 1979; Kindig et al., 2002; Copp et al., 2009). (3) When RBC flux during exercise is thus increased up to threefold and fractional O₂ extraction increases from approximately 25% to 80%, longitudinal capillary recruitment will occur in order to maintain the increased O₂ exchange capacity. (4) As mentioned before, the extremely low intramyocyt PO₂ seems an important "O₂ depleted region" for myoglobin O₂ desaturation in order to improve intramuscular O₂ passive diffusion (Gayeski and Honig, 1985, 1987; Honig et al., 1997; Voter and Gayeski, 1995; Richardsson et al., 1995a,b; 1998). (5) The main regulator of capillary haemodynamics could be its endothelial surface layer (glycolax) whereas mechanical alterations of this layer, for example during exercise, may be important to change blood-myocyte O₂ flux. It has already been shown that elimination (Desjardins and Duling, 1990) or modification of the endothelial surface layer (glycolax) by hyperglycemia (Zuurbier et al., 2005), changes capillary hematocrit and haemodynamics profoundly.

Fig. 3: Hypothetical mechanisms for a differential approach towards capillary haemodynamics compared to the classical view of capillary recruitment (Poole et al., 2011).



In a century full of major technological and microscopic advancements, the “classical” theory of capillary recruitment during exercise maintains to be dogmatic in human physiology. With great respect to the enormous accomplishments of Adolf Eugen Fick and Auguste Krogh, it is of major importance to stay critical and challenge these dogmatic theories. Especially because these observations are of great interest in pathological populations, where peripheral oxygenation is limited due to microvascular and/or muscle deficiencies. Therefore, it remains crucial to gain more insights on these peripheral oxygenation mechanisms.

3. PERIPHERAL OXYGENATION IN CLINICAL POPULATIONS

Some clinical populations can be recognized by an altered peripheral oxygenation pattern. One example of a pathology where peripheral oxygenation pattern is disturbed as a consequence of mitochondrial malfunctioning, is Mitochondrial Myopathy (MM). Oxygen consumption and peripheral oxygenation during exercise are even frequently used as a diagnostic tool in this population. The pathogenesis of this disease, as well as peripheral oxygenation patterns in these MM patients are discussed below. Less obvious is the connection between a disturbed peripheral oxygenation pattern and Chronic Fatigue Syndrome (CFS). However, seen the fact that exercise intolerance and muscle pain and weakness are of frequent occurrence in most CFS patients, multiple studies have started focusing on muscle abnormalities. Recently, the Institute of Medicine (2015) have even proposed Severe Exercise Intolerance Syndrome (SEID) as a new label for these group of patients, thereby emphasizing the crucial importance of these exercise related problems.

3.1 Mitochondrial myopathy

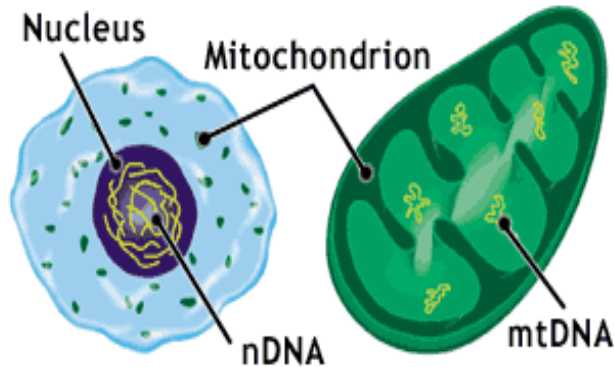
3.1.1 Prevalence, aetiology and diagnosis

Mitochondrial diseases are multisystem disorders clinically characterized by a large variety of signs and symptoms as a consequence of (severe) mitochondrial dysfunction. The prevalence of mitochondrial disorders is 1/5000 live births (Thorburn, 2004), whereas the prevalence of pathogenic mitochondrial DNA (mtDNA)-mutations is reported as high as 1/200 live births (DiMauro, 2011; Elliott et al., 2008). Initial research to investigate mitochondrial diseases began in the late 1980's with the discovery of large scale mtDNA mutations and point mutations as a cause of some severe diseases (Colombo et al., 1988; Sing et al., 1989; Tanaka et al., 1991).

Mitochondria are the sole cell organelles controlled by a dual genome system (mitochondrial and nuclear). Thirteen of the 87 structural proteins in these complexes are encoded by the mitochondrial genome (mtDNA), whereas approximately 1500 nuclear encoded genes (nDNA)

are thought to play a role in the synthesis of these complexes (Calvo et al., 2006). The location of mtDNA and nDNA in the cell is presented in Figure 4.

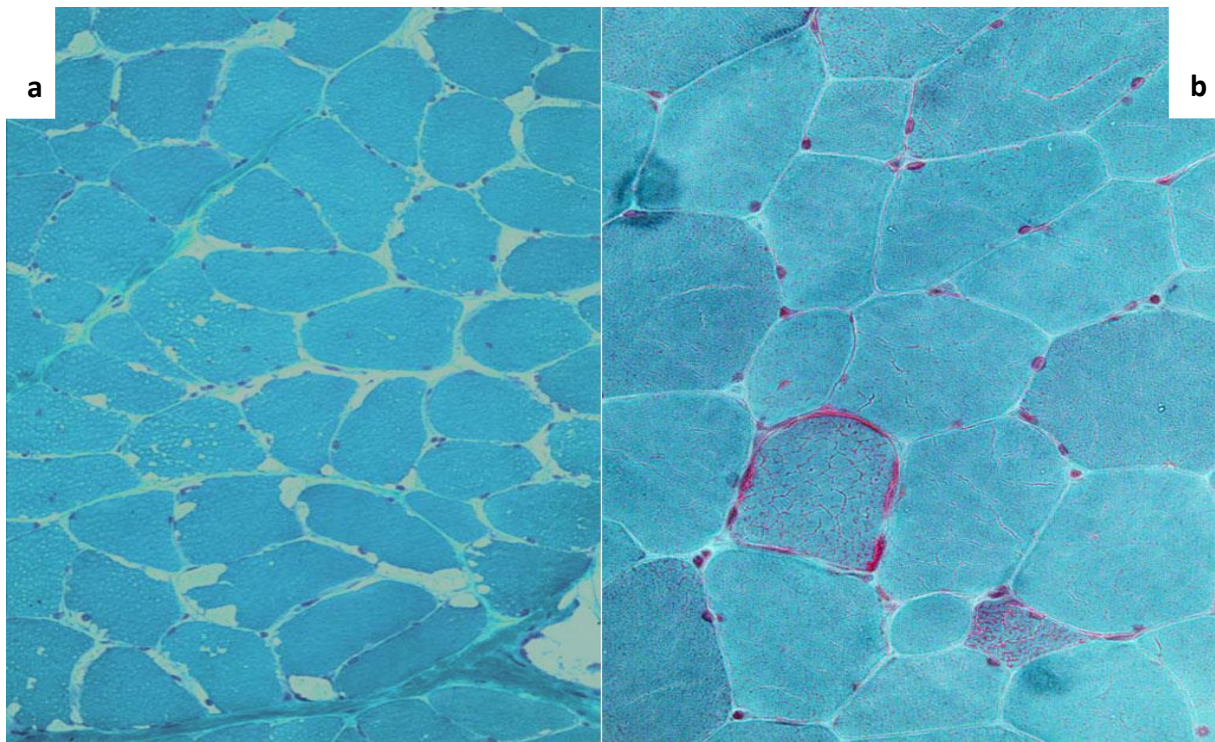
Fig. 4: Schematic presentation of where nDNA and mtDNA is located in the cell.



One deletion or mutation in an important mtDNA or nDNA sequence, responsible for one of these complexes or enzymes, may have severe consequences on mitochondrial functioning. Pathogenic mtDNA mutations are usually heteroplasmic, which means that a mixture of pathogenic mutant and healthy wild-type mtDNA can be found within the same cell. The amount of wild-type mtDNA can vary from tissue to tissue whereas tissue failure is expected when the amount of pathogenic mutant mtDNA is higher than 60-85% (“threshold phenomenon”) (Goodfellow, 2014). This threshold depends on which mutation is present in which cell type (Durham et al., 2007). A cell type with a higher O₂ dependence, e.g. muscle tissue, will exceed this threshold sooner in comparison with other tissues. Clinical phenotypes that can be associated with a specific OXPHOS defect genotype vary enormously. Some genotypes are associated with a specific phenotype such as Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (MELAS), Myoclonic Epilepsy with Ragged Red Fibres (MERRF), and Alpers-Huttenlocher. Unfortunately, the correlation between genotype and clinical phenotype is poor in most patients (Tarnopolsky and Raha, 2005). Considering the fact that mitochondria are crucial in several physiological processes and are present in many tissues -all possessing a different “threshold phenomenon”-, an enormous heterogeneity can be observed in the spectrum of mitochondrial diseases. In case skeletal muscle tissue is the predominantly affected tissue, the term mitochondrial myopathy (MM) is used.

The gold standard for diagnosing OXPHOS defects in MM patients remains a morphological, histochemical and biochemical analysis of skeletal muscle biopsy samples (Bourgeois and Tarnoplosky, 2004). One of the clinical features, indicative for MM, are ragged red fibres (RRF) in skeletal muscle tissue after red dye infusion in biopsy samples. In Figure 5, an example is presented of healthy skeletal muscle tissue and skeletal muscle tissue where ragged red fibers are abundantly present. This histopathological hallmark is a result of a compensatory mitochondrial proliferation at the ultra-structural muscle fibre level as a consequence of the intracellular mitochondrial impairment. Moreover, paracrystalline inclusions will be created, representing the crystallization of mitochondrial creatine kinase. As such, elevated creatine kinase (CK) values in venous blood samples are a common feature in MM patients.

Fig. 5: A typical example of a healthy skeletal muscle biopsy sample (a) and ragged red fibres in a skeletal muscle biopsy sample (b), indicative for mitochondrial myopathy.



For practical reasons, however, it is not feasible to perform a skeletal muscle biopsy in each patient suffering from exercise intolerance and/or excessive fatigue. There is a need for a non-invasive and non-exhaustive screening tool (Tarnopolsky et al., 1998). Several cycle ergometry protocols while measuring VO_2 kinetics, lactate values and blood flow have been proposed in the literature as screening tool for detection of MM patients. However, the results of these

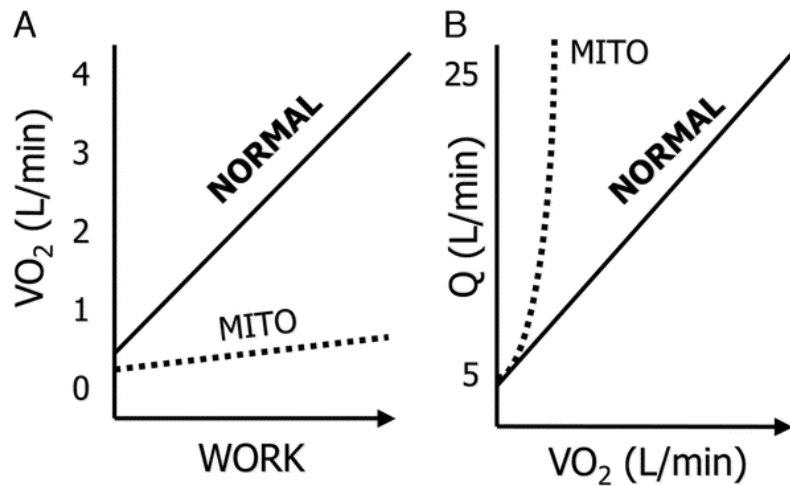
tests were variable (Dandurand et al., 1995; Dysgaard-Jepesen et al., 2003; Finsterer and Milvay, 2004; Grassi et al., 2009; Siciliano et al., 1999; Taivassalo et al., 2003). Three major problems could be identified when using whole body exercise tests as a screening tool. (i) These tests lack sensitivity (63% - 75%) and specificity (70% - 90%) to replace the skeletal muscle biopsy method (Tarnopolsky, 2004), in particular because in this way, 25% - 37% of the people will get a wrong diagnosis and 10% - 30% are not correctly excluded from having MM. (ii) Whole body maximal exercise induces a high cardiorespiratory load which can be dangerous as cardiomyopathy is a frequent symptom in this MM population. (iii) A good correlation between heteroplasmy level and exercise (in)tolerance was observed, indicating that individuals with a low heteroplasmy level will show no exercise intolerance and will be falsely declared healthy (Jepesen et al., 2003b). Hence, while exercise intolerance and excessive fatigue are key features of this pathology, muscle biopsy remains the gold standard for diagnosis.

3.1.2 Exercise tolerance spectrum in mitochondrial myopathies

As mentioned before, the aerobic production of ATP in skeletal muscle tissue, which takes place in the mitochondria, is (severely) reduced. The severity of this mitochondrial malfunctioning depends on which mutation and what amount of mutant mtDNA is present within the myocytes. At whole body level, tachycardia and dyspnoea are frequently occurring symptoms in this patient population (Haller and Vissing, 2009). Moreover, some studies have shown a significantly reduced maximal workload and peak oxygen consumption (VO_{2peak}) during an incremental cycle protocol in MM patients compared to healthy control subjects (Taivassalo et al., 2003; Dysgaard-Jepesen et al., 2003; Grassi et al., 2009). The reduced VO_2 in these patients is a result of a decrease in Q and/or $D_{a-v}O_2$ while a normal physiological response to incremental exercise intensity is an increased VO_2 , resulting from an increased Q and $D_{a-v}O_2$. These previously mentioned symptoms do, however, occur in other patient populations and chronic inactive subjects as well. It may be interesting to look for commonalities and divergences between these populations and the MM group. Mitochondrial dysfunction is a feature that has been hypothesized in other pathologies like cerebral palsy (Peterson et al., 2012), metabolic syndrome (Hawley and Lessard, 2007; Boushel et al., 2007), Chronic Obstructive Pulmonary Disease (COPD) (Gagnon et al., 2014), Peripheral Arterial

Occlusive Disease (PAOD) (Pipinos et al., 1999), Chronic Fatigue Syndrome (CFS) (Smits et al., 2011),... The frequently asked question considering this mitochondrial dysfunction as a disease hallmark, was the fact whether this presumed dysfunction primarily originated from the pathology or not. This was in particular because these patient populations were generally recognized as physically inactive subjects (Peterson et al., 2012; Pipinos et al., 1999; Batista-Amorin et al., 2014; Gagnon et al., 2014; Hawley and Lessard, 2007; Boushel et al., 2007; Smits et al., 2011). A significant decline in physical activity could be a secondary cause for reduced muscle mitochondrial functioning (Boushel et al., 2007). Hence, it has been shown that chronic inactivity leads to a decreased mitochondrial density and citrate synthase activity whereas oxidative phosphorylation (mitochondrial function) remains intact (Boushel et al., 2007; Hood et al., 2006). A disturbed oxidative phosphorylation (mitochondrial dysfunction), which does not occur in physically inactive subjects, is the central diagnostic hallmark in MM patients. As such, a reduced mitochondrial density and a disturbed mitochondrial functioning could be a discriminating factor between muscle dysfunction as a consequence from chronic physical inactivity and MM. In MM patients, there is a disturbed peripheral oxygenation where O_2 extraction will be blunted as a consequence of mitochondrial dysfunction at muscle level. Subsequently, Q will abundantly increase in order to compensate this reduced $D_{a-v}O_2$ (Taivassalo et al., 2003). A majority of MM patients exhibited such a blunted ability to increase $D_{a-v}O_2$, resulting in a so called “hyperkinetic circulatory response” (Haller and Vissing, 2009; Taivassalo et al., 2002; Taivassalo et al., 2003). Such a hyperkinetic circulatory response is a second hallmark that differs MM patients from physically inactive subjects, where O_2 supply was rather limited. The disturbed Q relation in MM patients is presented in Figure 6.

Fig. 6: A schematic presentation of this hyperkinetic circulatory response to exercise. In Figure A, the increase in VO_2 is presented in MM patients and healthy subjects with increasing intensity. In figure B, a hyperkinetic cardiovascular response was found in MM patients as an attempt to compensate the reduced O_2 extraction (Tarnopolsky et al., 2005).



In addition, abnormal high Respiratory Exchange Ratio's (RER) were found in MM patients indicating an excessive carbon dioxide (CO_2) production (Taivassalo et al., 2003). This excessive CO_2 production may be a result of an increased bicarbonate buffering of protons in MM patients, probably due to elevated anaerobic energy delivery. Lactic acidosis has often been used as an indicator of impaired oxidative metabolism and as a diagnostic tool to screen mitochondrial disorders (Tarnopolsky and Raha, 2005). When mitochondrial dysfunction impairs the aerobic production of ATP, ATP will be produced using anaerobic glycolytic pathways, where glucose and glycogen will be converted to lactate and protons. Consequently, this increased amount of protons will bind to bicarbonate resulting in an elevated CO_2 production. Another anaerobic pathway to produce ATP, is the phosphocreatine pathway, where creatine acts as a transporter to deliver phosphates in the myocyte. The aerobic energy system is the main regulator of phosphocreatine recovery when exercise is finished. In MM patients, this post exercise phosphocreatine (PCr) recovery kinetics, will be delayed as a consequence of the malfunctioning mitochondria (Barbirolly et al., 1995; Fabrizi et al., 1996; Matthews et al., 1991).

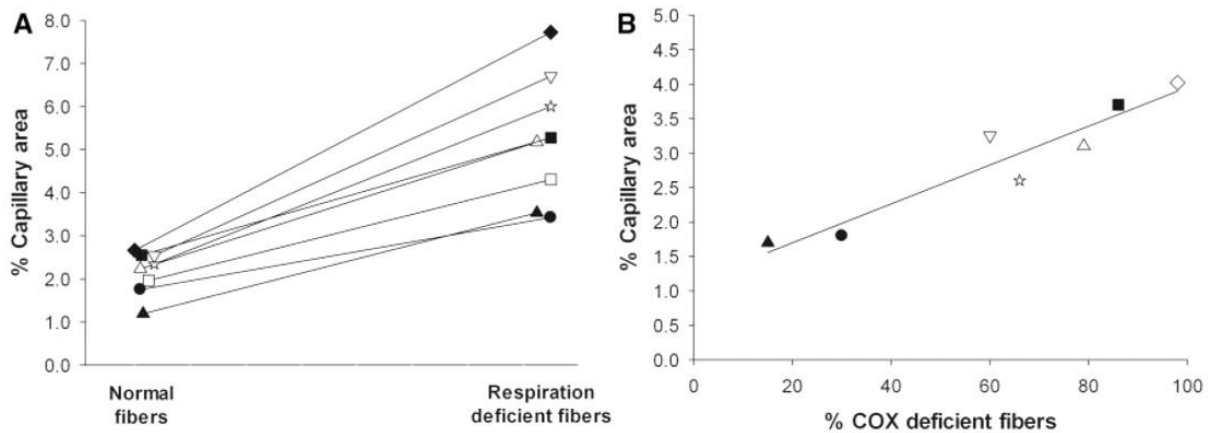
3.1.3 Peripheral oxygenation in mitochondrial myopathies

Considering the three problems we acknowledged before (i.e. sensitivity and specificity (i), danger of cardiomyopathy when performing whole body exercise (ii) and the problem of lower heteroplasmy levels in one subject (iii)), non-exhaustive exercise tests on isolated muscles should be preferred. In this way, peripheral oxygenation of investigated muscles are studied to gain more insight in peripheral adaptations and disturbed physiological responses to exercise. Hence, some forearm screening tests were developed to investigate peripheral oxygenation in small muscles, thereby imposing a low cardiovascular load (Hänisch et al., 2006; Jensen et al., 2002; Taivassalo et al., 2002; Van Beekvelt et al., 1999). All these studies, except Van Beekvelt et al. (1999), used venous blood samples to observe O₂ desaturation values during exercise. Van Beekvelt et al. (1999) was, to our knowledge, the only study that non-invasively measured muscle VO₂ (VO_{2m}) for comparison between MM patients and healthy subjects using NIRS. However, some methodological concerns can be raised with the interpretation of the absolute NIRS values and the influence of subcutaneous adipose tissue in this study (Van Beekvelt et al., 2001). The overall finding was that O₂ desaturation during and after exercise was significantly reduced in the MM population, indicating an impaired O₂ extraction at microvascular level. The heterogeneity of the desaturation values within the MM patients was however, quite large (Hänisch et al., 2006; Jensen et al., 2002; Taivassalo et al., 2001).

It is well known that peripheral oxygenation of skeletal muscles is limited in this MM patient population due to mitochondrial defects. Previously, it was mentioned that physical inactivity, which is a possible consequence of this mitochondrial dysfunction could deteriorate disease severity by a decreasing mitochondrial density (Taivassalo et al., 2006). However, contradicting results in pathologic mtDNA amount have been found in training studies, with an overall improvement in O₂ extraction, VO_{2peak}, peak work capacity and a normalisation of the hyperdynamic cardiovascular response (Taivassalo et al., 2001; Taivassalo et al., 2006). In Taivassalo et al. (2001), the mutation load of pathogenic mtDNA was increased after a training program while this was not the case in Taivassalo et al. (2006) with the same intensities. It was hypothesized that the severity of the lower mean mutation load (65 % vs 55 %) was responsible for this divergence (Taivassalo et al., 2006). The inverse relation between mtDNA

mutation load and skeletal muscle oxidative capacity implicates a disturbed relation between O_2 delivery and O_2 consumption during exercise. In MM patients, O_2 delivery relative to O_2 consumption can be three times as big as in healthy subjects (Taivassalo et al., 2002). Additionally, in a recent study, it has been observed that MM patients possess a mean capillary area 2.5 fold higher than healthy control subjects. This phenomenon is presented more clearly in Figure 7. A maladaptive capillarization is thus stimulated by the presence of an impaired mitochondrial metabolism. It is also important to mention that the extent to which this capillary density has increased, is directly associated with the severity of the disease (Taivassalo et al., 2012). Hence, Jeppesen et al. (2006) found no increase of capillary abundance in muscle fibres of MM patients with a more preserved phenotype. Taivassalo et al. (2012) suggested that a central mechanism could be responsible for the increased amount of capillaries surrounding oxygen deficient muscle fibres. According to this suggestion, PGC 1 α has been postulated to be such a central controlled activator of mitochondrial biogenesis (Lin et al., 2005). Moreover, PGC 1 α seems to augment capillary angiogenesis by increasing Vascular Endothelial Growth Factor (VEGF). This increase in VEGF was stimulated by co-activation of the Oestrogen Related Receptor- α (ERR- α) (Arany et al., 2008). The fact that MM patients showed an elevated PGC 1 α amount (Adhietty et al., 2007), probably due to the typical mitochondrial proliferation in ragged red fibres, could be a possible explanation for this disproportionate capillary density.

Fig. 7: A schematic presentation of the relation between capillary area and the amount of deficient muscle fibers in MM patients (Taivassalo et al., 2012).



In this section, sufficient evidence was provided to show that nDNA or mtDNA mutations and deletions may severely affect mitochondrial respiration, thereby restraining aerobic energy delivery. When exercise intolerance and excessive fatigue are the general symptoms, MM should be suspected in patients. Despite some general distinctions which are frequently occurring in physical inactive subjects and other diseases like COPD, PAOD, CVS, ... (e.g. lower VO_{2peak} , tachycardia, dyspnoe, elevated serum lactate values,...), it seems that an altered peripheral oxygenation is the most striking symptom in this patient population. As such, a disproportional muscle perfusion and capillary area has been observed in MM patients as a consequence of the limited O_2 extraction at muscle level. These effects are however, less pronounced in MM patients with a lower mutation load. Considering the fact that this is quite a “young” research area, little is known about these mild mitochondrial diseases. Moreover, the majority of the screening tests, discussed in this part, were intensive or invasive protocols. A standard non-invasive and non-exhaustive test protocol which measures skeletal muscle perfusion and O_2 extraction would be of great interest as a screening tool or follow up of these patients.

3.2 Chronic Fatigue Syndrome (CFS)

3.2.1 Prevalence, aetiology and diagnosis

Different syndromal definitions have been developed for the description of Chronic Fatigue Syndrome (CFS). Of these, the CDC criteria (Center for Disease Control and Prevention) (Fukuda et al., 1994) are most frequently used. It has led to different labels, including myalgic encephalomyelitis (ME) and more recently, severe exercise intolerance syndrome (SEID) (Institute of Medicine, 2015). This depends, among other considerations, on the framework which was used for aetiology and pathogenesis. A major factor in this variability consists of the focus on a pure biological or biopsychosocial model. Hence, a wide prevalence range has been reported between 0.006% and 3%, depending on which criteria are used for diagnosis. Moreover, epidemiological studies reveal that Caucasian women, older than 30 years, are especially susceptible for developing CFS (Afari et al., 2003).

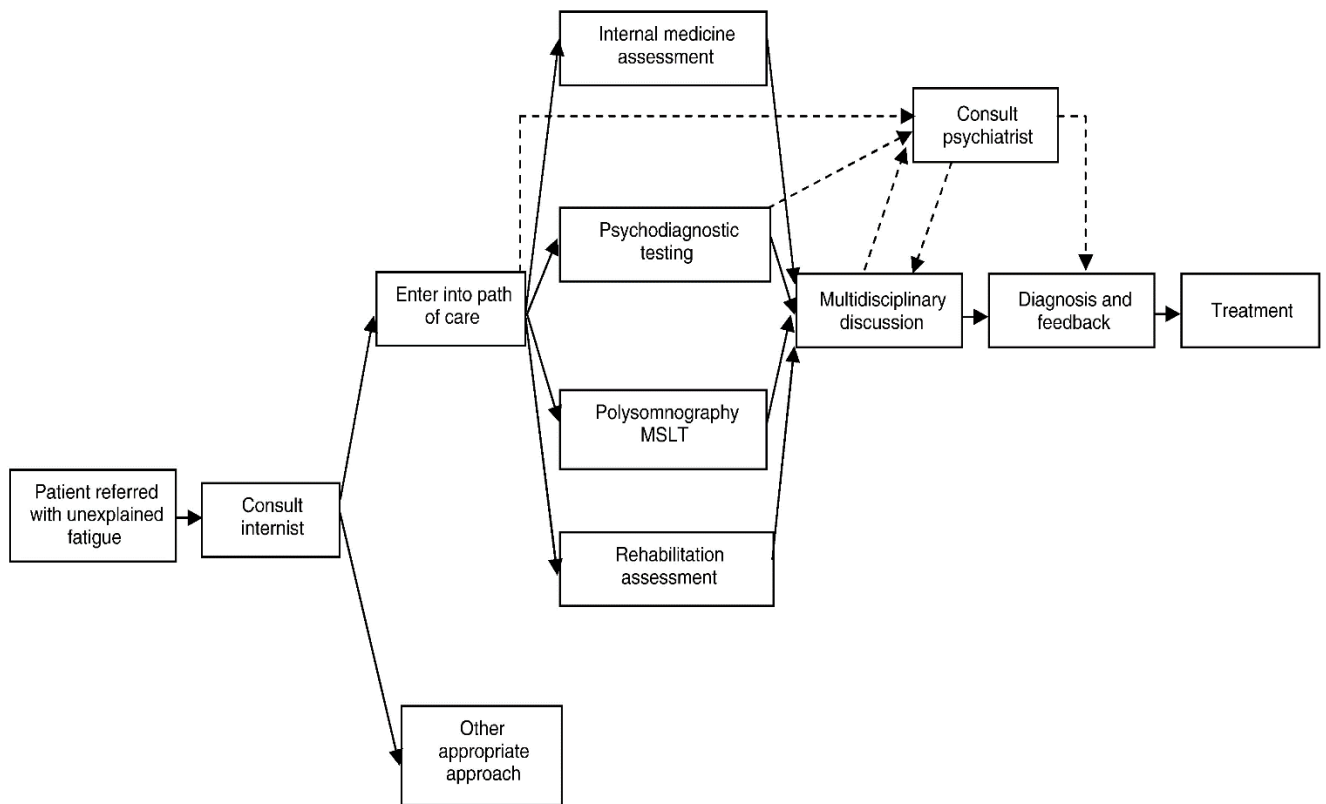
CFS is characterized by unexplained fatigue lasting for longer than six months which is not due to continuing exertion and does not improve by rest. Essentially, the presence of an underlying disease needs to be excluded (Fukuda et al., 1994). Besides this major criterion of long lasting unexplained fatigue, a diagnosis of CFS requires at least 4 out of 8 minor criteria. These minor criteria include post-exertional malaise lasting for at least 24 h, sore throat, tender cervical or axillary lymph nodes, muscle pain, multi-joint pain without swelling or redness, headache of a new type, pattern or severity, memory and concentration impairment and unrefreshing sleep (Fukuda et al., 1994). Also, fibromyalgia (FM) has been frequently diagnosed as a comorbidity for CFS (Buchwald and Garrity, 1994; Buchwald, 1996). This disorder can be described as widespread and chronic muscle pain in different tender points. Possible muscle abnormalities have been studied extensively in both CFS and FM pathogenesis.

The aetiology of CFS remains controversial and largely unknown. There are different hypotheses, either focusing on psychologic (Abbey et al., 1991) or biologic determinants (Myhill et al., 2009; Morris and Maes, 2014). The controversy on causes of CFS continues in the absence of compelling evidence for a particular aetiology. This is illustrated by the analysis of possible causes of post-exertional malaise, major feature of this illness. Many hypothetical

mechanisms have been postulated in the past, to explain the reduced exercise capacity and post-exertional malaise in CFS patients. These speculative mechanisms have often been related to autonomic dysfunction or central sensitisation which could have major implications on exercise tolerance and recovery. Central sensitisation can be defined as a hyperexcitability of the central nervous system with an inadequate pain inhibition after exercise (Meeus et al., 2013). It represents an increased “central” response to “peripheral” stimuli, thereby generating hyperalgesia (increased pain from a stimulus that usually provokes pain) and allodynia (pain due to a stimulus that does not usually provoke pain). Nosologically, according to Clauw (2010), Central Sensitisation Syndrome (CSS) might be even more appropriate to use than CFS/FM in this patient population, in particular in a subpopulation with predominantly FM and pain as presenting symptom. Many speculative mechanisms have been investigated as possible sources for this inadequate pain modulation after exercise. As such, Brain Derived Neurotrophic Factor (BDNF) has been postulated as a key feature in this chronic pain situation as it plays a major role in nociception (Nugruha et al., 2012). BDNF belongs to the neurotrophine family, which is involved in different neuroplasticity processes like modulating mood and pain perception. It has been shown that inhibition of BDNF signal transduction can inhibit central pain sensitisation (Kerr et al., 1999; Pezet et al., 2002). Although this central sensitisation model has been studied extensively the last few years, it cannot explain all the CFS symptoms (i.e. postexertional malaise and muscle stiffness) (Kulshreshtha and Deepak, 2013). Autonomic dysfunction comprises the symptoms and effects that could be related to the inadequate functioning of the autonomic nervous system (ortho- and parasympathetic functioning) and is another explanatory hypothesis for some syndromal manifestations. This autonomic dysregulation may originate from an altered and inadequate stress response, probably due to chronic stress (Meeus et al., 2014). Chronic stress owns the ability to reduce parasympathetic activity. Despite contradictory reports about autonomic dysregulation, it might be interesting to make a clear distinction between CFS patients and CFS patients with a fibromyalgia comorbidity when focussing on autonomic dysregulation (Kulshreshtha and Deepak, 2013). There is a general scientific consensus about the sympathetic “hyper” activity and/or parasympathetic dysfunction in both populations (Reyes del Paso et al., 2012; Frith et al., 2012; Meeus et al., 2013). The difference between both populations, however, might be due to the deficits in pain inhibition to acute stressors by the cardiovascular system in the FM

and not in the CFS population (Meeus et al., 2013; Reyes del Paso et al., 2011). In FM patients, a decreased stroke volume, myocardial contractility and baroreceptor sensitivity has been determined (Reyes del Paso et al., 2010; 2011). This is quite important as the cardiac baroreflex system is an important mediator in the relationship between blood pressure (autonomic nervous system) and pain (central sensitisation). Hence, it has been shown that experimental activation (stretch) of the baroreceptors induces antinociceptive effects (Rau and Elbert; 2001). Although differences have been found between CFS/FM patients and healthy control subjects with respect to autonomic dysfunction or central sensitisation, it remains unclear what causes these syndromal disturbances. Various hypothetical aetiologies have been proposed in the literature, i.e. psychological (stress), neuroendocrinological, virological and immunological mechanisms (Van Cauwenbergh et al., 2014). Many neuroendocrinological alterations have been observed in CFS/FM patients such as increases in nerve growth factor and BDNF and decreases in serotonin, norepinephrine and dopamine (Clauw et al., 2010). Furthermore, in a systematic review of Nijs et al. (2014), studies on the responses to exercise were systematically and qualitatively analyzed to draw some conclusions concerning these possible immunologic and metabolic causes of this post-exertional malaise and fatigue. The conclusions were threefold: (i) There is no sufficient evidence for a post exercise decreased amount in cytokines in CFS patients. (ii) CFS patients have a more pronounced response in the complement system with a higher elastase amount after exercise. Elastase is a proteolytic enzyme produced by monocytes and neutrophils during inflammatory responses. A higher amount after exercise is thus an indication for an excessive inflammatory response. (iii) More and more evidence exists for an earlier and longer lasting oxidative stress during and after exercise in these CFS patients. However, these observations rather describe effects than a true cause. A more logical approach is to integrate different factors into a biopsychosocial explanatory model, incorporating vulnerability, triggering and perpetuating factors. This holistic approach (Wessely et al., 1999; Mariman et al., 2013) can be translated in practice into a multidisciplinary assessment and includes internal medicine assessment, psychodiagnostic screening, rehabilitation assessment and polysomnography combined with a multiple sleep latency test in order to screen for primary or comorbid sleep disorders. Subsequently, a diagnostic decision will be made in a multidisciplinary discussion (Wessely et al., 1999; Mariman et al., 2013). An organigram of the diagnostic process is presented in Figure 8.

Fig. 8: Diagnostic process of patients with long lasting, unexplained fatigue. Full lines indicate systematic, interrupted lines indicate optional steps (Mariman et al., 2013).



3.2.2 Exercise tolerance spectrum in CFS patients

Exercise intolerance and post-exertional malaise are major symptoms in CFS patients. Moreover, all symptoms will deteriorate during and after (intensive) exercise, indicating exercise related abnormalities in these patients (Nijs et al., 2014). These general symptoms are combined with a perceived muscle weakness and pain in many CFS patients (Fulle et al., 2000). Nonetheless these clear symptom descriptions, contrasting results have been found regarding reduced muscle strength (Fulcher and white, 2000; Kent-Braun et al., 1993) and VO_{2max} values during exercise. A reason for this lacking uniformity could be the possible existence of a subgroup suffering from an intracellular immune deregulation which could be related to a reduced exercise capacity. Snell et al. (2002) showed that CFS patients with such a deregulated immune pathway (2', 5' oligoadenylaat (2-5A) synthetase/RNA L-pathway) had a significantly reduced exercise capacity compared to CFS patients with normal intracellular

immune function. The underlying hypothetical mechanism for this association could be that such a deregulated immune pathway could lead to a channelopathy by affecting the ATP sensitive potassium channels, thereby inducing muscle weakness (Nijs et al., 2004). When investigations report VO_{2max} in these patient populations, caution is advised when interpreting these results. Especially because reaching VO_{2max} requires a “levelling of” and it has been shown that CFS patients are not able to reach this “levelling of” (Astorino et al., 2000; Day et al., 2003). Therefore, the use of “ VO_{2peak} ” values in this context would be more appropriate. Some studies showed normal values for aerobic power in CFS patients (Sisto et al., 1996; Rowbottom et al., 1998; Kent-Braun et al., 1993) while others demonstrated a reduced aerobic capacity (Montague et al., 1989; De Becker et al., 2000). The latter study of De Becker et al. (2000) was carried out on a large sample size of 427 CFS patients, thereby challenging the methodological problem of a limited number of patients. Peak heart rate as well as heart rate at the anaerobic threshold during an incremental exercise protocol was significantly decreased in CFS patients, thereby indicating a suboptimal cardiac functioning. A speculative reason for this reduced cardiac function could be a disturbed autonomic system or a viral presence in CFS patients (Pagani et al., 1994). Ortho- and parasympathetic dysregulation (i.e. reduced parasympathetic and elevated orthosympathetic activity) could explain an increased resting heart rate and a significantly reduced peak heart rate during exhaustive exercise (Cordero et al., 1996; Freeman and Komaroff, 1997; Pagani et al., 1994; Wilke et al., 1998). Furthermore, increased plasma lactate concentrations were found during submaximal exercise in CFS (Riley et al., 1990). Another feature characterizing the differential response towards fatiguing exercise is a delayed recovery in a CFS population (Paul et al., 1998; Meeus et al., 2014). A majority of CFS patients (with and without FM comorbidity) are complaining about muscle fatigue and an abnormal recovery after exercise, especially in the upper limb muscles (Ickmans et al., 2014). Hence, a significantly decreased recovery ratio was found after exhaustive handgrip exercise in the CFS populations with FM comorbidity. In contradiction with previous studies, no significant differences in recovery were found in the CFS population without FM comorbidity (Ickmans et al., 2014), indicating that conclusions are not readily generalizable over the whole spectrum of CFS with or without FM. The frequent contradictions on features of exercise intolerance may be due to heterogeneity in the CFS cohort or to a secondary cause, i.e. chronic inactivity. As was mentioned in previous part, these musculo-skeletal and cardiovascular manifestations are frequently occurring in other (physically

inactive) patient populations as well. Nevertheless, exercise intolerance and excessive fatigue are primary characteristics in this patient group with severe daytime dysfunction.

3.2.3 Peripheral oxygenation in CFS patients

The reduced exercise capacity in combination with muscle pain and weakness as a major symptom suggests a possible key role of skeletal muscle as a hotspot for this disease. As some studies found significant differences in VO_{2max} values between CFS patients and healthy control subjects, a disturbed peripheral oxygenation was suspected (McCully et al., 2003). There are three good reasons to investigate eventual peripheral oxygenation abnormalities in CFS patients. (i) As elucidated previously, there is moderate evidence for an increased and earlier production of oxidative stress (Reactive Oxygen Species (ROS)) in CFS patients, indicative for a possible mitochondrial dysfunction (Nijs et al., 2014; Fulle et al., 2000; Morris and Maes, 2014). (ii) A slower phosphocreatine (Pcr) recovery ratio after exercise was observed in CFS patients (McCully et al., 1996; McCully and Natelson; 1999), indicative for reductions in oxidative metabolism as well. (iii) The autonomous nervous system dysregulation (Montague et al., 1988) and vasomotor inactivity (Jones et al., 2010) could alter skeletal muscle blood flow during exercise.

Notwithstanding these hypothetical statements, contrasting results have been found when focusing on peripheral muscle oxygenation (McCully et al., 2003). Some studies showed a disturbed muscle metabolism (Arnold et al., 1984; Wagenmakers et al., 1988; Fulle et al., 2000) while others found no abnormalities (Kent-Braun et al., 1993; Barnes et al., 1993). Likewise, some studies found a reduced skeletal muscle blood flow during exercise in CFS patients (Bennett et al., 1991; McCully et al., 1996, 2004; McCully and Natelson, 1999) while others found no different pattern (McCully et al., 2003). A speculative explanation for this reduced skeletal muscle blood flow during and after exercise could be a reduced capillary density in CFS patients or an autonomous nervous system dysregulation (Lindh et al., 1995; Montague et al., 1988). Taking into account the mechanisms of peripheral oxygenation, this means that there will be a limited delivery of O_2 at muscle level compared to healthy control subjects. Furthermore, this reduced capillary density was found in combination with a larger proportion

of muscle fibre type II in CFS and FM patients (Lindh et al., 1995; Pietrangelo et al., 2009). These results were, however, not replicated in Smits et al. (2011), where equal muscle fibre type I and II composition were found in skeletal muscle biopsies of CFS patients and healthy control subjects. In the latter study, a decreased citrate synthase activity was found in CFS patients, indicating a reduced mitochondrial density. This finding does not differentiate between CFS as primary cause or physical inactivity as a secondary for reduced mitochondrial density.

Possible reasons for these divergent findings concerning peripheral oxygenation are threefold. First of all, there are some major differences in methodological set up between studies to investigate muscle oxidative function in these patients, ranging from skeletal muscle biopsy samples in resting conditions (Fulle et al., 2000) to P-MRS spectrometry in resting conditions (Arnold et al., 1984) but also during or after exercise (Barnes et al., 1993; McCully and Natelson, 1999; McCully et al., 2003,2004), which is important to challenge the oxidative system. Secondly, skeletal muscle biopsy is the only gold standard method in order to demonstrate skeletal muscle oxidative dysfunction (Bourgeois and Tarnoplosky, 2004). A third possibility to explain the different findings is the heterogeneity within the investigated CFS population and the possible mediating factor of chronic inactivity. In a lot of previously mentioned studies, this heterogeneous oxygenation response of the CFS patients has been presented as an explanation for not finding significant differences (McCully et al., 2003, 2004; McCully and Natelson, 1999; Barnes et al., 1993). Recently, Miller et al. (2015) found reduced oxygenation responses using NIRS in CFS patients compared to healthy control subjects during dynamic exercise. To our knowledge, this was the first study using NIRS to investigate oxygenation responses in CFS patients. However, these studies show in general, a large standard variability concerning VO_{2peak} , aerobic power, peripheral blood flow and muscle metabolism, within the CFS population.

3.3 Is there a connection between both diseases (MM and CFS)?

The central problem in the MM population is a genetic defect (mtDNA and/or nDNA) causing mitochondrial dysfunction in skeletal muscle tissue. The severity of this dysfunction depends on the location of the targeted DNA sequence and on the heteroplasmy amount of the mtDNA,

if the mtDNA is affected (Di Mauro, 2010). This mitochondrial dysfunction has, however been hypothesized to be a key element of muscle abnormalities in CFS patients as well (Fulle et al., 2000; Morris and Maes, 2013). In this context, it is interesting to recite some clinical features which are similar between both patient populations. Exercise intolerance and post-exertional malaise are some of these overlapping symptoms in MM and CFS patients. Additionally, it is quite remarkable that skeletal muscle is the targeted area in MM patients and a suspected hot spot in CFS too. General symptomatology of MM as well as the overlapping and differing manifestations are presented in table 1

Table 1: Similarities and differences in the wide variety of symptoms in mitochondrial disorders and CFS (Morris and Maes; 2013).

Organs	Symptoms in mitochondrial disorder	Symptoms in CFS
Muscles	muscle weakness	yes
	Cramps	yes
	Pain	yes
Brain	mental retardation	–
	epilepsia	–
	migraine	yes
	dementia	neurocognitive symptoms
	neuro-psychiatric disorders	sometimes depression
	autistic behaviors	–
Nervous system	neuropathic pain	yes
	dysautonomia	yes
Heart	cardiomyopathy	–
Ears	hearing loss	–
Eyes	optic atrophy	–
	retinitis pigmentosa	–
	blindness	–
	ptosis	–
Endocrine system	diabetes	–
Liver	liver failure	–
Lungs	respiratory problems	–
General	sometimes fatigue	key symptom
	exercise intolerance	yes
	diminished cardiac response to exercise	yes
Course of symptoms	waxing and waning	yes
	progressive course	yes

The studies on mitochondrial dysfunction in CFS patients showed inconsistent results on structural muscle abnormalities. Some studies found mitochondrial degeneration as well as fusion and branching of the mitochondrial cristae in CFS patients (Behan et al., 1991, 1993; Behan, 1992). Also, there is moderate evidence for an increased amount of ROS in skeletal muscle tissue as a possible representation of mitochondrial dysfunction (Fulle et al., 2000; Morris and Maes, 2013). The latter studies of Fulle et al. (2000) and Behan et al. (1991; 1993; 1992) were performed at rest on skeletal muscle biopsy samples (m. vastus lateralis). Mitochondrial dysfunction can be the result and the source of the increased ROS amount as it is a vicious circle where deficient mitochondria produce more ROS, thereby damaging more mitochondria (Meeus et al., 2013). In contrast, Barnes et al. (1993) found no consistent bioenergetic abnormalities during and after exercise in CFS patients compared to MM patients and healthy control subjects. In resting skeletal muscle, MM patients showed significantly

lower PCr/ATP and PCR/P_i ratios compared to CFS patients and healthy controls while no differences were found between CFS patients and healthy control subjects. This is an indication of an impairment of the ATP energy reserve phosphates in MM patients. Furthermore, significantly prolonged recovery half times for [PCr], [Pi] and [ADP] were found in MM patients while no general differences were found in the CFS population. Again, a large heterogeneity was observed in the CFS population, indicating that there may be subgroups suffering from mitochondrial dysfunction, while others do not. In Figure 9, free cytosolic [ADP] at the end of exercise in healthy control subjects, CFS and MM patients are presented. CFS patients have a very heterogeneous response to exercise with a wide range and more variability as compared to controls. This insight requires another way of thinking towards oxygenation responses in a CFS population. Possibly, some studies found mitochondrial dysfunction in CFS patients as a consequence of a small sample size rather than an altered physiological response to exercise. For example, Fulle et al. (2000) found significant differences in mitochondrial function and oxidative damage in only 6 CFS patients while Barnes found no consistent differences in a group of 46 CFS patients. Moreover, both studies were difficult to compare as Fulle et al. (2000) carried out analyses on skeletal muscle biopsy samples at rest while Barnes et al. (1993) investigated the bio energetic response to exercise of these CFS patients. Also, within this group of 46 patients, large differences were found between individual bioenergetics (See figure 9). It should be questioned whether it would be beneficial to carry out extra analyses in the individuals with abnormal bio energetic responses. These extra analyses are also required because it is impossible to differentiate mitochondrial

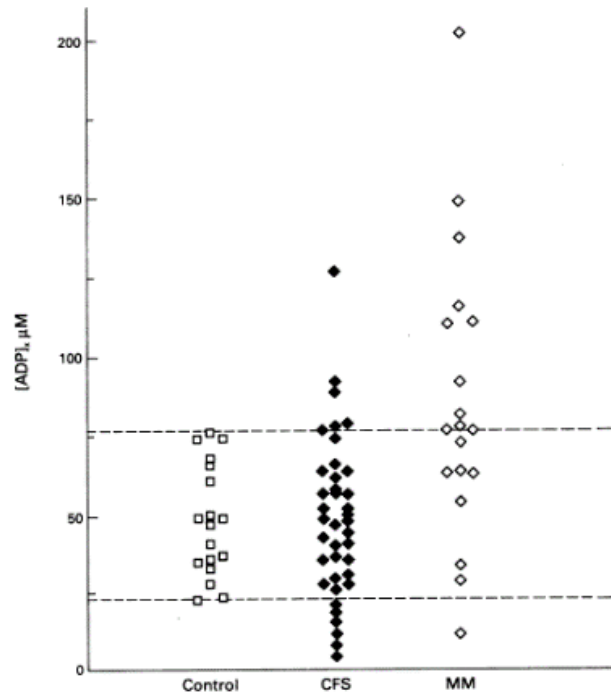


Fig. 9: Distribution histogram of the free [ADP] at the end of exercise in healthy control subjects, MM and CFS patients. The dotted lines show the upper and lower limits of the control scores (Barnes et al., 1993).

dysfunction from a decreased mitochondrial content by P-MRS analysis (Smits et al., 2011). This is also essential to differentiate between an organic origin (i.e. mtDNA, nDNA) or a possible consequence of chronic inactivity (Boushel et al., 2007). In the study of Smits et al. (2011), it was found that mitochondrial function was intact in CFS patients as compared to MM patients. The mitochondrial density however, was significantly decreased in CFS patients, thereby indicating that skeletal muscle biopsy analysis is important to draw the right conclusion. Profound analysis of responses to exercise could possibly throw another light on the heterogeneity within the CFS cohort. Additionally, one should be aware of the frequent symptom similarities between both CFS, MM and physically inactive subjects (Smits et al., 2011; Boushel et al., 2007, Taivassalo et al., 2006). There are two important exercise related markers that differentiate MM pathology from the other populations, i. e. a hyperdynamic cardiovascular response and mitochondrial dysfunction (Smits et al., 2011; Taivassalo et al., 2001; 2006; 2012). With respect to the exercise response of CFS subjects, it is hard to differentiate between a primary (disease) or a secondary (chronic inactivity) cause.

In MM, mitochondrial dysfunction during and after exercise has been clearly described and exercise intolerance can be clearly related to the disease severity. These problematic oxygenation responses to exercise are a consequence of genetic defects affecting mitochondrial function. However, little is known about eventual mild types and acquired types of MM (Morris and Maes, 2013). Notwithstanding these well described MM peripheral oxygenation responses, a lot of inconsistent findings are presented on eventual similar responses in CFS patients. Further assessment on this topic is essential, preferably with optimal methodological experiments to investigate peripheral oxygenation responses in CFS and MM patients and healthy subjects.

4. MEASURING PERIPHERAL OXYGENATION

As discussed in the first part of this dissertation, according to Fick's law,- there are two determinants determining the peripheral O₂ consumption or peripheral oxygenation at muscle level, i.e. microvascular O₂ extraction and skeletal muscle blood flow. To gain more insight in these peripheral oxygenation patterns in healthy and clinical populations, it is essential to obtain valid and reliable measurements of these two determinants. Also, it is important to emphasize that exhaustive exercise and invasive testing should be avoided to improve the accessibility of the measurements and exercise protocols. A critical overview of the available literature with respect to this subject is presented below.

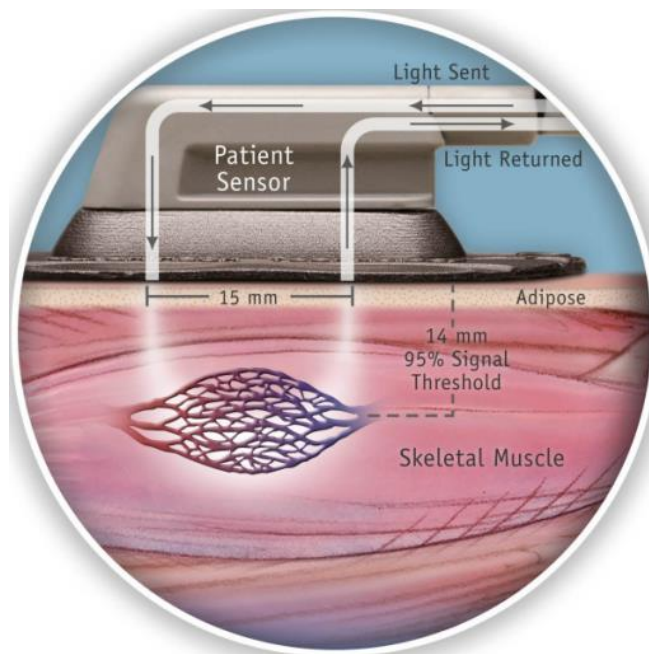
4.1 Microvascular O₂ extraction

The gold standard to monitor O₂ extraction ($D_{a-v}O_2$) at rest or during exercise is the difference of O₂ content in arterial (PaO₂) and venous blood gasses (PvO₂). During exercise at moderate or maximal intensity however, it is complicated to quantify PaO₂ to the investigated muscle. In this case, venous effluent PvO₂ will decrease because more O₂ will be extracted to the exercising muscle (Pirnay et al., 1972; Costes et al., 1996). As such, the difference between this post exercise PvO₂ and the PvO₂ at rest was used multiple times to present a representative value of the extracted O₂ to the muscle. This "Seldinger technique" (Seldinger et al., 1953) consists of the placement of a catheter which was percutaneously inserted in the investigated vein. A major disadvantage, in particular when performing exercise, is the invasive character of this technique. Therefore, some other techniques have been proposed to investigate muscle peripheral oxygenation. Since the introduction of the ³¹P-MRS to measure muscle oxidative metabolism in 1981, it has become the non-invasive gold standard to measure peripheral O₂ extraction. Post exercise phosphocreatine (PCr) resynthesis rate measured by ³¹P-MRS has been recognized as one of the most reliable and non-invasive parameters for quantification of muscle oxidative function (Chance et al., 1985; Kemp et al., 1993; Lodi et al., 1997; McCully et al., 1993). The major disadvantage when using this latter technique is the fact that it is very difficult and expensive to use when performing exercise. Also, it is impossible to differentiate a decreased mitochondrial density from a disturbed

mitochondrial functioning when using this technique in patient populations (Smits et al., 2011).

Near infrared spectroscopy (NIRS) is a frequently used method to evaluate skeletal muscle oxygenation *in vivo* at rest and during exercise (Ferrari et al., 1992; Hamaoka et al., 1996; 2003; McCully et al., 1991) which is an indication of the balance between O_2 delivered to and consumed within tissues (Esaki et al., 2005). This non-invasive optical technique is based on the penetration of NIR light throughout biological tissue such as skin, adipose tissue and muscle where it is absorbed by iron or copper centres of haemoglobin, myoglobin and mitochondrial cytochrome-c oxidase (Piantadosi and Duhaylongsod, 1994). Consequently, the concentration of oxygenated and deoxygenated haemo- and myoglobin (oxy[Hb+Mb] and deoxy[Hb+Mb]) can be determined by NIRS. Furthermore, total haemoglobin (tHb) and O_2 saturation (SmO_2) can be calculated. In Figure 10, the functioning of NIRS is schematically presented.

Fig. 10: Schematic presentation of the working NIRS mechanism (Beilman et al., 2009). The emitted light will be absorbed by oxy[Hb+Mb] and deoxy[Hb+Mb] (at different wavelengths).

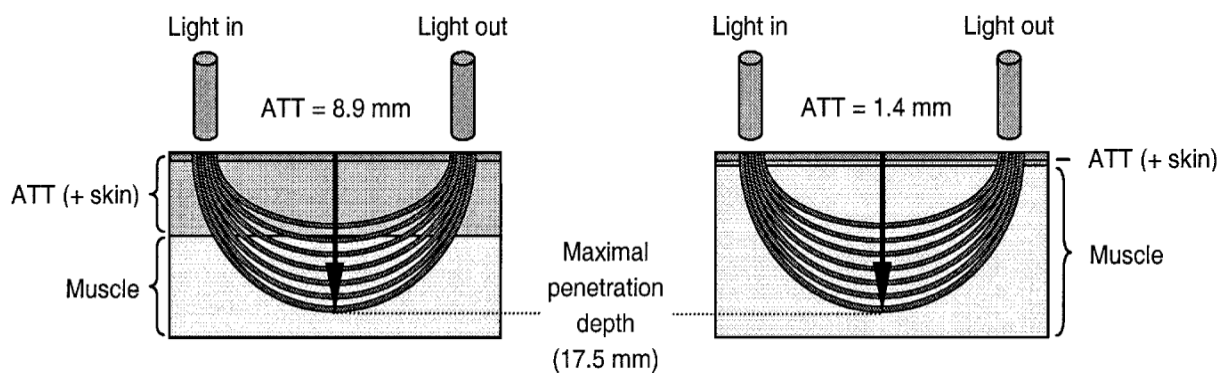


The validity of this technique has been established in some studies (Mancini et al., 1994; Wilson et al., 1989; Esaki et al., 2005; Hamaoka et al., 2000), but questioned in others (Costes et al., 1996; MacDonald et al., 1999) comparing muscle deoxygenation with venous PO₂ (de-)saturation measurements or ³¹P-MRS (Sako et al., 2001). The reason for this discrepancy when validating NIRS, could be a consequence whether or not these values were physiologically calibrated by applying cuffs on the studied limb (Esaki et al., 2005). This “physiological calibration” consists of an occlusion of the artery at the studied limb. Consequently, NIRS oxy[Hb+Mb] and SmO₂ will decrease and deoxy[Hb+Mb] will increase until steady state minimal and maximal values are reached. As such, values are physiologically calibrated and can be expressed relatively towards these minimal or maximal values. Another important issue which can be addressed when using NIRS, is what values are reliable to use when estimating O₂ extraction. Deoxy[Hb+Mb] has been frequently used as the most important NIRS marker to quantify microvascular O₂ extraction (Mancini et al., 1994; Grassi et al., 2007).

As elucidated previously, exhaustive exercise should be avoided in MM and CFS patients, due to the possible comorbidity of cardiomyopathy in MM patients and the fact that disuse of leg muscles is an important issue in MM and CFS patients. Also, complaints of upper limb muscle pain and exercise intolerance are a common feature in CFS patients (Ickmans et al., 2014). Therefore, it would be very interesting to apply NIRS technology on upper limb muscles during exercise in these patient populations. Despite its major advantages (e.g. the non-invasive nature and low cost) when using NIRS, there are still some methodological issues that need serious consideration. A first problem affecting NIRS in skeletal muscle tissue is subcutaneous Adipose Tissue Thickness (ATT) (See figure 11). As such, some studies have demonstrated that NIR light measurements were attenuated with increasing ATT values (Van Beekvelt et al., 2001; Cooper et al., 2010; Messere et al., 2013; Grieger et al., 2013). Ferrari et al. (2011) even stated that NIRS can only be used in lean subjects which strongly limits the clinical application of this tool. This statement was written supposing that a lot of patient populations have a larger subcutaneous ATT layer. It has been shown however, that the possible influence of subcutaneous ATT on NIRS signalling at rest is significantly reduced in forearm muscles compared to other skeletal muscles (Grieger et al., 2013). Moreover, some correction

algorithms have been developed to reduce this influence (Niwiyama et al., 2000; Bowen et al., 2013). Notwithstanding these corrections, it remains crucial to keep focusing on these methodological issues as this device offers major advantages to measure peripheral O₂ extraction non-invasively in clinical populations at a low cost.

Fig. 11: Schematical presentation of the possible influence of subcutaneous ATT on NIRS of skeletal muscle tissue (Van Beekvelt et al., 2002).

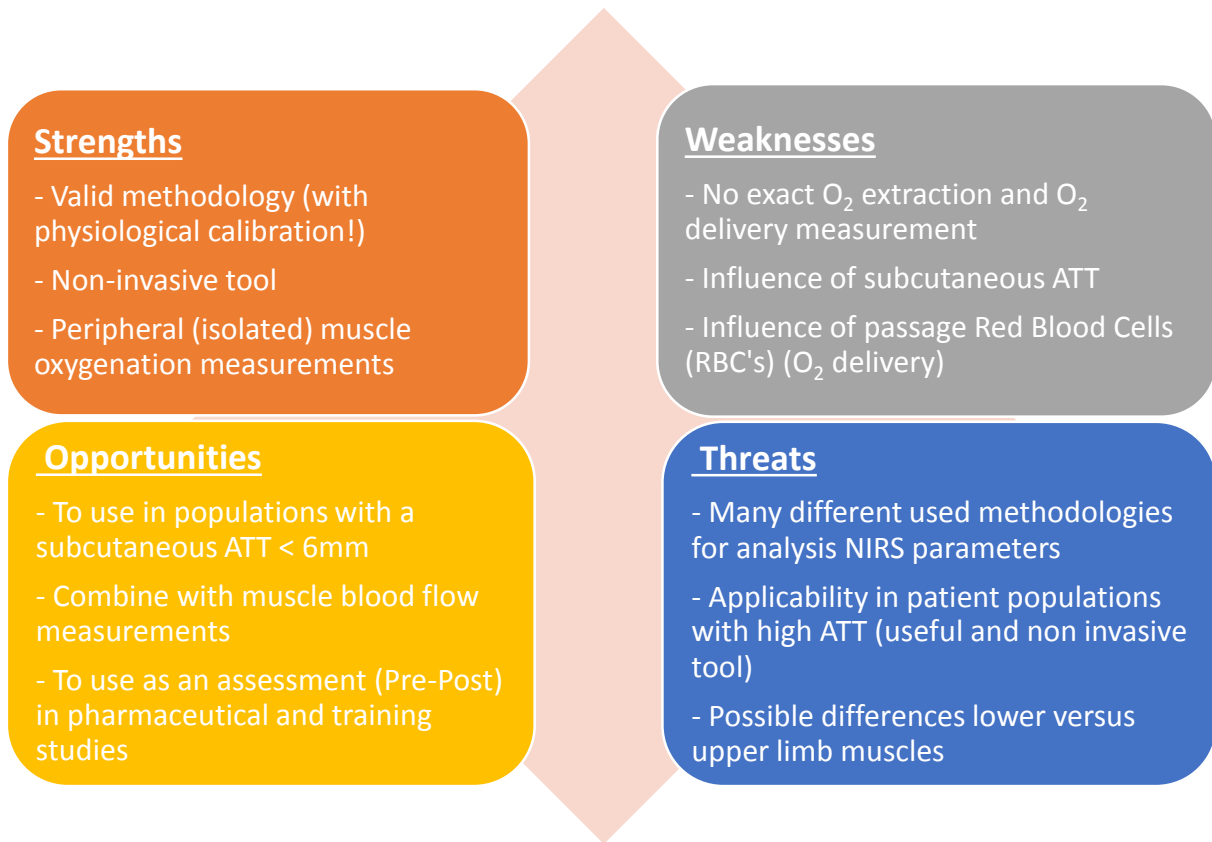


A second issue, although on a different level, is the possible influence of O₂ delivery on NIRS parameters (Ferrari et al., 2011). This issue has been poorly defined in the past and is of major importance when using this device in patient populations. Of course there are some indirect cardiovascular markers which could have a major impact on this O₂ delivery at muscle level. Blood pressure is such a marker which is associated with the vasomotor response at the start of exercise. Such a “normal” response is a sympathetic mediated general vasoconstriction and vasodilation to the active muscle. The fact that blood pressure owns the ability to influence tissue oxygenation was shown in Georger et al. (2010), where norepinephrine intake induced a higher blood pressure resulting in a higher tissue oxygenation. Furthermore, some blood properties such as haematocrit may have a major impact on NIRS derived oxygenation parameters as well. Hence, it has been shown that transfusion of red blood cells led to an increased tissue oxygenation (Yuruk et al., 2011; Torella et al., 2003).

Notwithstanding the ongoing debate between groups preferring to use deoxy[Hb+Mb] values (Delorey et al., 2004; Koga et al., 2011) to estimate microvascular O₂ extraction whereas other groups are using SmO₂ values, stating that deoxy[Hb+Mb] is sensitive to changes in blood volume (Hamaoka et al., 2011), deoxy[Hb+Mb] is considered the most reliable value to

measure microvascular O_2 extraction. This was also shown in other studies (Ryan et al., 2012; Binzoni et al., 2010; Van Beekvelt et al., 2001), where VO_{2m} was calculated from the oxy[Hb+Mb] and deoxy[Hb+Mb] time constants during arterial occlusion. In the study of Ryan et al. (2012), a correction was implemented for changes in blood volume in order to exclude possible influences of this variable. Hence, it is important that totHb values are constant (by a synchronous increase in deoxy[Hb+Mb] and decrease in oxy[Hb+Mb]) when using this method. Moreover, Ryan et al. (2012) showed a significant association between absolute VO_{2m} values and the previously mentioned subcutaneous ATT influence. However, when using relative values (%) towards the ischemic calibration, it was possible to exclude subcutaneous ATT influences (Ryan et al., 2012). In Figure 12, you can find a SWOT analysis about the use of NIRS to estimate peripheral oxygenation of skeletal muscle.

Fig 12: SWOT analysis of the NIRS device to estimate peripheral oxygenation in skeletal muscles.

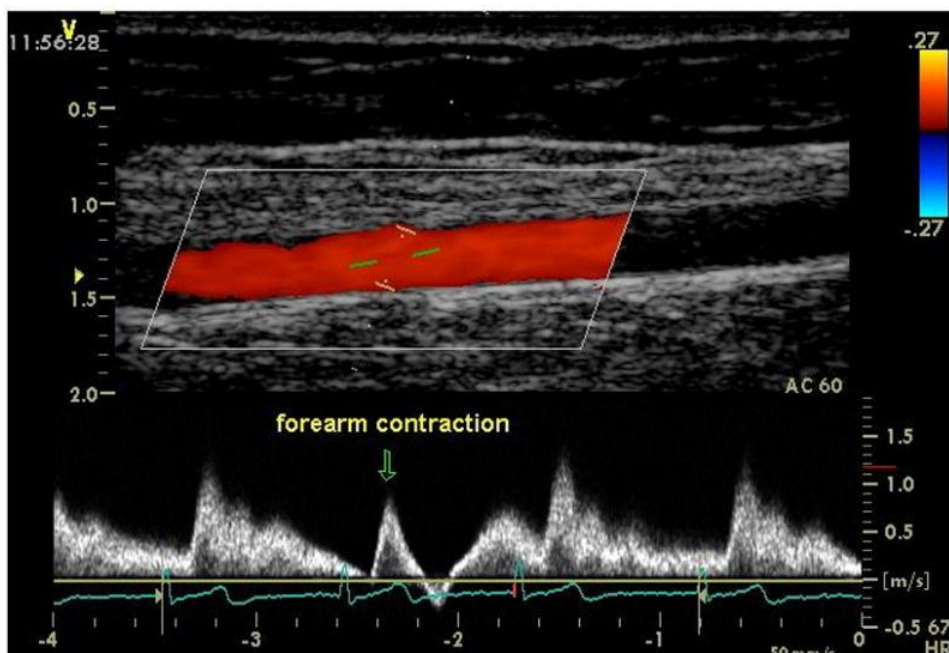


4.2 Skeletal muscle blood flow

In contrast to the measurement of O₂ extraction, there is no gold standard non-invasive method to measure skeletal muscle perfusion. As such, it remains problematic to investigate validity of new measurement methods as there is no ideal assessment of skeletal muscle blood flow (Casey et al., 2008). In general, three techniques have been developed to assess arterial inflow to, venous outflow from or local muscle blood flow. (i) The indicator methods (Jorfeldt and Wahren, 1971; Anderson and Saltin, 1985; Ganz and Swan., 1974) where a dye or a cold saline solution is infused in the blood, assuming that indicator concentration or temperature deflection is proportional to the flow rate of the blood. (ii) Venous occlusion plethysmography (Bygdeman and Pernow, 1978), where venous occlusion is applied to the studied limb while arterial inflow is not limited. The subsequent increase of the limb cross sectional area is a reliable measure for arterial inflow of the studied limb. (iii) Doppler Ultrasound measurements which are based on assessing the erythrocyte reflection to transmitted sound waves of a

specific frequency (Gill, 1979; 1985). This assessment is obtained from the intraluminal space of the artery, which can be determined visually on the Doppler Ultrasound apparatus. As such, blood flow to the limb can be calculated by multiplying the cross sectional area of the artery by the blood velocity under the probe (Radegran, 1997). This latter technique has been mentioned as the most sensitive and accurate technique in two reviews assessing blood flow measurement methodologies (Radegran, 1999; Casey et al., 2008). The unique feature when using this device is a high temporal resolution of limb perfusion, allowing to accurately measure changes in limb blood flow when performing exercise. Moreover, this technique has its non-invasive character as a major advantage compared to the other techniques. It is however, very important to carry out these measurements in a fixed position of the limb to avoid changes due to movement (Radegran, 1999). When respecting all these methodological issues, this technique has a strong reliability (Shoemaker et al., 1996). In Figure 13, Doppler Ultrasound measurements are presented.

Fig. 13: Doppler Ultrasound image of the brachial artery during forearm exercise. The waveforms underneath the image represent the mean blood velocity in the artery (Casey et al., 2011).



In general, two non-invasive methods were presented which enable us to assess microvascular O_2 extraction and skeletal muscle blood flow. The non-invasive character combined with the feasibility of these techniques should be optimal to use in patient populations. Surprisingly, both techniques (i.e. NIRS and Doppler Ultrasound) have not been combined frequently in previous investigations.

5. GAPS IN THE CURRENT LITERATURE

In the first section of this dissertation, a short introduction of peripheral oxygenation mechanisms was presented, thereby demonstrating that the “classical view” on these issues is changing. Of course, this has a major impact on the way how certain diseases with microvascular or muscular complications should be analysed. Therefore, an overview was presented of two diseases in particular, where peripheral oxygenation abnormalities are suspected, i.e. MM and CFS.

In the second section, various investigations were presented showing clear peripheral oxygenation abnormalities in the MM population as a consequence of mitochondrial malfunctioning. In general, two major problems could be raised when considering peripheral oxygenation responses in MM, based on this literature. (i) There is a need for combining non-invasive and non-intensive screening tools to reliably measure peripheral oxygenation. Previous exercise protocols were in most of the cases whole body exercises, thereby imposing a high cardiopulmonary load. Especially for follow up and interventional studies of MM patients, this would be of great interest in future studies. (ii) Reliable testing protocols with sufficient specificity and sensitivity are of great interest as a lot of previous work showed a large heterogeneity in the MM cohort. In this context, it is important to highlight the problem of MM patients with a moderate heteroplasmy amount in skeletal muscle tissue. Unlike this MM population, there is a lot of controversy about muscle and peripheral oxygenation abnormalities in CFS patients. An overall conclusion that can be found in almost all studies investigating exercise responses in the CFS cohort, is the fact that there is a compelling heterogeneity within this population concerning muscle and peripheral oxygenation abnormalities. Again, two crucial issues can be raised towards this research area, where future work is essential. (i) Non-invasive and non-intensive exercise should be preferred to exclude

possible deconditioning bias in these patients. (ii) It should be suitable to analyze individual results of every patient and not only the mean values of the heterogeneous CFS cohort. As such, this heterogeneity could be investigated more profoundly. The reason for comparing these MM and CFS patient populations is that they have some possible commonalities and differences in exercise intolerance symptomatology and peripheral oxygenation responses to aerobic exercise. A clear decrease in O₂ extraction due to mitochondrial dysfunction is one of the key mechanisms in the MM pathology. Although there have been some contradictory reports, this can be a common feature with a subgroup of CFS patients, probably due to a decreased mitochondrial density. Smits et al. (2011) stated that the major difference between MM and CFS patients is a mitochondrial dysfunction and a decreased mitochondrial density. According to the scientific literature, blood flow during exercise should be a major difference between both populations with a hyperdynamic cardiovascular response in MM patients and a decreased muscle blood flow in CFS patients, probably due to autonomic dysregulation, a decreased capillary density or a different muscle fibre type composition.

In the third part of this dissertation, some techniques were presented to estimate peripheral O₂ extraction and skeletal muscle blood flow. However, there are still some methodological issues that can be addressed towards the NIRS technique. (i) The influence of subcutaneous ATT is still a major issue influencing the NIR signal, especially when testing pathological populations. (ii) The reliability of this technique should be investigated on every new skeletal muscle and protocol where it is used to measure peripheral O₂ extraction. (iii) It has not been clearly defined which parameters are influenced by O₂ delivery and to what extent. Doppler ultrasound has been shown as the most sensitive technique to non-invasively measure skeletal muscle blood flow at rest and during exercise.

6. RESEARCH OBJECTIVES AND GENERAL OUTLINE OF THE DISSERTATION

Based on these problems, the aim of this research was to develop a reliable, non-invasive and non-exhaustive exercise screening tool for people suffering from malfunctioning peripheral oxygenation. In this context, the focus of this research was not only to study these parameters in MM patients suffering from clear mitochondrial dysfunction, but in CFS patients as well.

Therefore, an Incremental Cyclic Contraction Protocol (ICCP) was developed, performing handgrip exercise, with NIRS and Doppler Ultrasound measurements on forearm muscles. This protocol was based on dynamic rhythmic contractions as it is well known that this type of exercise predominantly activates oxidative metabolism (Poole et al., 2010).

AIM 1: Investigate the reliability of physiologically calibrated NIRS parameters during ICCP.

As indicated previously, various or no results have been found concerning validity and reliability of the NIRS technique when applied on small muscle groups. In this ICCP, relative deoxy[Hb+Mb] values during dynamic exercise were analyzed relative to their arterial occlusion maximal values. The reliability of these measurements on forearm muscles during ICCP was investigated in healthy subjects in **study 1**. We hypothesized that these relative deoxy[Hb+Mb] values would be reliable measurements to estimate O₂ extraction.

AIM 2: To investigate possible influence of subcutaneous ATT and blood flow on NIRS measurements.

When using the previously described NIRS technique (Aim 1), one should be aware of the methodological issues that remain to be problematic in the analysis of these measurements. In this context, the aim of this study was to investigate the relation between absolute and physiologically calibrated NIRS values on the one hand, and subcutaneous ATT and blood flow on the other hand. Hence, we hypothesized in **study 2** that the relative values of deoxy[Hb+Mb] would not be influenced by subcutaneous ATT and blood flow, and would therefore, be a reliable measure to use in pathological populations.

AIM 3: To investigate post-occlusive and relative deoxy[Hb+Mb] in forearm muscles during ICCP in MM patients.

As an attempt to develop a non-invasive and non-exhaustive exercise protocol, it was hypothesized that MM patients would have a different deoxy[Hb+Mb] pattern during this ICCP, due to mitochondrial dysfunction. Also, because post-occlusive NIRS responses have already been studied extensively, we wanted to compare these deoxy[Hb+Mb] responses

during and after the arterial occlusion in an MM population and a healthy control group in **study 3**. Moreover, the relative deoxy[Hb+Mb] values during ICCP were studied in **study 4**. In both studies, we hypothesized a different pattern in the MM cohort compared to healthy matched control subjects.

AIM 4: To use this ICCP deoxy[Hb+Mb] and FBF measurements as a screening tool for mitochondrial dysfunction in CFS patients.

As a last part in this research, this study aimed to detect peripheral oxygenation abnormalities in individuals suffering from CFS. In **study 5**, Doppler US measurements were added to the ICCP to obtain insight in skeletal muscle blood flow during exercise. Distinctively from other scientific reports, the aim was to analyze all individual results more profoundly. Hence, CFS patients with a disturbed peripheral oxygenation pattern were further investigated by taking blood, muscle and skin biopsy samples. The aims of this dissertation are summarized in Figure 14.

In general, the aim of this research was to create a framework for CFS and MM patients complaining about exercise intolerance, muscle pain or weakness and post-exertional malaise. The foundation of this framework was the development of an accessible exercise test that didn't evoke anxiety to participate or severe exhaustion. Moreover, to counteract eventual deconditioning effects, due to the disease, handgrip exercise was used in this protocol. This was in particular because disuse of leg muscles is an important issue in MM and CFS patients. Also, complaints of upper limb muscle pain and exercise intolerance are a common feature in CFS patients (Ickmans et al., 2014). The first step was to deal with methodological barriers when using these non-invasive techniques. The second step was to investigate whether patients with a clearly demonstrated mitochondrial dysfunction could be identified using this protocol. Last but certainly not least, it was the purpose to investigate peripheral oxygenation interference in a CFS population where these complaints occur frequently. The discussion part offers a critical review on this framework and postulates whether it could be used in future experiments.

Fig. 14: Overview of the studied population, used techniques and experimental aims in the different scientific reports (Healthy Subjects= HS; Mitochondrial Myopathy patients= MM; Chronic Fatigue Syndrome patients= CFS; Deoxyhaemoglobin and –myoglobin= deoxy[Hb+Mb]; Incremental Cyclic Contraction Protocol= ICCP; Adipose Tissue Thickness= ATT; Near Infrared Spectroscopy= NIRS).

	Study 1	Study 2	Study 3	Study 4	Study 5
Population	HS		MM / HS		CFS / HS
Methods	NIRS	NIRS Doppler US	NIRS	NIRS	NIRS Doppler US
Experimental aims	Investigate reliability of relative deoxy[Hb+Mb] during ICCP	Assess possible influence of ATT and BF on absolute and relative NIRS values	Compare post occlusive hyperemic deoxy[Hb+Mb] response	Compare relative deoxy[Hb+Mb] values during ICCP	Compare relative deoxy[Hb+Mb] values and FBF during ICCP

PART II

ORIGINAL RESEARCH

STUDY 1

RELIABILITY OF NEAR INFRARED SPECTROSCOPY (NIRS) FOR MEASURING FOREARM OXYGENATION DURING INCREMENTAL HANDGRIP EXERCISE

Bert Celie, Jan Boone, Rudy Van Coster, Jan Bourgois

Journal: Eur J Appl Physiol. 2012; 112(6):2369-74

ABSTRACT

The purpose of this study was to test the reliability of a new handgrip exercise protocol measuring forearm oxygenation in twenty healthy subjects on two occasions. The retest took place 48h later and at the same time of day. The incremental exercise consisted of 2-min steps of cyclic handgrip contraction (1/2Hz) separated by 1 min of recovery. The exercise started at 20%MVC, was increased with 10%MVC each step and was performed until exhaustion (69,5% MVC and 73%MVC). Near Infrared spectroscopy (NIRS) was used to measure deoxygenation (deoxy[Hb+Mb]) and oxygen saturation (SmO₂) in the forearm muscles. Prior to the exercise protocol an arterial occlusion of the forearm was performed until deoxy[Hb+Mb] did no longer increase. Maximal increase in deoxy[Hb+Mb] during 10s of each exercise bout was expressed relative to the occlusion amplitude. ICC was used to examine the test-retest reliability. Significant ICC's were reported at 50% ($r= 0.466$; $p=0.017$) and 60% MVC ($r=0.553$; $p=0.005$). The group mean of the maximum increase in oxygen extraction was $45.6 \pm 16.7\%$ and at the retest $44.9 \pm 17.0\%$ with an ICC of $r=0.867$ ($p<0.001$) which could be classified (Landis and Koch 1979) as almost perfect. The absolute SmO₂-values showed reliable ICC's for every submaximal intensity except at 60%MVC. An ICC of $r=0.774$ ($p<0.001$) was found at maximal intensity. The results of the present study show that deoxy[Hb+Mb] and SmO₂ responses during this protocol are highly reliable and indicate that this protocol could be used to get insight into deoxygenation and oxygen saturation in a population with low exercise tolerance.

INTRODUCTION

Near infrared spectroscopy (NIRS) is a well established technique to measure tissue oxygenation. In biological tissues like bone, muscle and skin, the NIRS-signal can be affected by absorbing and scattering of the NIR-light at a particular wavelength. Three molecules account for most of the NIRS light absorption: oxygenated and deoxygenated haemoglobin (Hb), myoglobin (Mb) and cytochrome oxidase c (Boushel et al., 2000). The concentration of cytochrome oxidase can be neglected. No distinction can be made between haemoglobin (Hb) and myoglobin (Mb) because there is an overlap of the NIR spectrum. In the past decade this technique has frequently been used to measure blood perfusion and oxygenation index of

several tissues. The validity of NIRS as an appropriate technique to measure oxygenation status at the level of microcirculation has been demonstrated in the past, both in the brain and in muscle tissue (Huppert et al., 2006; Mancini et al., 1994; Mehargoul-Schipper et al., 2002). Beside validation of NIRS other studies have shown that NIRS provides a reliable technique for measurement of the oxygenation status of active muscles during exercise (Kell et al., 2004; Pereira et al., 2005). Significant test-retest intraclass correlations (ICCs) ($r=0.69-0.84$) were found for total blood volume and oxygenation index of the erector spinae muscle during a static endurance protocol (Kell et al., 2004). Limb oxygenation measurements in the vastus lateralis during knee extensions at slow ($r=0.73-0.76$) and fast ($r=0.85-0.97$) velocities were shown to be reliable (Pereira et al., 2005). Considering the muscle oxygen saturation (SmO_2) measurements with NIRS, reliable results were found in a recent study (Tew et al., 2010). Blood volume and SmO_2 in the vastus lateralis at rest were affected by changes in skin blood flow. However SmO_2 during exercise was not influenced. Significant test-retest ICCs ($r=0.93$ and $r=0.96$) for the rest and end-exercise periods respectively, were reported for muscle oxygen saturation (SmO_2) (Tew et al., 2010). This means that SmO_2 is relevant and reliable to observe in an exercise protocol.

These previous studies all focused on perfusion and oxygenation in large muscle groups. However little is known about the reliability of NIRS measurements in small muscles. Muthalib et al. (2010) tested the reliability of NIRS measuring the M. biceps brachii oxygenation. They concluded that the level of reliability is acceptable. Their protocol consisted of sustained isometric contractions at 30% and 100% of the maximal voluntary contraction (MVC) for 10 seconds and of repeated isometric contractions (1s contraction, 1s relaxation). A second study of Van Beekvelt et al. (2002) established the reliability of measuring local muscle oxygen consumption (mVO_2) in the forearm muscles. In this study however our interest is not the mVO_2 , but rather the oxygenation measurements in the forearm muscles. Also the arterial occlusion in Van Beekvelt et al. (2002) was used to calculate the mVO_2 and was performed on the subjects directly after the handgrip exercise. In the present study we wanted to test the reliability of deoxygenation measurements on the forearm muscles during an incremental handgrip protocol, without calculating the mVO_2 . We focused on the deoxy[Hb+Mb] because it is often considered as an accurate indication for oxygen extraction into the muscle (Grassi

et al., 2006). Our study was different to Van Beekvelt et al. (2002) both in that the arterial occlusion occurred prior to the exercise and also in our interest in deoxy[Hb+Mb] as an indication for arterio-venous O₂ difference in the local muscle, which is only one parameter of Fick's law. Further research to establish the NIRS reliability on small muscles is necessary as it could be used to study muscle oxygenation without inducing a high cardiac stress in several populations. The purpose of this study is to test the reliability of the NIRS on forearm muscles in a newly developed incremental exercise protocol

METHODS

Subjects

Twenty healthy subjects (8♂, 12♀) participated in this study, and their mean (\pm SD) age, height, and body mass were 24.4 ± 7.9 yr, 172.6 ± 8.2 cm and 66.5 ± 9.5 kg, respectively. Skinfold thickness at the forearm was measured at the location under the NIRS probe using a skinfold caliper (Holtain Ltd, Crymmych, UK). The mean skinfold thickness was 6.0 ± 2.2 mm at the proximal section of the forearm. All subjects reported no health problems or upper extremity injuries. Subjects were asked not to perform strenuous exercise 48h prior to and during the test-retest experimental period. The study conformed to the recommendations of the local Human Research Ethics Committee in accordance with the declaration of Helsinki. A written informed consent was signed by each subject.

Near Infrared Spectroscopy (NIRS)

During the exercise test, muscle tissue oxygenation was measured with a near infrared spectroscopy (NIRS) system (Oxiplex TS, ISS, Champaign, Illinois, USA). This system is based on an infrared light absorption method, where the infrared light is emitted at different wavelengths. The NIRS-probe consisted of eight light-emitting diodes operating at wavelengths 750 and 830 nm and one detector fibre bundle (source-detector distance = 2.0-3.5cm). The deoxy[Hb+Mb] was stored at a frequency of 25 Hz and afterwards digitally averaged into 1s-values. The probe was positioned longitudinally on the M. flexor carpi radialis

and ulnaris and on the M. flexor digitorum superficialis and secured with Velcro straps around the upper arm. Pen marks were made over the skin to detect movement of the probe during the exercise and in order to ensure that the probe could be positioned in exactly the same location during the retest. This retest took place 48h after the first test at the same time of day.

Study design and protocol

Preceding the test the maximal voluntary contraction (MVC) force was determined on the hydraulic handgrip dynamometer (Saehan corporation, Masan, Korea) for all subjects (best of three attempts). Each attempt was terminated when the force showed a clear stagnation or a decrease. Between every attempt there was a five minute rest period. The NIRS probe was placed on the M. flexor carpi radialis and ulnaris and a pneumatic cuff on the upper arm. The subjects performed the complete exercise protocol in supine position. The protocol started with an arterial occlusion of the forearm. There was no specific timeframe for this occlusion period as it was executed until a steady state in deoxy[Hb+Mb] was reached. This occlusion was carried out with a cuff inflated at about 260mmHg on the upper arm. After the occlusion there was a 5 minute rest period before the exercise task started. This task consisted of 2 minute periods of an incremental cyclic contractions protocol (ICCP) at $\frac{1}{2}$ Hz (1 second contraction, 1 second relaxation) at different intensities of maximal voluntary contraction (% MVC). These contraction periods were separated by a 60-s rest period. The work intensity was increased by 10% MVC each step. This protocol was executed until exhaustion was reached and the subjects were not able to produce the required force. The cyclic contractions for all subjects used the same (dominant) hand in each period and in each of the two sessions, which were separated by 48 hours.

Data analysis

When muscle tissue is exposed to an arterial occlusion, acute local hypoxia is induced. Following Fick's law muscular oxygen consumption (VO_{2m}) is the resultant of the product of muscle blood perfusion (Q_m) and oxygen extraction ($\Delta a-v O_2$). To determine the mean values

within this protocol a smoothing procedure was performed in which the 30-s and 10-s mean values were determined by means of a moving average (Boone et al., 2010). The amplitude of the deoxy[Hb+Mb] response (i.e. the difference between the highest 30-s average of deoxy[Hb+Mb] during the occlusion and the 30-s average of deoxy[Hb+Mb] preceding the occlusion) was used as an index for maximal O₂-extraction and was set to 100%. The changes in deoxy[Hb+Mb] during each work step (i.e., the mean of the maximum 10-s) were expressed relative to this amplitude.

The muscle oxygen saturation (SmO₂) is a calculation of $\text{oxy[Hb+Mb]} / (\text{oxy[Hb+Mb]} + \text{deoxy[Hb+Mb]})$. Because the NIRS instrument provides absolute measurements of these parameters and the oxy[Hb+Mb] is set relative to the tot[Hb+Mb], values can be reported without the need for a physiological calibration using arterial occlusion. At every intensity (%MVC) interval the 10-s mean minimum values of the SmO₂ were subtracted from the 30-s baseline value before the exercise protocol started.

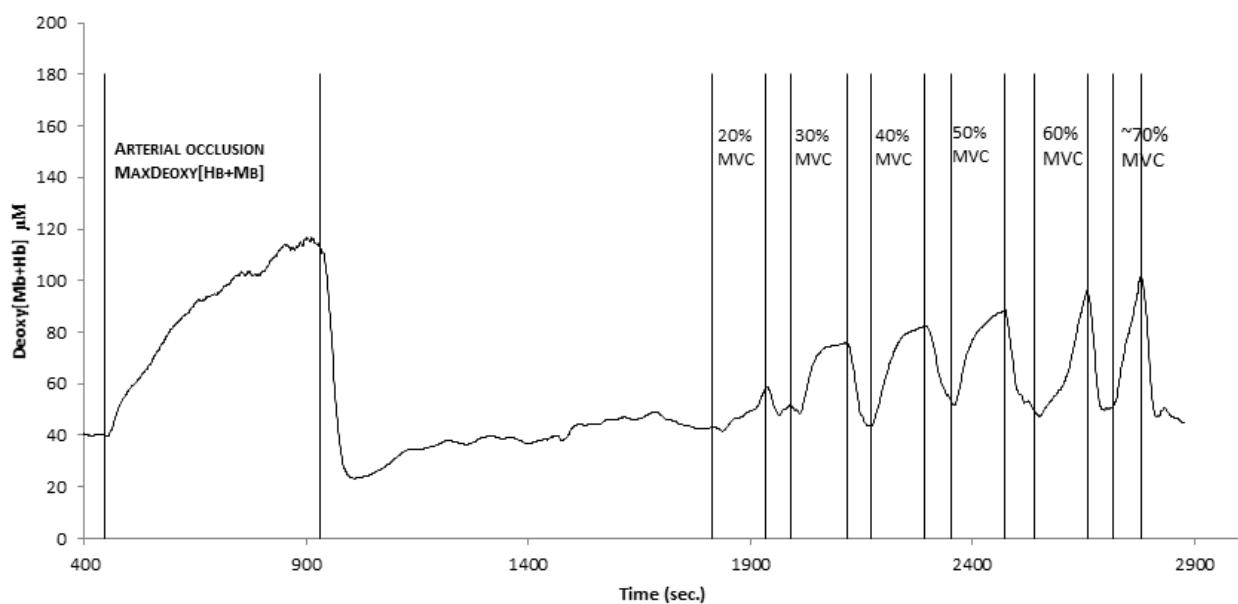
Statistical analysis

To measure significant differences in the MVC and amplitude of the occlusion steady state a paired samples T-test was used. The same analysis was used to compare the increase (in %) in deoxy[Hb+Mb] as a function of the amplitude in deoxy[Hb+Mb] and the decrease in SmO₂ between the test and the retest for each work step. In addition single measure intraclass correlation coefficients (ICCs) and the kappa test for agreement were used to examine the test–retest reliability of this new protocol. The ICCs were interpreted following Landis and Koch's (1977) benchmarks of 0.00–0.20 slight, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 substantial, 0.81–1.0 almost perfect. Significance was set at P<0.05. Statistical computations were performed using SPSS® software (version 18; SPSS Lead Technologies Inc., Chicago, IL, USA). All data are presented as mean ± SD.

RESULTS

No significant differences ($p=0.188$) were found between the MVC at the test ($x=492 \pm 125$ N 10.72) and at the retest ($x=475 \pm 127$ N). The mean final step when the exercise was finished was at 69 % MVC for the first test and 73 % MVC for the retest. Typical deoxy[Hb+Mb] outputs as a response on the arterial occlusion and the incremental cyclic contractions protocol (ICCP) are shown in figure 1. The mean amplitude of the maximal deoxy [Hb+Mb] during the arterial occlusion between the test ($x=44.05 \pm 5.22\mu\text{M}$) and the retest ($x=47.16 \pm 4.65 \mu\text{M}$) showed no significant difference ($p=0.156$). For the amplitude of the arterial occlusion an ICC of $r=0.952$ ($p<0.001$) was found.

Fig.1: Typical changes in the values of deoxygenated haemoglobin and myoglobin (Deoxy[Hb+Mb]) of one subject during arterial occlusion and the incremental cyclic contractions protocol (ICCP) until exhaustion.



The submaximal and maximal deoxy[Hb+Mb] values (in %) at the test and the retest are presented in figure 2 and figure 3. No significant differences were found for the mean values of the submaximal increase (in %) in deoxy[Hb+Mb] for 20% ($p=0.419$), 30% ($p=0.331$), 40%

($p=0.947$), 50% ($p=0.12$) and 60% MVC ($p=0.894$). The ICC and p -values are presented in table 1 for all submaximal and maximal increases relative to the occlusion value. In addition no significant differences were found ($p=0.907$) for the increase in deoxy[Hb+Mb] (in %) during the final bout (i.e. 69.5%-73%) between the test ($n=45.6 \pm 16.7\%$) and the retest ($n=44.9 \pm 17\%$). For the maximal increase in oxygen extraction during the incremental cyclic contractions protocol (ICCP) an ICC of $r=0.873$ ($p<0.001$) was found between the test and the retest values (see figure 4). The pattern (in %) at these intensities at the test and the retest is given in figure 2. Following Landis and Koch's (1977) benchmarks the reliability for the submaximal intensities is slight for an intensity of 30%, fair for the intensities of 20% and 40% MVC and moderate for the intensities of 50% and 60%. For the maximal O_2 -extraction (in %) the reliability of the measurements is almost perfect (Landis and Koch 1977).

Table 1: ICC and p -values for the deoxy[Hb+Mb]-values relative to the maximum value (during arterial occlusion) at the submaximal and maximal intensities.

	20%MVC	30%MVC	40%MVC	50%MVC	60%MVC	69.5%-73%
ICC	0,321	0,025	0,334	0,466	0,553	0,873
P-value	0,078	0,457	0,069	0,017	0,005	<0,001

Fig. 2: Mean submaximal percentual deoxy[Hb+Mb] values at different intensities (% MVC) at the test and the retest for all 20 subjects. Trends of significant ICC's ($\$ \leq 0.1$) and ICC's at the significance levels of $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$ are presented between the test and retest.

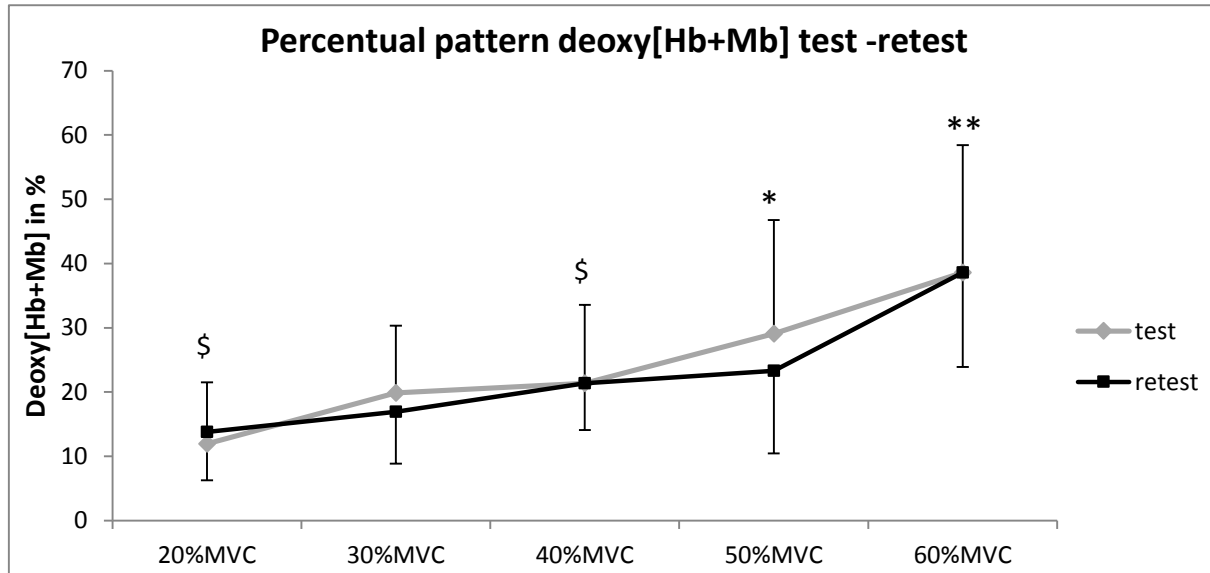


Fig. 3: Mean maximal percentual deoxy[Hb+Mb] values at the maximal intensity at the test and retest for all 20 subjects. ICC at the significance level $***p \leq 0.001$ is presented between the test and the retest.

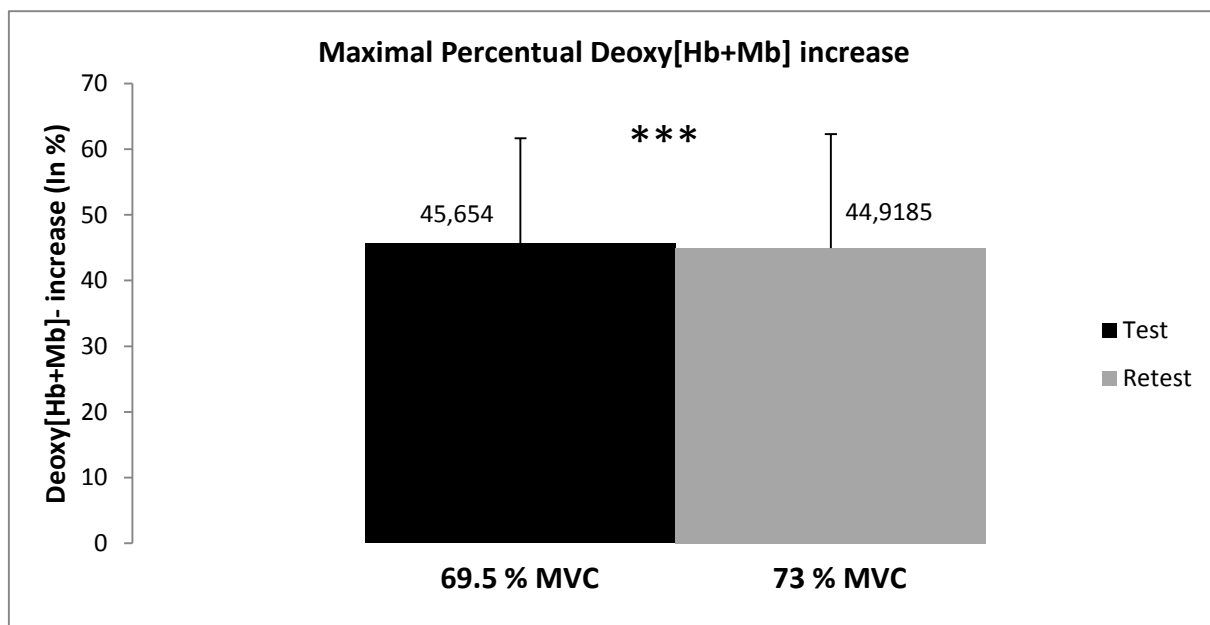
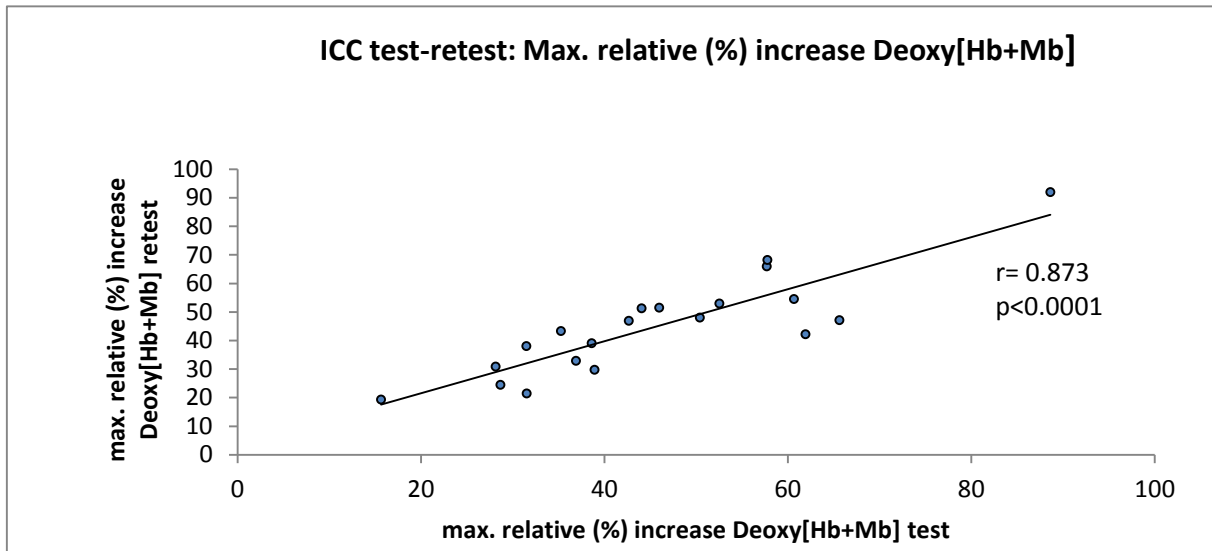


Fig. 4: The ICC between the maximal deoxy[Hb+Mb] increase (in %) between the test and the retest.

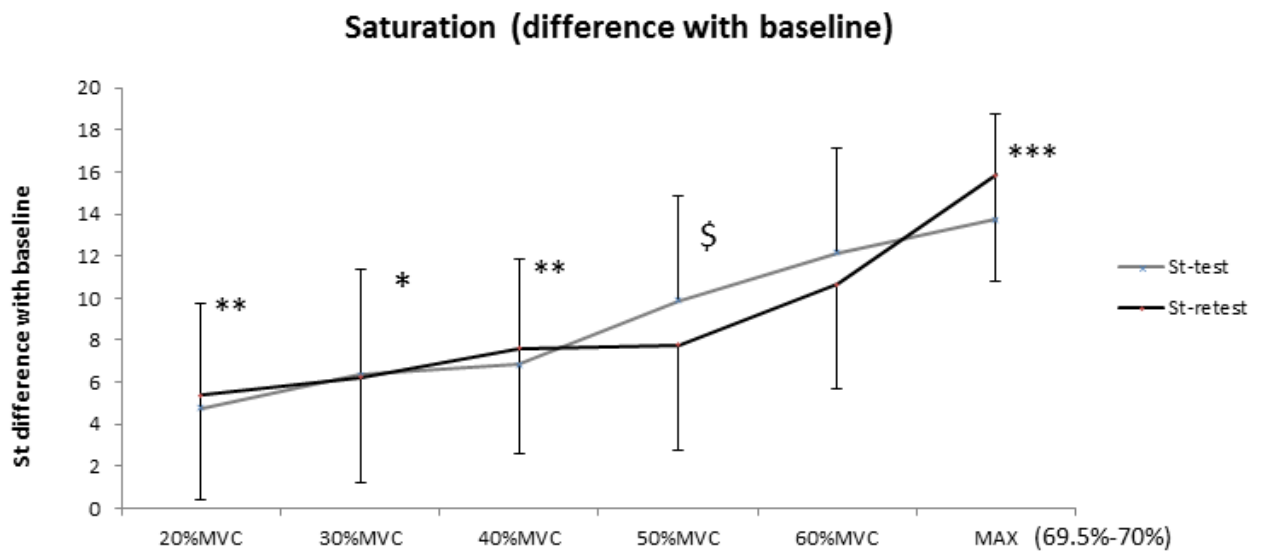


The submaximal and maximal SmO_2 -values (Subtracted from baseline) at the test and the retest are presented in figure 5. No significant differences were found for the SmO_2 -values between the test and the retest. The ICC and p-values are presented in table 2 for all submaximal and maximal decreases in SmO_2 . Following Landis and Koch's (1977) benchmarks the reliability for the submaximal intensities is fair for the intensities of 30%, 50% and 60% MVC, moderate for the intensity of 40% and substantial for 20%MVC. For the maximal SmO_2 the reliability of the measurements is substantial (Landis and Koch 1977).

Table 2: ICC and p-values for the SmO₂ (differences between the lowest 10-s mean values and 30-s baseline values) at the submaximal and maximal intensities.

	20%MVC	30%MVC	40%MVC	50%MVC	60%MVC	69.5%-73%
ICC	0,602	0,39	0,579	0,345	0,22	0,774
P-value	0,002	0,04	0,003	0,063	0,171	<0,001

Fig. 5: Mean submaximal and maximal absolute SmO₂-values (subtracted from baseline values) (St) at different intensities (% MVC) at the test and the retest for all 20 subjects. Trends of significant ICC's ($\$ \leq 0.1$) and ICC's at the significance levels of $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$ are presented between the test and retest.



DISCUSSION

To our knowledge, this is the first study reporting a significant test-retest ICC for the deoxy[Hb+Mb] and SmO₂ response in forearm muscles with near infrared spectroscopy (NIRS). Other researchers have concluded that NIRS is a reliable tool for measuring haemodynamics, but specific to large muscles (limb) or brain (Kell et al., 2004; Pereira et al., 2005). The main finding of our study was that according to our protocol NIRS is a reliable tool for measuring deoxygenation in small muscles. The deoxy[Hb+Mb] was studied, to gain a better insight into peripheral extraction mechanisms. The only calculation in this study was to compare the increases of deoxy[Hb+Mb] relative to the maximal deoxygenation. It is important to mention that this arterial occlusion was very important in our protocol as a maximal deoxygenation value. The difference between our protocol and other studies is that the occlusion was maintained until a steady state of the deoxy[Hb+Mb] was reached. Our underlying rationale for the use of this protocol is that because it is expressed relatively, it can be used for comparison between individuals. This is because other factors (i.e. adipose tissue) can influence the absolute value. In our opinion an arterial occlusion standardized in time is not effective because the time to reach a steady state differs greatly between individuals. When an arterial occlusion is induced, muscle blood perfusion (Q_m) of the forearm is stopped. As a consequence local available oxygen is consumed to respond to the metabolic demands of the forearm muscles at that moment. The increased O₂-extraction from the blood into the muscle can be observed in our deoxy[Hb+Mb]- output because more oxygen is released from the haemoglobin molecules. The maximal increase (in %) in the protocol was the most important output from this test: a high value represents in fact a high extraction rate of oxygen from the blood into the muscle.

In the research of Van Beekvelt et al. (2002) the occlusion was imposed for 45-s and the muscular VO₂ (mVO₂) was calculated. The protocol and the outcome that was examined were very different in this study compared to the study of Van Beekvelt et al. (2002). In this research our interest was not to calculate the mVO₂, but to determine the oxygen extraction in the forearm muscles. Van Beekvelt et al. (2002) used the 45-s arterial occlusion to calculate the mVO₂. In our study, as mentioned previously, the occlusion had a different purpose: as a

maximal deoxy[Hb+Mb]-value to analyze the other data relative to, and not as a calculation parameter. Despite the fact that both studies investigated the reliability of NIRS on forearm muscles, both outcome and methods were very different.

Grassi et al. (2006) were the only researchers that used a similar protocol and outcome to compare the oxygen extraction between a mitochondrial myopathy and a control population. The fact that this protocol was executed on a cycle ergometer was a major difference compared to our study. The deoxy[Hb+Mb] increases in the incremental cycloergometric test were also examined relative to the maximal steady state deoxy[Hb+Mb]. Grassi et al. (2006) concluded that the NIRS showed an impaired oxygen extraction in a mitochondrial myopathy and a McArdle population. Our method is very easy to execute and could be useful to study oxygen extraction reliably in a population with a high exercise intolerance as well as in a healthy population.

The fact that substantial reliability was found considering the oxygen saturation supports the fact that reliable results were found with the deoxy[Hb+Mb]-values. The SmO_2 is a calculation of $oxy[Hb+Mb] / Tot[Hb+Mb]$ or $oxy[Hb+Mb] / (oxy[Hb+Mb]+deoxy[Hb+Mb])$. Although the Deoxy[Hb+Mb] output is the most important as a measure for microvascular oxygen extraction, it is useful to compare the SmO_2 as well. Physiological calibration using arterial occlusion is not necessary. The relation between the SmO_2 and the deoxy[Hb+Mb] values are inverse. When the deoxy[Hb+Mb] values are increasing, the SmO_2 -values decrease. The fact that the decrease of SmO_2 shows similar reliability with the deoxy[Hb+Mb] values supports the fact that the measurements of forearm oxygenation during this protocol are reliable.

To our knowledge Van Beekvelt et al. (2002) is the only study that addressed the reliability of NIRS on the forearm muscles, using a different protocol and outcome. Using this protocol, this is the first study that finds a highly reliable outcome for measuring the deoxy[Hb+Mb] relatively and the SmO_2 . In conclusion we propose that this protocol can be used to compare muscle deoxygenation and oxygen saturation between different subjects.

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STUDY 2

POSSIBLE INFLUENCES ON THE INTERPRETATION OF FUNCTIONAL DOMAIN (FD) NEAR INFRARED SPECTROSCOPY (NIRS): AN EXPLORATIVE STUDY

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ABSTRACT

Objectives: The influence of subcutaneous adipose tissue (ATT) and oxygen (O₂) delivery has been poorly defined in frequency domain (FD) near infrared spectroscopy (NIRS). Therefore, the aim of this study was to investigate the possible influence of these variables on all FD NIRS responses using a reliable protocol. Moreover, these influences were also investigated when using relative oxy- and deoxyhaemoglobin and –myoglobin (oxy[Hb+Mb] and deoxy[Hb+Mb]) values (in %).

Methodology: In part 1 of this study, a regression analysis was carried out for ATT and maximal/minimum oxy[Hb+Mb], deoxy[Hb+Mb], oxygen saturation (SmO₂) and total haemoglobin (totHb) amplitudes during an incremental cyclic contraction protocol (ICCP) in a group of 45 subjects. Moreover, the same analysis was carried out between subcutaneous ATT and the relative oxy- and deoxy[Hb+Mb] values (in percentage). In part 2 of this study, a regression analysis was performed for peak forearm blood flow (FBF) during ICCP and the absolute and relative NIRS values in a group of 37 subjects.

Results:

Part 1: Significant exponential correlation coefficients were found between ATT and deoxy[Hb+Mb] ($r=0.53$; $p<0.001$), oxy[Hb+Mb] ($r=0.57$; $p<0.001$) and SmO₂ amplitudes ($r=0.57$; $p<0.001$). No significant relations were found between ATT and relative oxy[Hb+Mb] ($r=0.37$; $p=0.07$) and deoxy[Hb+Mb] ($r=0.09$; $p=0.82$).

Part 2: Significant positive correlation coefficients were found between force at exhaustion and maximal FBF ($r=0.66$; $p<0.001$), maximal differences in deoxy[Hb+Mb] ($r=0.353$; $p=0.032$) and totHb ($r=0.512$; $p=0.002$) while no significant correlation coefficients were found between these maximal force values and maximal differences in oxy[Hb+Mb] ($r=-0.267$; $p=0.111$) and SmO₂ ($r=-0.267$; $p=0.111$). Significant linear correlation coefficients were found between FBF and deoxy[Hb+Mb] ($r=0.51$; $p=0.001$), oxy[Hb+Mb] ($r=-0.50$; $p=0.001$), SmO₂ ($r=-0.54$;

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$p=0.001$) and totHb amplitude ($r=0.61$; $p<0.001$). No significant correlations were found when using relative oxy[Hb+Mb] ($r=-0.01$; $p=0.957$) and deoxy[Hb+Mb] ($r=-0.02$; $p=0.895$).

Conclusion: Based on these findings, caution is advised when using NIRS values, as subcutaneous ATT and O₂ delivery significantly influence NIRS measurements. To eliminate these influences, use of relative deoxy[Hb+Mb] is advised, especially in clinical settings and/or in people with a higher subcutaneous ATT layer.

INTRODUCTION

Near infrared spectroscopy (NIRS) is a frequently used method to evaluate muscle oxidative metabolism and haemodynamics *in vivo* at rest and during exercise (Ferrari et al., 2011; Hamaoka et al., 2011). This noninvasive optical technique is based on the penetration of NIR light throughout biological tissue such as skin, adipose tissue and muscle where it is absorbed by iron or copper centers of haemoglobin, myoglobin and mitochondrial cytochrome-c oxidase (Piantadosi and Duhaylongsod, 1994). In this way, the concentration of oxygenated and deoxygenated haemo- and myoglobin (oxy[Hb+Mb] and deoxy[Hb+Mb]) can be determined with NIRS. In general, there are three types of NIRS, i.e. continuous wave (CW), frequency domain (FD) and time domain (TD) NIRS. The difference between CW spectroscopy, which is the most widely used oximetry approach, and FD spectroscopy, is the technical complexity. Moreover, only the FD technique offers the absolute characterization of the tissue optical properties from which it is possible to obtain absolute concentration values of oxy[Hb+Mb] and deoxy[Hb+Mb] (Ferrari et al., 2011). The validity of this technique has been established in some studies (Mancini et al., 1994; Hamaoka et al., 2000; Wilson et al., 1989; Esaki et al., 2005) but questioned in other studies (Costes et al., 1996; MacDonald et al., 1999) comparing muscle deoxygenation with venous blood oxygen (de-)saturation measurements or phosphorous magnetic resonance spectroscopy (P-MRS) (Sako et al., 2001).

In general, two major issues need consideration when using NIRS, i.e. subcutaneous adipose tissue thickness (ATT) and changes in blood volume (Hamaoka et al., 2011). Because the majority of earlier studies investigating this matter, used the CW NIRS technique, these issues are poorly/less well defined when using FD spectroscopy. The possible influence of subcutaneous ATT is an important factor determining the NIRS signal (Van Beekvelt et al., 2001; Messere and Roatta, 2013; Grieger et al., 2013; Cooper et al., 2010). Ferrari et al. (2011) stated that the NIRS technique is therefore constrained to lean subjects, strongly limiting the clinical application of this tool. Although some correction algorithms have been developed (Bowen et al., 2013; Niwiyama et al., 2000) to measure muscle oxygenation independent from ATT, limited data are available regarding varying interventions such as arterial occlusion to counteract these influences (Hamaoka et al., 2011). A second point that merits consideration

is the influence of O₂ supply on NIRS values as little is known about this issue. When focusing on microvascular O₂ extraction, some investigators prefer to report deoxy[Hb+Mb] as it seems to be the best indicator of O₂ extraction when total haemoglobin (totHb) remains stable (Delorey et al., 2004; Koga et al., 2011). Other investigators however, prefer to use O₂ saturation (SmO₂) as deoxy[Hb+Mb] is sensitive to blood volume changes during exercise (Hamaoka et al., 2011). All these previous studies did, however, investigate this matter using CW modalities. The possible influence of ATT and changes in blood volume on these FD measurements are, to our knowledge, not known.

Due to the fact that possible influence of subcutaneous ATT and changes in blood volume are poorly defined, especially on FD NIRS, the purpose of this study was twofold. The first aim was to investigate the influence of subcutaneous ATT on all FD NIRS responses. Given the fact that we carried out limb ischemia as a physiological calibration in our protocol (Celie et al., 2012), we also investigated the influence of ATT on the relative oxy[Hb+Mb]- and deoxy[Hb+Mb] values (i.e. maximal responses during exercise towards the arterial occlusion (AO) maximal value). The second aim was to explore the relation between limb O₂ delivery and FD NIRS values to gain more insight about the influence of limb blood flow on all NIRS responses during exercise. According to earlier studies, investigating the relation between ATT and CW NIRS, we hypothesized that absolute FD NIRS responses would decrease with increasing subcutaneous ATT. Also, we hypothesized that increasing limb O₂ delivery would influence the FD NIRS responses during exercise. However, when using relative oxy[Hb+Mb] and deoxy[Hb+Mb] values, we hypothesized that these influences could be eliminated.

METHODS

Subjects

In part 1 of this study, the relation between subcutaneous ATT and the NIRS responses was studied in healthy subjects (21♀, 24♂). The mean age, body weight and height was respectively 28 ± 11 years; 73.5 ± 13.9 kg and 1.76 ± 0.1 m (mean values \pm SD).

In part 2 of this study, the relation between forearm blood flow (FBF) and the NIRS responses was studied in healthy subjects (14♀, 23♂). The mean age, body weight and height was respectively 26 ± 6 years; 67.7 ± 11.4 kg and 1.76 ± 0.09 m. The subjects were fully informed of the study protocol and of any risk associated with the experiments before giving their written consent for participation. The study was approved by the ethical committee at the Ghent University Hospital. Procedures were in accordance with the recommendations of the Helsinki Declaration.

Study design and protocol

In part 1 of the study, before the start of the incremental cyclic contraction protocol (ICCP)²⁰ maximal voluntary contraction (MVC) force (expressed in Newton (N)) in the dominant arm was determined using a hydraulic handgrip dynamometer (Saehan corporation, Masan, South-Korea) for all subjects (best of three attempts). Determination of the MVC force was performed without warming up and they had to sustain their maximum force for at least 2 sec. Between every attempt a 5 minute resting period was included. Then the NIRS probe was placed on the M. flexor carpi ulnaris and the M. flexor digitorum superficialis, where subcutaneous ATT was measured using a skinfold caliper (Holtain Ltd., Crymmych, U.K.). After the placement of the NIRS probe on the muscles, a pneumatic cuff was placed around the ipsilateral upper arm. All subjects performed the complete exercise protocol in supine position. This protocol started with a forearm arterial occlusion by inflating the cuff to 260mmHg. The duration of the occlusion was not specified as it was executed until a steady state in deoxy[Hb+Mb] was reached. However, the mean duration of the arterial occlusion in

this study was 420 ± 112 sec. After the occlusion, there was a resting period before baseline conditions were restored and then the ICCP started. The ICCP consisted of 2 minute periods during which incremental cyclic contractions were performed at $\frac{1}{2}$ Hz (1 second contraction, 1 second relaxation), at different intensities of maximal voluntary contraction (% MVC). The contraction periods were separated by a 60-s rest period. The work intensity started at 20% MVC and increased by 10% MVC each step. This protocol was executed until exhaustion was reached and the subjects were no longer able to produce the required force. A typical NIRS output of one subject during this ICCP is presented in fig. 1. The duration of this test procedure was about 1h and the reliability of this protocol has been established in a previous study (Celie et al., 2012).

Additional to the experimental procedure of part 1 of the study, brachial artery Doppler US measurements (on the forearm muscles) were executed in part 2 of the study to determine FBF. As such, baseline FBF values were recorded for all subjects preceding the exercise test and during the ICCP.

Measurements

Near Infrared Spectroscopy (NIRS)

Skeletal muscle tissue oxygenation was measured with a FD near infrared spectroscopy (NIRS) system (Oxiplex TS, ISS, Champaign, Illinois, USA). This system is based on an infrared light absorption method, where near-infrared light is emitted at different wavelengths. The NIRS-probe consisted of eight light-emitting diodes operating at two wavelengths, 690 and 830nm, and one detector fibre bundle (source-detector distance = 2.0 - 3.5cm). The deoxy[Hb+Mb] data were stored at a frequency of 25Hz and afterwards digitally averaged into 1s-values. The probe was positioned longitudinally on the M. flexor carpi ulnaris and M. flexor digitorum superficialis of the dominant arm and secured with Velcro straps. Pen marks were made on the skin to detect probe displacement during the exercise.

Doppler Ultrasound

In part 2 of the study, the ultrasound Doppler system (Vivid 7, GE Vingmed Ultrasound, Horten, Norway) was used to measure FBF which is the preferred method during exercise compared to plethysmography (Casey et al., 2008). A M12L linear probe was used to image the brachial artery. The brachial artery was insonated approximately midway between the antecubital and axillary regions, medial to the biceps brachii. Brachial arterial diameter (BAD) was measured during peak systolic momentum at a perpendicular angle along the central axis of the scanned area (2D longitudinal view) to optimize spatial resolution. Velocity measurements were obtained from angle-corrected longitudinal spectral Doppler images. The angle between the direction of blood flow and the direction of the ultrasound beam was kept at ≤ 60 degrees. Also, a velocity-time integral was used to average 10 cardiac cycles for blood flow velocity measurements. Blood flow was calculated using BAD (cm) and mean blood velocity (v_{mean} cm/sec):

$$\text{Blood flow (ml min}^{-1}\text{)} = v_{\text{mean}}(\text{cm s}^{-1}) \{ \pi (\text{vessel diameter (cm)} / 2)^2 * 60 (\text{s min}^{-1})$$

Data analysis

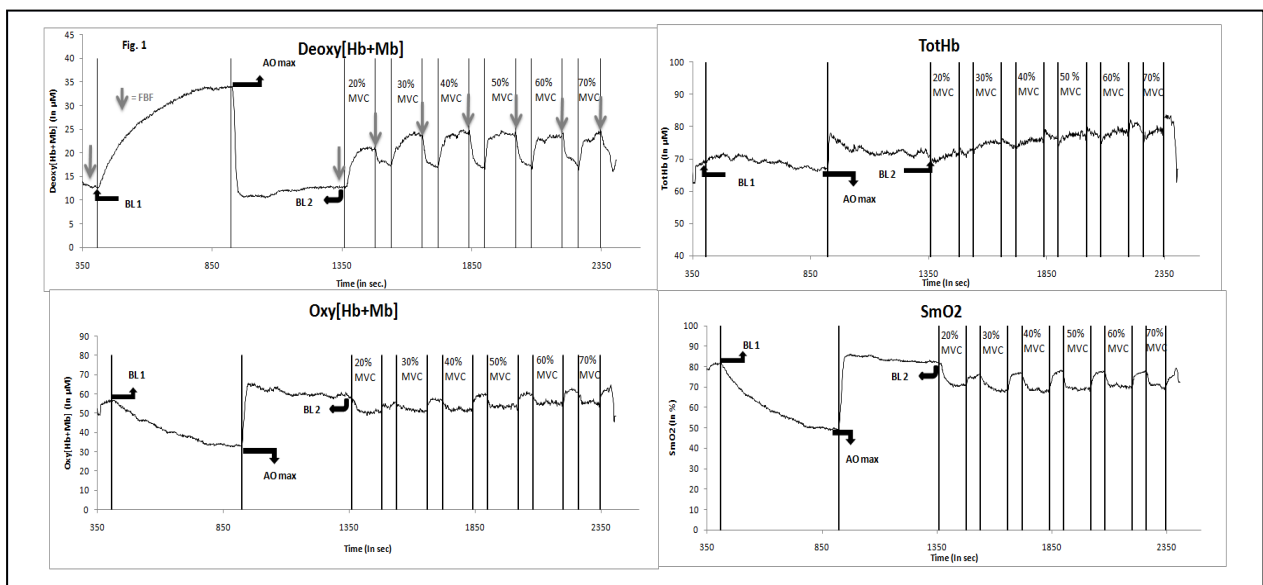
In part 1 and 2 of the study, we analyzed the mean values of oxy[Hb+Mb], deoxy[Hb+Mb], SmO₂ and totHb using a smoothing procedure in which the 10-s mean values were determined by means of a moving average. At every work bout during the ICCP, the amplitude of the NIRS-response was calculated by subtracting the baseline value (i.e., the mean 30s value prior to the start of the ICCP) from the steady state values during the exercise bout (i.e., the highest 10s average). For the statistical analysis we only included the maximal amplitudes of the responses obtained during the completed work bout with the highest intensity. Because oxy[Hb+Mb] and SmO₂ are decreasing during exercise towards baseline resting values, these are negative values. In Figure 1, typical oxy[Hb+Mb], deoxy[Hb+Mb], SmO₂ and totHb values are presented during arterial occlusion and ICCP. In addition to the amplitude of these values, the relative responses of oxy[Hb+Mb] and deoxy[Hb+Mb] were calculated by expressing the

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maximal absolute amplitude during ICCP relative to the amplitude from the arterial occlusion (i.e. difference between baseline values and highest 30 s mean values during AO). As such, we used these values in percentage for statistical analysis with subcutaneous ATT (Celie et al., 2012).

In part 2 of the study, FBF (expressed in ml min^{-1}) was measured preceding ICCP and after every incremental step (% MVC) in the protocol. ICCP had FBF baseline values subtracted so as to calculate the response to exercise compared to resting conditions. The maximal difference during ICCP (peak FBF- baseline FBF) were used for statistical analysis with the oxy[Hb+Mb], deoxy[Hb+Mb], totHb and SmO₂ amplitudes and the relative oxy[Hb+Mb] and deoxy[Hb+Mb] values, as described previously. In Figure 1, an individual pattern is presented whereas a schematic summary is given of all measurements.

Fig. 1 Example of a typical deoxy[Hb+Mb], oxy[Hb+Mb], totHb and SmO₂ output of one subject during the incremental cyclic contractions protocol (ICCP). All measurements (e.g. forearm blood flow (FBF) in study 2) are presented schematically. In study 2, FBF measurements were always combined with brachial artery diameter (BAD) measurements.



Statistical analysis

In part 1 of the study, a regression analysis was performed for ATT and maximal/minimal oxy[Hb+Mb], deoxy[Hb+Mb], SmO₂ and totHb amplitudes calculating Pearson's R square. Also, a regression analysis was used to investigate the relationship between ATT and the relative maximal/minimal oxy[Hb+Mb] and deoxy[Hb+Mb] values (in %). When statistical significance was observed, a best fit analysis was performed. This was executed using Sigmaplot 10 with SigmasStat 3.5 software (Systat Software Inc., San José, USA) to find the best fit (between) model, a linear or an exponential/logarithmic model.

In part 2 of the study, the same statistical analysis was executed to study the relationship between the force at the maximal reached intensity during ICCP (%MVC), FBF (O₂ delivery) and the NIRS responses. Significance was set at P<0.05. Statistical computations were performed using SPSS® software (version 18; SPSS Lead Technologies Inc., Chicago, IL, USA). All data are presented as mean ± SD.

RESULTS

1. Subcutaneous ATT

The best fit between subcutaneous ATT and all significant NIRS responses was an exponential/logarithmic fit ($Y = Y_0 + a(1 - e^{-(X-X_0)/b})$). All results of this best fit model analysis are presented in table 1. A significant exponential/logarithmic correlation coefficient was found between subcutaneous ATT ($\mu=6.5 \pm 3.4$ mm) and maximal amplitude in absolute deoxy[Hb+Mb] ($\mu=36.1 \pm 17.7$ μ M) ($r=0.53$; $p<0.001$), oxy[Hb+Mb] ($\mu=-20.2 \pm 22.5$ μ M) ($r=0.57$; $p<0.001$) and SmO₂ ($\mu=-18.2 \pm 13.7$ %) ($r=0.57$; $p<0.001$). No significant relation was found between ATT and maximal difference in totHb ($\mu=33.2 \pm 22.4$ μ M) ($r=-0.34$; $p=0.3$). All correlations relating these NIRS responses to subcutaneous ATT are presented in Figure 2. When using relative oxy[Hb+Mb] ($\mu=-33.5 \pm 30.1$ %) ($r=0.37$, $p=0.07$) or deoxy[Hb+Mb] ($\mu=55.3 \pm 20.3$ %) ($r=0.10$; $p=0.82$) values (in %) towards the arterial occlusion maximal values, no

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significant relation was found with subcutaneous ATT. These correlation coefficients are added in Figure 3.

Table 1: Results of the ‘best fit model’ for a linear and an exponential/logarithmic model (SEE= Standard Error of estimate; RSS= Residual Sum of Squares):

		R-Square	SEE	RSS	F-value	p-value
Deoxy[Hb+Mb]	Linear	0.22	33.1	48205	12.2	0.01
	Exponential	0.28	31.8	44509	16.9	0.0002
Oxy[Hb+Mb]	Linear	0.22	20.1	16613	11.7	0.0014
	Logarithmic	0.28	19.3	15304	16.2	0.0002
SmO ₂	Linear	0.25	10.7	4807	14.4	0.0005
	Logarithmic	0.30	10.2	4509	18.6	<0.0001

Fig. 2: Correlation coefficients between subcutaneous ATT and maximal oxy[Hb+Mb], deoxy[Hb+Mb], totHb and SmO₂ responses. Pearson’s R and p- values are included as well as the intercept values where significant.

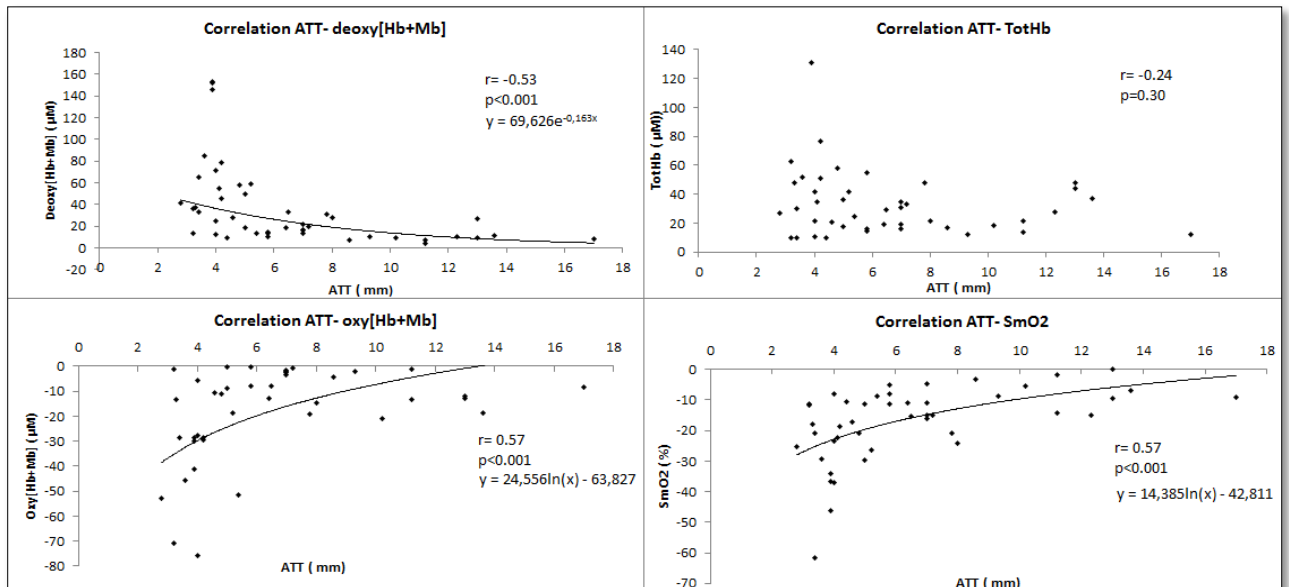
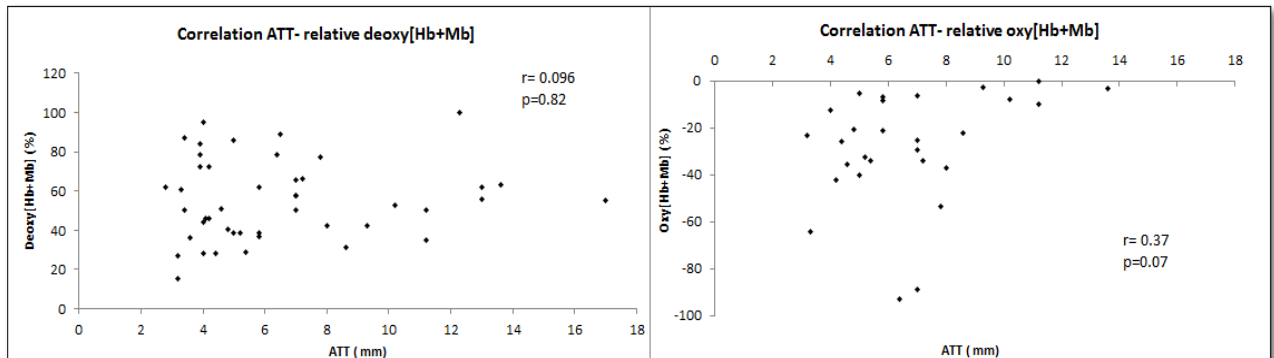


Fig. 3: Correlation between subcutaneous ATT and maximal relative deoxy[Hb+Mb] and oxy[Hb+Mb]. Pearson's correlation coefficients and p-values are included.



2. Maximal voluntary contraction (MVC) force and forearm blood flow (FBF)

The mean maximal force output at exhaustion for the entire population was respectively $326 \pm 107\text{N}$, which was at a mean intensity (\pm SD) of $65 \pm 10\%$ MVC. Significant positive correlation coefficients were found between the force, reached at the maximal intensity and maximal FBF ($\mu=419.8 \pm 245\text{ ml/min}$) ($r=0.66$; $p<0.001$), maximal differences in deoxy[Hb+Mb] ($\mu=82 \pm 73.8\ \mu\text{M}$) ($r=0.353$; $p=0.032$) and maximal differences in totHb ($\mu=296.4 \pm 199\ \mu\text{M}$) ($r=0.512$; $p=0.002$) while no significant correlation coefficients were found between this maximal force and maximal differences in oxy[Hb+Mb] ($\mu=-58.6 \pm 53.9\ \mu\text{M}$) ($r=-0.267$; $p=0.111$) and SmO_2 ($\mu=-29.7 \pm 18.8\%$) ($r=-0.267$; $p=0.111$). Also, no significant relation between maximal force at exhaustion and the relative values (towards arterial occlusion maximum values) of deoxy[Hb+Mb] ($\mu=51.8 \pm 25.1\%$) ($r=-0.20$; $p=0.231$) and oxy[Hb+Mb] ($\mu=-35.9 \pm 20.9\%$) ($r=-0.154$; $p=0.369$) were found.

Significant positive correlation coefficients were found between maximal FBF and maximal amplitudes in deoxy[Hb+Mb] ($r=0.511$; $p=0.001$) and totHb ($r=0.609$; $p<0.001$) while significant negative correlation coefficients were found between maximal FBF and maximal amplitudes in oxy[Hb+Mb] ($r=-0.503$; $p=0.001$) and SmO_2 ($r=-0.537$; $p=0.001$). All correlations relating NIRS amplitudes to FBF are presented in Figure 4. When using relative deoxy[Hb+Mb], no significant relation was found with FBF ($r=-0.022$; $p=0.895$). This same result was found for

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relative oxy[Hb+Mb] values ($r=-0.009$, $p=0.957$). These correlation coefficients are shown in Figure 5.

Fig. 4: Correlation between maximal FBF and maximal/minimal oxy[Hb+Mb], deoxy[Hb+Mb], totHb and SmO₂ responses during ICCP. Pearson’s correlation coefficients and p- values are included as well as intercept values.

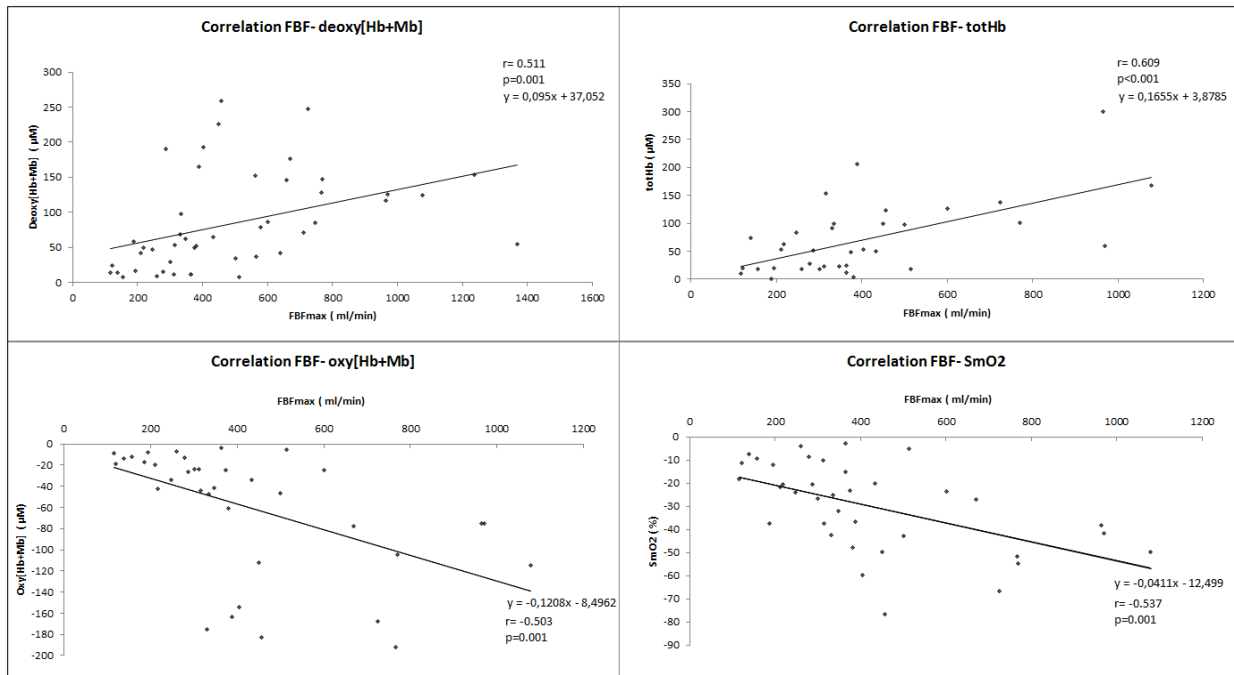
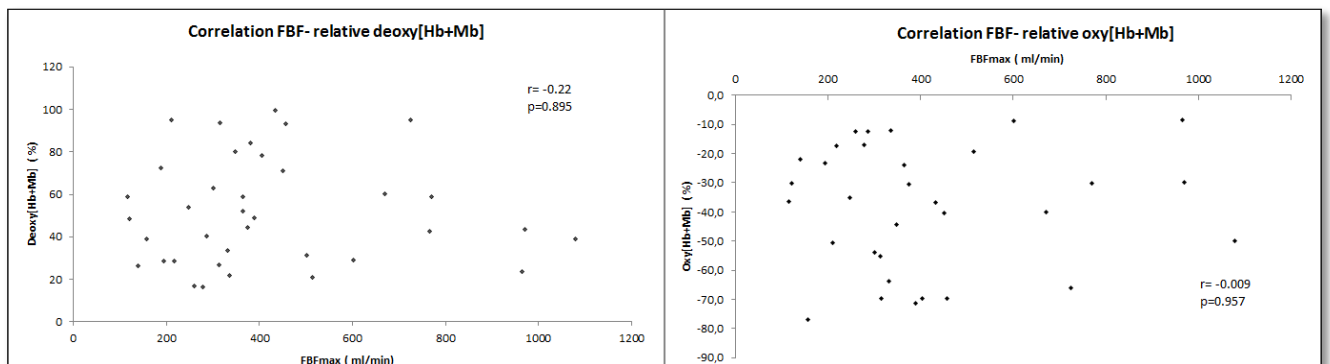


Fig. 5: Correlation between maximal FBF and relative maximal oxy[Hb+Mb] and deoxy[Hb+Mb]. Pearson’s correlation coefficients and p- values are included.



DISCUSSION

In the present study, the influence of ATT on the responses of a frequency domain (FD) NIRS was investigated. It was observed that the maximal oxy[Hb+Mb], deoxy[Hb+Mb] and SmO₂ responses to an incremental handgrip test were related to the thickness of the skinfold at the location of the probe, indicating that ATT might blunt the NIRS responses to exercise. However, when the oxy[Hb+Mb] and deoxy[Hb+Mb] responses were expressed relative to the amplitude values obtained during arterial occlusion, the impact of ATT was no longer present.

Additionally, to obtain insight into the interpretation of the absolute and relative NIRS responses, the relationship with FBF and MVC was studied. It was observed that MVC as well as FBF correlated significantly with the amplitude of the NIRS responses. Similar to the subcutaneous ATT influence on the NIRS signal, this relationship disappeared when using relative oxy[Hb+Mb] and deoxy[Hb+Mb].

1. Subcutaneous ATT

In part 1 of the study, a significant negative correlation coefficient was found between subcutaneous ATT layer and deoxy[Hb+Mb] which is an indication that maximal deoxy[Hb+Mb] amplitudes were attenuated with higher subcutaneous ATT. Additionally, significant positive correlation coefficients were found between ATT and oxy[Hb+Mb] and SmO₂ amplitudes in forearm muscles during ICCP. Hence, this positive relation means that both variables were also influenced by subcutaneous ATT during exercise. On the contrary, no significant relation was found with totHb which is, however, in contradiction with other studies showing a significant negative relation between totHb measurements and ATT (Van Beekvelt et al., 2001; Grieger et al., 2013; Bowen et al., 2013). This could be due to the fact that a different NIRS technology was used in these studies, whereas CW spatially resolved spectroscopy obtains a scaled absolute value of tHb (Ferrari et al., 2011). In this study FD NIRS was used, where tHb values are derived from the absolute concentration values of oxy[Hb+Mb] and deoxy[Hb+Mb] (Ferrari et al., 2011). Another plausible explanation for these divergent findings could be the fact that the correlation between ATT and totHb is significantly

weaker in forearm muscles compared to leg muscles, probably due to a smaller muscle tissue thickness (Grieger et al., 2013). In contrast to the study of Grieger et al. (2013), we found a significant correlation coefficient between SmO_2 and ATT in forearm muscles. These opposite results could be clarified by the fact that we measured minimal saturation values during exercise, whereas Grieger et al. (2013) used resting baseline values. Considering the significant correlation coefficients for all NIRS responses except totHb, it can be stated that a higher subcutaneous ATT layer resulted in an attenuated absolute oxy[Hb+Mb], deoxy[Hb+Mb] and SmO_2 signal. Taking into account these results, the statement of Ferrari et al. (2011) saying that the NIRS technique is strongly constrained to lean subjects, could be confirmed. A possible explanation for the blunting influence of ATT on NIRS responses is the reduced absorption and/or scattering in muscle tissue (Wolf et al., 2003). This latter investigation has shown that, whereas ATT was between 6 and 14 mm, the NIRS absorption coefficient (μ_a) quickly decreased while the NIRS scattering coefficient (μ_s) remained constant (Wolf et al., 2003). In this study however, mean subcutaneous ATT (\pm SD) for all subjects was 6.5 ± 3.4 mm. Based on the conclusions drawn in Wolf et al. (2003), this indicates that the NIRS absorption coefficient (μ_a) was decreased in this study. This of course limits the clinical application of this tool in diverse patient populations. Therefore, it is interesting that the relative values of oxy[Hb+Mb] and deoxy[Hb+Mb] did not correlate significantly with ATT values. As such, when using relative oxy[hb+Mb] and deoxy[Hb+Mb] values, subcutaneous ATT does not influence the outcome and so, these relative values could be used in clinical populations with a higher subcutaneous ATT. In this context, it is important to highlight the role of arterial occlusion as a physiological calibration. When validating the NIRS technique for measuring microvascular O_2 extraction, in some studies (Mancini et al., 1994; Hamaoka et al., 2000; Wilson et al., 1989; Esaki et al., 2005) an arterial occlusion was performed in the studied limb as a physiological calibration. In the other studies where NIRS validation was not confirmed to measure microvascular O_2 extraction (Costes et al., 1996; MacDonald et al., 1999), no physiological calibration was carried out. Consequently, the use of absolute NIRS values, without arterial occlusion to measure O_2 extraction, seems not valid. When using FD NIRS in clinical populations, it is strongly advised to use the relative values using a physiological calibration. As such, relative deoxy[Hb+Mb] values (in percentage) could be an interesting parameter of microvascular O_2 extraction rate in different populations.

2. Forearm blood flow (FBF)

Another issue concerning FD NIRS use is the possible influence of exercise intensity (MVC) and limb blood flow (O_2 delivery) on all responses. In part 2 of this study, it has been shown that MVC correlated significantly with FBF and all absolute NIRS responses. Moreover, forearm blood flow (FBF) correlated significantly with the amplitude of the NIRS responses. To discuss this issue it is important to emphasize that O_2 delivery should differentially influence the different NIRS responses. Only one preceding study focused on the relation between O_2 delivery and NIRS values during exercise (Fadel et al., 2004). In accordance with the study of Fadel et al. (2004), totHb correlated significantly positive with FBF during rhythmic handgrip exercise. TotHb has frequently been used in earlier studies as an indicator of blood volume/flow changes (Ferrari et al., 2011; Cettolo et al., 2007). The results of this study confirms the appropriate use of totHb as a marker for changes in muscle blood volume/flow. Furthermore, for the interpretation of our results, it is important to emphasize that the individual intensity during this ICCP depends on the MVC. When individual MVC values are higher, O_2 demand at muscle level during ICCP will be proportionally increased as well. According to Fick's law, skeletal muscle blood flow (Q_m) and microvascular O_2 extraction ($\Delta a-v O_2$) will be higher during ICCP in subjects with a higher MVC in order to meet the increased O_2 demand. In our opinion it is obvious that higher MVC values can be associated closely with higher maximal FBF values. The linear increase in limb blood flow to match O_2 supply to O_2 demand has been demonstrated in leg muscles (Calbet et al., 2007; Mortensen et al., 2007; Richardson et al., 1995) and in forearm muscles (Green et al., 2005).

The rationale for this methodological paper concerning NIRS, is its possible application in clinical populations. In this context, the relationship between NIRS responses on the one hand and maximal FBF and MVC on the other hand is rather complicated. The absolute maximal values correlated significantly with maximal force at exhaustion and maximal FBF, whereas the relative oxy[Hb+Mb] and deoxy[Hb+Mb] did not. A higher force at exhaustion implicates an improved functional capacity of the muscle to contract at a higher load. The higher maximal FBF value is an indication of the improved functional capacity at muscle level, probably due to an increased capillary density and modifications in dilator function related to endothelial

change (Delp and Laughlin, 1997; Sinoway et al., 1986; Smolander, 1994; Snell et al., 1987). Whereas a maximal force at exhaustion during ICCP is higher, FBF values will be increased too in these individuals, probably to have an expanded passage of O₂, free fatty acids and glucose during sustained dynamic contractions. It could be possible that individuals with a higher MVC may have a higher amount of capillaries that influences the NIR penetration at muscle level. In these individuals, capillary blood flow will be increased at the site where deoxy[Hb+Mb] is measured. This higher amount of capillary O₂ supply at the site of the NIRS probe means that haemoglobin flux will be increased. As such, larger deoxy[Hb+Mb] increases will be possible during exercise compared to subjects with a lower haemoglobin flux at the measurement site. Consistent with this explanation, absolute deoxy[Hb+Mb] measurements will depend on intensity and capillary haemoglobin flux. It is however questionable, whether it is favorable to measure this improved functional capacity rather than clinical related peripheral problems in some pathologic populations. Therefore, it is interesting that the relationship between intensity (MVC) and O₂ delivery on the one hand and NIRS responses on the other hand disappears by using relative values. In our opinion, the use of absolute or relative oxy[Hb+Mb] and deoxy[Hb+Mb] depends on the experimental purpose of the study. When searching for functional adaptations in certain sports or other populations with a very low subcutaneous ATT, one could use absolute NIRS values. However, when testing clinical populations to detect certain muscle abnormalities, one should definitely use relative values because it excludes eventual differences in muscle oxygenation due to an improved functional capacity of the muscle.

In conclusion we can state that NIRS remains a very interesting and useful tool to study haemodynamics. However, it is very important to be aware of some practical and methodological issues when using this device. Subcutaneous ATT has a significant influence on all FD NIRS responses except totHb and FBF influences all FD NIRS responses. It appears that these influences are not different as described in other studies using CW spectroscopy. Moreover, based on these findings, caution could be advised to use NIRS values. To eliminate these influences, use of relative deoxy[Hb+Mb] could be strongly advised, especially in clinical settings and/or in people with a higher subcutaneous ATT layer.

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STUDY 3

FOREARM MUSCLE OXYGENATION DURING AND FOLLOWING ARTERIAL OCCLUSION IN HEALTHY SUBJECTS AND MITOCHONDRIAL MYOPATHY

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ABSTRACT

The aim was to study whether mitochondrial myopathy induces different oxygenation (deoxy[Hb+Mb] and oxy[Hb+Mb]) responses during and following arterial occlusion. In 10 mitochondrial myopathy patients (MMpatients) (age: 29 ± 7 years; body mass: 59.9 ± 15.7 kg; height: 166.2 ± 11.4 cm) and age- and gender-matched healthy subjects (age: 28 ± 9 years; body mass: 72.7 ± 16.9 kg; height: 174.4 ± 8.7 cm) arterial occlusion was performed by inflating a cuff to 240mmHg. Deoxy[Hb+Mb] and oxy[Hb+Mb] were registered during (AOoxy and AOdeoxy) and following (POdeoxy and POoxy) arterial occlusion. Amplitude of AOdeoxy did not differ ($p=0.47$) between MMpatients ($44.9 \pm 28.0 \mu\text{M}$) and healthy subjects ($38.6 \pm 22.8 \mu\text{M}$). The time constant of the exponential model was greater in MMpatients ($263.4 \pm 49.1\text{s}$ vs. $200.3 \pm 73.7\text{s}$, $p=0.03$). Following cuff release, in both populations a transient increase in total[Hb+Mb] was observed induced by different kinetics of POoxy and POdeoxy. The increase in POoxy (TD= $6.6 \pm 2.2\text{s}$ and $11.9 \pm 3.5\text{s}$; $\tau=3.8 \pm 1.4\text{s}$ and $6.4 \pm 2.9\text{s}$ for MMpatients and healthy subjects, respectively) was faster ($p<0.001$ for TD and τ) compared to the decrease in POdeoxy (TD= $13.2 \pm 3.6\text{s}$ and $26.5 \pm 4.6\text{s}$; $\tau=-6.2 \pm 2.2\text{s}$ and $-9.6 \pm 2.4\text{s}$ for MMpatients and healthy subjects, respectively). POoxy and POdeoxy showed faster kinetics ($p<0.001$ and $p<0.01$ for TD and τ , respectively) in MMpatients compared to healthy subjects. MMpatients display altered oxygenation responses during and following arterial occlusion reflecting pathology related changes in the relationship between muscle blood flow and oxygen uptake.

KEYWORDS: oxygenation, arterial occlusion, O₂ supply, O₂ extraction, mitochondrial myopathy

INTRODUCTION

The relationship between muscle blood flow (Q_m) and oxygen uptake (VO_{2m}) is an important determinant of exercise tolerance in healthy and patient populations. A thorough understanding of this relationship provides insight into cardiovascular and muscular adjustments to changes in metabolic demand. In healthy subjects a tight coupling between O_2 supply and O_2 demand has been observed, while in patient populations this relationship is often disturbed inherent to the pathologic condition either affecting the central (i.e., cardiovascular and pulmonary) and/or peripheral (i.e., muscle) response (Poole et al., 2011). In patients suffering from mitochondrial myopathy (MMpatients) one or more genetic defects impair the oxidative phosphorylation system (OXPHOS), resulting in a reduced capacity to increase muscle O_2 extraction during exercise and in this way disturbing the functioning of the aerobic metabolism. Furthermore, a hyperdynamic cardiovascular response has been shown as a compensation for the limited O_2 extraction exercise (Dandurand et al., 1995; Taivassalo et al., 2002).

Evaluation of the capacity to increase muscle O_2 extraction in patients suffering from MM can be performed directly by the invasive measurement of the arterio-venous O_2 difference ($C_{a-v}O_2$) across the exercising muscles (Linderholm et al., 1969; Taivassalo et al., 2002), or indirectly based on the Fick-principle with measurements of whole-body VO_2 and blood flow (Q) (Taivassalo et al., 2003). Since the validity of the non-invasive determination of Q during maximal exercise has been questioned (Warburton et al., 1999) and considering practical issues when using invasive methods, the need has emerged to develop new techniques to estimate the functional capacity for muscle O_2 extraction in MM patients. Near-infrared spectroscopy (NIRS) is a non-invasive technique that measures the concentration of oxygenated and deoxygenated haemoglobin and myoglobin (deoxy[Hb+Mb] and oxy[Hb+Mb]), thus providing information on the oxygenation at the muscle level. Especially deoxy[Hb+Mb] seems to be useful for functional evaluation in MMpatients since it has been shown that deoxy[Hb+Mb] can be considered as a reflection of microvascular O_2 extraction (Delorey et al., 2003; Grassi et al., 2003; Ferreira et al., 2005).

The hyperemic response following brachial artery occlusion at rest is often used to evaluate the relative health of the vasculature (Rossi et al., 2008; Suzuki et al., 2008; Takase et al., 2008; Schechter et al., 2009). Bopp et al. (2011) suggested that using NIRS during and following brachial occlusion might have significant (clinical) implications. This non-invasive technique can provide insight into the dynamic relationship between convective delivery of O₂ to the microvasculature and diffusion of O₂ from capillary to myocyte (i.e., the relationship between Q_m and VO_{2m}). Previously, this relationship has proven insightful for understanding the determinants of exercise tolerance in both healthy and patient populations, such as MMpatients (Poole et al., 2011).

Therefore, the purpose of the present study was to test if muscle oxygenation responses, measured with NIRS, during and following arterial occlusion at rest would differ between MMpatients and age- and gender-matched healthy subjects. We hypothesized that 1) MMpatients would display slower deoxygenation kinetics during arterial occlusion compared to healthy subject, reflecting an impaired aerobic capacity, and 2) the reoxygenation kinetics (oxy[Hb+Mb]) following arterial occlusion would be faster in Mmpatients, mimicking the hyperdynamic cardiovascular response to exercise seen in these patients (Dandurand et al., 1995; Taivassalo et al., 2002).

METHODS

Subjects

We studied two groups of subjects. The first group consisted of ten patients suffering from mitochondrial myopathy (MMpatients), based on biochemically findings. The second group consisted of ten age- and gender-matched untrained healthy subjects. The present study was approved by the ethics committee of the Ghent University Hospital. Prior to the start of the experiments, the subjects were fully informed of any risk associated with the experiments before giving their written consent for participation and all experiments were in accordance with the recommendations of the Helsinki Declaration. All patients were diagnosed as having MM based on the results of biochemical analysis in a skeletal muscle biopsy, using

spectrophotometrical assays (De Vriese et al., 2006), which remains the gold standard for diagnosis of mitochondrial diseases and by Blue Native Polyacrylamide Gel Electrophoresis (Van Coster et al., 2001).

Experimental protocol

On arrival at the laboratory the subjects had some time to adjust to the laboratory conditions. First the anthropometric characteristics (body weight and height) and the skinfold thickness at the location of the probe were registered. Afterwards the subjects were asked to lie down on a bed and they remained in supine position throughout the entire protocol. The NIRS-probe was positioned longitudinally on the M. flexor carpi and ulnaris and on the M. flexor digitorum superficialis of the right arm. Pen marks were made over the skin to detect movement of the probe during the experimental procedures. The NIRS-probe was secured with Velcro straps and a black blanket was placed over the probe to ensure that the ambient light could not penetrate the tissue and disturb the measurement. An inflatable cuff was placed on the upper right arm to induce the arterial occlusion. The subjects remained in this resting position for a few minutes until a stable baseline value at rest was obtained for the NIRS-parameters (deoxy[Hb+Mb] and oxy[Hb+Mb]). Then the cuff was quickly (within 3s) inflated to 240 mmHg in order to induce an arterial occlusion of the brachial artery obstructing the blood flow to the muscles of the lower arm (under the NIRS-probe). The arterial occlusion was maintained until deoxy[Hb+Mb] and oxy[Hb+Mb] reached a clear steady state without a specific pre-set time frame. The cuff was then released and deoxy[Hb+Mb] and oxy[Hb+Mb] was recorded for three minutes to follow the reoxygenation kinetics. All subjects were tested in an air-conditioned laboratory at 21° C.

Measurements

Throughout the arterial occlusion, muscle tissue oxygenation was measured with a near infrared spectroscopy (NIRS) system (Oxiplex TS, ISS, Champaign, USA). This system is based on an infrared light absorption method, where infrared light is emitted at different wavelengths. The NIRS-probe consisted of eight light-emitting diodes operating at

wavelengths 750 and 830 nm and one detector fibre bundle (source-detector distance = 2.0-3.5 cm). The deoxy[Hb+Mb] was stored at a frequency of 25 Hz and afterwards digitally averaged into 1s-values.

Data analysis

The 1 s-values of deoxy[Hb+Mb] and oxy[Hb+Mb] were plotted as a function of time. An exponential model (1) was fit to the deoxy[Hb+Mb] and oxy[Hb+Mb] response to the arterial occlusion (AOdeoxy and AOoxy).

$$Y(t) = Y_0 + A (1 - e^{-(t-TD)/\tau})$$

in which Y_0 is the baseline value (i.e., prior to the start of the arterial occlusion), A is the amplitude of the deoxy[Hb+Mb] and oxy[Hb+Mb] response, TD is the time delay and τ is the time constant.

Since Bopp et al. (2011) showed that the deoxy[Hb+Mb] and oxy[Hb+Mb] response immediately following arterial occlusion follow a sigmoid pattern, these response were also fit by means of a Gompertz sigmoid function.

$$Y(t) = Y_0 + Ae^{-e^{-(t-TD)/\tau}}$$

in which Y_0 represents the baseline value (i.e., the steady state level of deoxy[Hb+Mb] and oxy[Hb+Mb] to the arterial occlusion), A is the amplitude of the response, TD is the time delay and τ represents a time constant. Curve fitting was performed in Sigmaplot 10 with SigmaStat 3.5 software (Systat Software Inc., San José, USA). Additionally, the parameter estimates were plotted against the thickness of the skinfold at the location of the NIRS-probe and Pearson product correlation was performed. If this correlation was significant, the slope of the correlation line was used to correct the parameters affected by skinfold thickness (i.e., Y_0 and A)(Koga et al., 2011).

Statistical analysis

Descriptive statistics were calculated (mean \pm SD) for Y_0 , A, TD and τ of the exponential (AOoxy and AOdeoxy) and Gompertz (POoxy and POdeoxy) models. The parameter estimates for AOoxy, AOdeoxy, POoxy and POdeoxy were compared between the two groups and two NIRS-signals (oxy[Hb+Mb] vs. deoxy[Hb+Mb]) by means of Repeated Measures ANOVA (2x2) in SPSS 14.0. Post hoc analysis was performed by means of Tukey tests. Statistical significance was set at $P < 0.05$.

RESULTS

The patients' (3 women, 7 men) age, height and body weight were respectively 29 ± 7 years, 166.2 ± 11.4 cm and 59.9 ± 15.7 kg. The healthy subjects' age, height and body weight were respectively 28 ± 9 years, 174.4 ± 8.7 cm and 72.7 ± 16.9 kg. Dominant forearm skinfold thickness under the NIRS probe was respectively 8.2 ± 4 mm and 12.3 ± 8.5 mm for MMpatients and healthy subjects.

1. Arterial occlusion

The arterial occlusion lasted on average 504 ± 129 s in the MM patients and 476 ± 102 s in the healthy subjects ($P=0.42$). In Figure 1 the deoxy[Hb+Mb] response of a representative patient and healthy subjects is displayed. The exponential model provided a good fit to both AOoxy and AOdeoxy ($R^2=0.98 \pm 0.01$ and 0.99 ± 0.01 in MMpatients and healthy subjects, respectively). The amplitude (A) and baseline (Y_0), but not the kinetic parameters (TD and τ) of both AOoxy ($R=-0.47$, $P=0.034$ and $R=-0.55$, $P=0.040$ for Y_0 and A, respectively) and AOdeoxy ($R=-0.45$, $P=0.043$ and $R=-0.56$, $P=0.029$ for Y_0 and A, respectively) were significantly correlated with the skinfold thickness at the location of the probe. Table 1 gives the mean parameter estimates, where necessary corrected for skinfold thickness, for the exponential model for the MMpatients and healthy subjects.

The baseline Y_0 and amplitude A of AO_{oxy} and AO_{deoxy} did not differ significantly between Mmp patients and healthy subjects ($P=0.64$ and $P=0.71$, respectively). For TD there was no significant difference between the groups ($P=0.41$), nor the signals ($oxy[Hb+Mb]$ vs. $deoxy[Hb+Mb]$) ($P=0.72$). However, for τ there was a significant main effect of group ($P=0.03$), indicating that τ was significantly longer in patients compared to healthy subjects.

Fig. 1: The pattern of $deoxy[Hb+Mb]$ during arterial occlusion in a representative patient (grey dots) and healthy subject (black dots). The cuff was inflated to 240mmHg at time 0s.

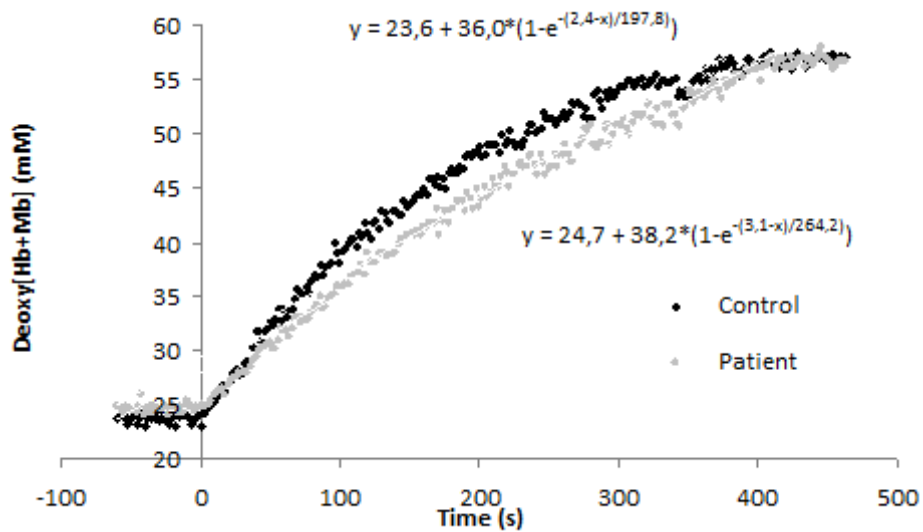


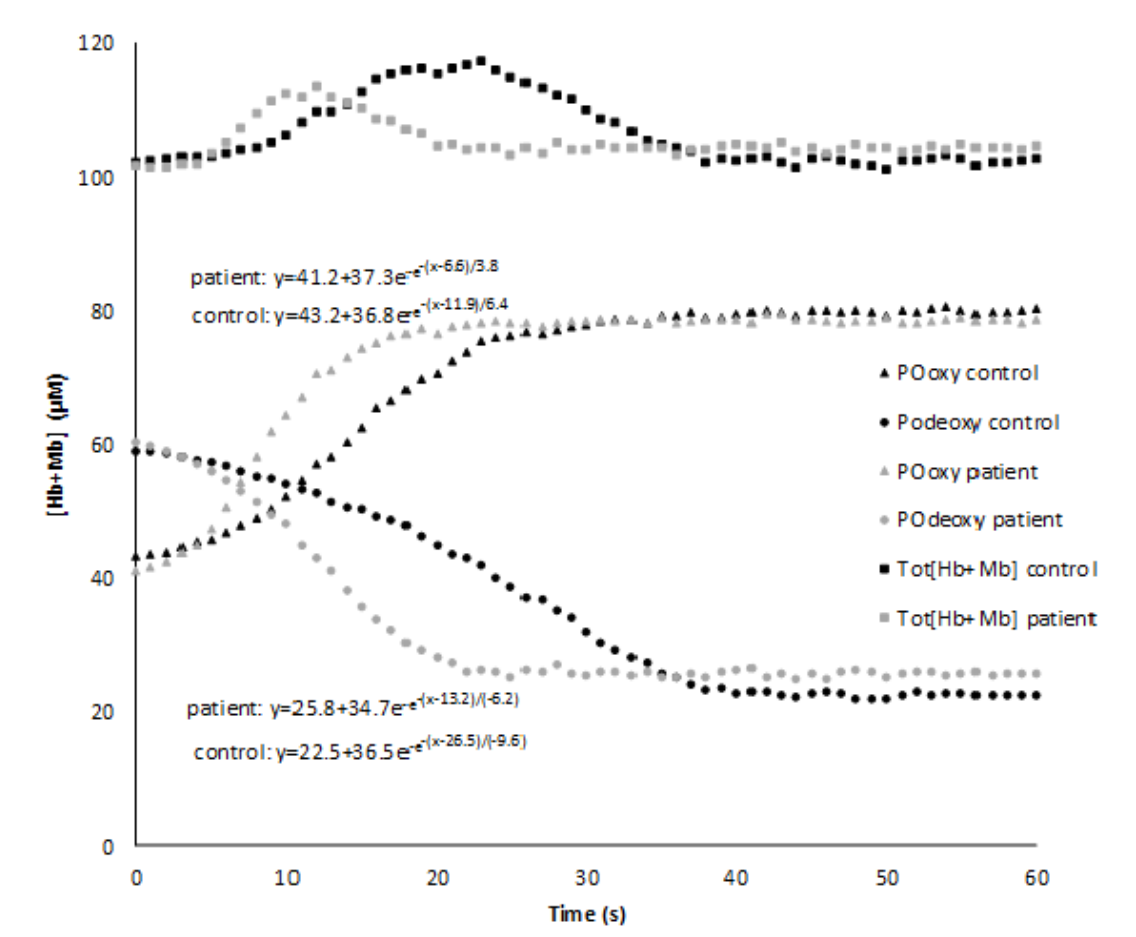
Table 1 (Exponential parameters to AOoxy and AOdeoxy): Mean parameter estimates for the exponential model fitted to AOoxy and AOdeoxy in patients and controls. * indicate significantly different from the healthy subjects ($P < 0.05$). Y_0' and a' represent Y_0 and a corrected for skinfold thickness.

	AOoxy		AOdeoxy	
	Patients	Healthy subjects	Patients	Healthy subjects
Y_0 (μM)	82.4 ± 15.2	81.4 ± 13.7	26.8 ± 14.8	23.7 ± 11.8
Y_0' (μM)	93.4 ± 13.8	89.5 ± 12.4	33.9 ± 12.1	27.8 ± 10.0
a (μM)	43.7 ± 29.7	37.9 ± 21.4	44.9 ± 28.0	38.6 ± 22.8
a' (μM)	52.9 ± 22.4	49.4 ± 19.1	53.1 ± 20.4	49.9 ± 19.3
TD (s)	6.2 ± 2.3	4.7 ± 1.7	5.5 ± 1.7	3.8 ± 1.0
τ (s)	258.4 ± 52.3 *	199.7 ± 69.8	263.4 ± 49.1 *	200.3 ± 73.7

2. Post-occlusion

The responses of oxy[Hb+Mb] (POoxy), deoxy[Hb+Mb] (POdeoxy) and total[Hb+Mb] (tot[Hb+Mb]) for a representative healthy subject and a patient are shown in Figure 2. Because tot[Hb+Mb] for both healthy subjects and patients was not constant through the early post-occlusion response, this implied that the amplitude and/or kinetics of the responses of POoxy and POdeoxy differed (Bopp et al., 2011).

Fig. 2 (POoxy and POdeoxy): The pattern of deoxy[Hb+Mb], oxy[Hb+Mb] and tot[Hb+Mb] immediately following arterial occlusion (time 0 represents the release of the cuff) for patient and healthy subject



The Gompertz function provided a good fit to both POoxy and POdeoxy ($R^2=0.95 \pm 0.02$ and 0.98 ± 0.02 , respectively). The amplitude (A) for POoxy ($R=-0.56$, $P=0.024$) and baseline (Y_0) and A for POdeoxy ($R=-0.35$, $P=0.041$ and $R=-0.48$, $P=0.029$, respectively) were significantly correlated with skinfold thickness at the location of the probe.

For both POoxy and POdeoxy response (Table 2), Y_0 (and Y_0') did not differ between the two test groups ($P=0.81$) but Y_0 was higher in POoxy compared to POdeoxy ($P<0.01$). For the amplitude of the response (A [and A']) there was no significant main effect of signal ($P=0.44$) nor of group ($P=0.65$), pointing out that the amplitude of POoxy did not differ from POdeoxy and that there was no difference between MMpatients and the healthy subjects. However, the kinetic parameters differed between signals (POoxy vs. POdeoxy) and groups (patients vs. healthy subjects). For TD, there was a main effect of both signal ($P<0.001$) and group ($P<0.001$). Post hoc analysis showed that TD was significantly higher in POdeoxy compared to POoxy and that TD was significantly higher in the healthy subjects compared to the patients. Similar results were obtained for the time constant (τ), i.e., there was a significant main effect of signal ($P<0.001$) and of group ($P<0.01$). The post hoc analysis revealed that $b \tau$ was higher (i.e., slower response) in POdeoxy compared to POoxy and that $b \tau$ was higher (i.e., slower) in the healthy subjects compared to MMpatients.

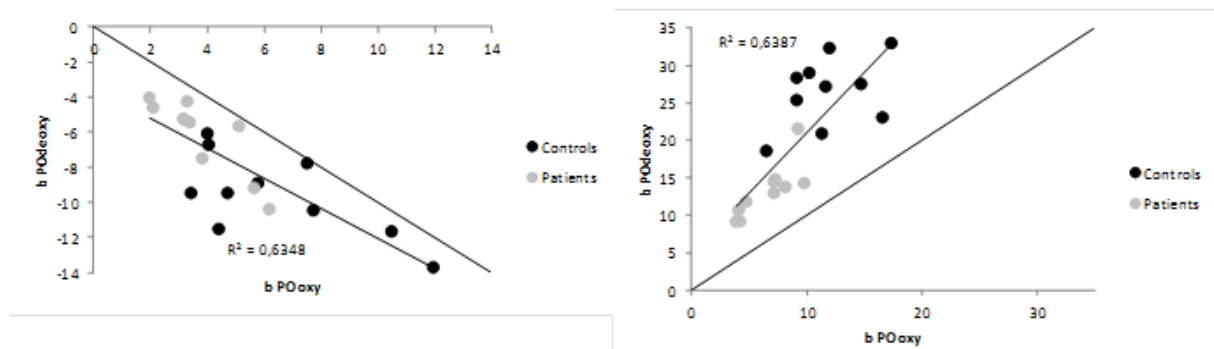
Table 2 (Gompertz parameters to PO₂ and PO₂)

	PO ₂		PO ₂	
	Patients	Healthy subjects	Patients	Healthy subjects
Y ₀	39.5 ± 20.7 #	45.8 ± 18.9 #	25.8 ± 10.6	22.5 ± 6.5
Y ₀ '			25.3 ± 9.4	29.6 ± 6.5
a	38.9 ± 17.1	34.1 ± 17.4	40.4 ± 22.8	37.7 ± 20.8
a'	43.2 ± 11.1	51.3 ± 16.0	47.4 ± 16.0	53.5 ± 21.3
TD (s)	6.6 ± 2.2 *,#	11.9 ± 3.5 #	13.2 ± 3.6 *	26.5 ± 4.6
τ (s)	3.8 ± 1.4 *,#	6.4 ± 2.9 #	-6.2 ± 2.2 *	-9.6 ± 2.4

Mean parameter estimates for the Gompertz model fitted to the oxy[Hb+Mb] (POoxy) and deoxy[Hb+Mb] response (POdeoxy) following the arterial occlusion in patients and healthy controls. The Y_0' and a' represent the Y_0 and a corrected for skinfold thickness. * indicates significantly different from the healthy subjects; # indicates significantly different from POdeoxy ($P < 0.05$).

Even though the kinetic parameters (TD and τ) were significantly different between POoxy and POdeoxy (resulting in the non-constant tot[Hb+Mb]), they were significantly correlated when the entire study population (both patients and healthy subjects) was considered (Figure 3), Thus, subjects who had faster responses in one variable tended to have faster responses in the other as well.

Fig. 3 (Correlations of kinetic parameter of POoxy and POdeoxy): Correlations between the kinetic parameters (b and X_0) from the Gompertz model fit to the oxy[Hb+Mb] and deoxy[Hb+Mb] response immediately following arterial occlusion in the entire study population. * indicates significant correlations ($P < 0.05$)



DISCUSSION

The aim of the present study was to test if the relationship between O₂ supply and O₂ demand during and following brachial artery occlusion differed between healthy controls and patients suffering from mitochondrial myopathies, using NIRS. This technique has been used to describe the relationship between convective and diffusive O₂ flux and the oxygenation within skeletal muscle non-invasively during exercise and thus might provide important clinical information. In the present study the kinetics of deoxy[Hb+Mb] and oxy[Hb+Mb] during and post arterial occlusion were described in a patient group suffering from mitochondrial myopathy (MM) and in healthy sedentary individuals. It was observed that, although the amplitude of the deoxy[Hb+Mb] response during arterial occlusion (AOdeoxy) was unaffected, MM patients had a slower increase in deoxy[Hb+Mb] following the onset of the occlusion, consistent with our first hypothesis. Post-occlusion, in the entire study population the kinetics of oxy[Hb+Mb] (POoxy) were faster compared to those of deoxy[Hb+Mb] (POdeoxy), reflecting a transient increase in tot[Hb+Mb] response, suggestive of a hyperemic response in the microvasculature. Furthermore, the kinetics of both POoxy and POdeoxy were faster in MMpatients compared to healthy subjects, consistent with hypothesis 2.

Arterial occlusion

Arterial occlusion has been used to obtain the maximal deoxygenation (i.e., highest deoxy[Hb+Mb]) of skeletal muscle (Grassi et al., 2007; Celie et al., 2011). Since deoxy[Hb+Mb] can be considered as an expression of microvascular oxygen extraction (Delorey et al., 2003; Grassi et al., 2003; Ferreira et al., 2005), this technique has also been used to obtain representative values of maximal capacity for O₂ extraction, so that deoxy[Hb+Mb] values during exercise can be referenced to these maximal values (Grassi et al., 2007; Celie et al., 2011). In this way, near-infrared spectroscopy can provide insight into the capillary-to-myocyte O₂ exchange and on the relationship between convective and diffusive capacity at the level of the myocyte (Poole et al., 2011). In the present study it was observed that deoxy[Hb+Mb] during arterial occlusion at rest follows an exponential increase to a steady state level which required on average 6-8 minutes. It should be noted that this caused some

discomfort in some subjects but they were all able to maintain the occlusion until there was a clear leveling off in deoxy[Hb+Mb]. The present study showed non-invasively slower deoxy[Hb+Mb] kinetics in MMpatients compared to healthy controls. Given that there is no blood flow in this condition (i.e., Q_m is zero), and that the amount of O_2 in the tissues was similar between groups (as reflected by a similar tot[Hb+Mb] Y_0 and A values), these slower deoxygenation kinetics can be related to genetic defects impairing the mitochondrial subcomplexes and/or enzymes (i.e., the oxidative phosphorylation system (OXPHOS)), resulting in a slower VO_{2m} and thus a reduced rate of O_2 extraction. Surprisingly the amplitude of deoxygenation response did not differ between MMpatients and healthy subjects. However, due to the heteroplasmic nature of this mitochondrial disorder there is a mixture of wild type and mutant mtDNA in the muscle tissue of these patients which means that not all mitochondria at the muscle level are defective (Taivassalo et al., 2003; Durham et al., 2007; DiMauro, 2010). Some wild type mitochondria are able to take up and consume oxygen. Our hypothesis is that in these circumstances some of the patients' mitochondria are able to utilize oxygen, but since the density of functioning mitochondria is lower in the patient population, the rate of oxygen utilization (indicating resting metabolic rate) will be slower compared to healthy subjects. Interestingly, the final amplitude of change in deoxy[Hb+Mb] in the two groups was the same, suggesting that the total content of Hb and Mb in the tissue during the occlusion was similar in both groups.

Post-occlusion

In line with the studies of Bopp et al. (2011) and Kragelj et al. (2001) a transient increase in tot[Hb+Mb] was observed in body study populations, reflecting an increase in microvascular [Hb] and hematocrit, since presumably muscle [Mb] remained constant throughout the protocol (Davis and Barstow, 2013). The increase in hematocrit (as tot[Hb+Mb]) is likely associated with the transient increase in blood flow (Klitzman and Duling, 1979) although the precise temporal relationship may be variable (Kindig et al., 2002; Ferreira et al., 2006). Further, we observed a delay in rise in tot[Hb+Mb] during reactive hyperemia following cuff release in the present study. This delay is similar to that reported by Kindig et al. (2003) who showed a delayed increase in capillary hematocrit of 10-15 s following the onset of muscle

contractions. This delay reflected a faster rise in the velocity of red blood cells in the capillaries compared to the slower rise in red blood cell flux following the onset of contractions. For both groups in the present study, the deoxy[Hb+Mb] response was slower relative to that of oxy[Hb+Mb] following cuff release, similar to that reported by Bopp et al. (2011). The transient increase in tot[Hb+Mb] (and the different recovery kinetics of oxy[Hb+Mb] and deoxy[Hb+Mb]) upon cuff release are likely related to convective and diffusive characteristics at the level of the myocyte (Poole et al., 2011). Upon cuff release, blood flow into the forearm muscle increases rapidly (Burton and Johnson, 1972) due to the increased conductance occurring during the arterial occlusion from the release of vasodilators (Engelke et al., 1996; Gonzalez-Alonso, 2012), resulting in a fast recovery of microvascular O₂ pressure and increase in oxy[Hb+Mb], assuming a constant VO_{2m}. In contrast, the more sluggish decrease in deoxy[Hb+Mb] compared to that of oxy[Hb+Mb] in all subjects likely reflects a sustained O₂ extraction in this early transient phase.

On average, the patients' PO_{oxy} and PO_{deoxy} responses, and thus also tot[Hb+Mb], were faster than those of the healthy controls. This would suggest that the initial rise in blood flow and oxygen delivery following cuff release was faster than the rate of utilization of oxygen by the tissue (higher Q_m/VO_{2m} in the transition) (Delorey et al., 2003; Grassi et al., 2003; Boone et al., 2009). This faster rate of change in oxy[Hb+Mb], deoxy[Hb+Mb] and tot[Hb+Mb] in MMpatients compared to healthy controls likely reflects a hyperdynamic cardiovascular response that has been observed during exercise in patients with mtDNA mutations (Jeppesen et al., 2012). In contrast to chronic heart failure patients (Copp et al., 2010), in which O₂ delivery is impaired relative to VO_{2m}, resulting in impaired recovery of microvascular PO₂ following exercise, this hyperemic response in Mmpatients results in the opposite effect, i.e., a higher velocity of blood flow and faster recovery of microvascular O₂ pressure compared to healthy subjects. In addition, the higher blood flow, in concert with the impaired VO_{2m} in Mmpatients, results in faster deoxy[Hb+Mb] kinetics following the ischemic challenge. The slower rate of reoxygenation in the healthy controls (as expressed by a slower decrease in deoxy[Hb+Mb]) is consistent with the observation of Kime et al. (2003), who found that muscles with a higher oxidative capacity displayed a slower reoxygenation following 10s of maximal isometric contraction. Thus, the slower decrease in deoxy[Hb+Mb] post-occlusion in

the healthy subjects reflects a healthier muscle recovery. This is however, in contrast to the study of Bravo et al. (2012), in which slower deoxy[Hb+Mb] kinetics were observed in MMpatients compared to healthy subjects following high-intensity exercise. It should be noted however, that the test methodology is difficult to compare to our study, since it consisted of cycle exercise at 70% of the peak power (i.e., large muscle mass and cardiopulmonary effort).

It should be noted that the deoxy[Hb+Mb] responses measured with NIRS provide insight into the relative relationship between Q_m and VO_{2m} without being able to address either the convective or diffusive capacity separately. This implies that changes in the NIRS signals in response to challenges such as ischemia or exercise can either reflect changes in muscle blood flow and/or microvascular O_2 extraction. Thus, the ability of the NIRS technique, by itself, to identify the mechanisms which underlie the responses of these challenges is limited. It is recommended to combine NIRS with other measurements, such as the non-invasive measurement of Q_m with Doppler Ultrasound, to gain greater quantitative insight.

The results of the present study might have important clinical implications. First, it is shown that NIRS can potentially be used for non-invasive screening and functional evaluation of Mmpatients, in place of traditionally more invasive techniques. Second, this study furthers the understanding of the relationship of Q_m to VO_{2m} in healthy subjects and Mmpatients. This relationship has been considered as an important determinant of exercise tolerance since it provides information on the capacity of the cardiovascular and muscle systems to adjust to changes in metabolic demand. While we did not measure exercise responses or tolerance in the current study, the insights gained regarding the balance of Q_m to VO_{2m} following an ischemic challenge at rest may provide insight into the relative impairment of this relationship during exercise in patient populations such as Mmpatients. Testing of this hypothesis must await further experimentation.

Conclusion

In the present study it was found that the kinetic responses of muscle oxygenation (deoxy[Hb+Mb], oxy[Hb+Mb] and tot[Hb+Mb]) differs between patients suffering from mitochondrial myopathy and healthy subjects. MMpatients showed a more rapid and pronounced increase in O₂ supply upon cuff-release, in combination with impaired VO_{2m}, compared to healthy controls. These different kinetics of oxy[Hb+Mb] and deoxy[Hb+Mb] reflect the pathology (impaired VO_{2m}) and adaptation (enhanced Q_m). These results indicate that NIRS can provide important insight into muscle oxygenation and the relationship between Q_m and VO_{2m} in response to brachial artery occlusion in healthy and disease.

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STUDY 4

FOREARM DEOXYHEMOGLOBIN AND DEOXYMYOGLOBIN (DEOXY[HB+MB]) MEASURED BY NEAR-INFRARED SPECTROSCOPY (NIRS) USING A HANDGRIP TEST IN MITOCHONDRIAL MYOPATHY

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ABSTRACT

Objectives: The purpose of the present study was to test whether peripheral oxygenation responses measured with near infrared spectroscopy (NIRS) would differ between patients suffering from mitochondrial myopathy (MM) and healthy controls during an incremental handgrip exercise test.

Methodology: Two groups of subjects were studied: 11 patients with MM and 11 age- and gender-matched untrained healthy controls. A handgrip exercise protocol until exhaustion was used consisting of 2-min periods of work ($\frac{1}{2}$ Hz) at different intensities, separated by a 60-s rest period. The changes in deoxy[Hb+Mb] during each work step were expressed in percent to the maximum deoxy[Hb+Mb]-value measured during arterial occlusion in forearm muscles. A Repeated Measures ANOVA was used to compare the increase in deoxy[Hb+Mb] between MM patients and controls with increasing intensity.

Results: Statistical analysis revealed a significant difference between both populations ($p < 0.001$) indicating that the increase in deoxy[Hb+Mb] showed a significantly different pattern in the two populations. In the post hoc analysis significant lower deoxy[Hb+Mb] - values were found for MM patients at every intensity.

Conclusion: The results of the present study showed significant different skeletal muscle oxygenation responses, measured with an optical method as NIRS, between MM patients and age- and gender-matched healthy subjects at submaximal and maximal level during an incremental handgrip exercise. This optical method is thus a valuable tool to assess differences in peripheral oxygenation. Moreover, this method could be used as an evaluation tool for follow up in interventional pharmacological studies and/or rehabilitation programs.

KEYWORDS

Near Infrared Spectroscopy (NIRS), mitochondrial dysfunction, handgrip exercise, peripheral oxygenation

INTRODUCTION

The relationship between skeletal muscle blood flow (Q_m) and oxygen uptake (VO_{2m}) is an important determinant of exercise tolerance in healthy and patient populations. A thorough understanding of this relationship provides insight into cardiovascular and muscular adjustments to changes in metabolic demand. In healthy subjects a tight coupling between O_2 supply and O_2 demand has been observed, while this relationship is often disturbed in patient populations inherent to the pathologic condition either affecting the central (i.e., cardiovascular and pulmonary) and/or peripheral (i.e., skeletal muscle) response (Poole et al., 2011). Since the validity of non-invasive determination of blood flow (Q_m) and arterio-venous O_2 difference ($C_{(a-v)}O_2$) during exercise has been questioned (Warburton et al., 1999) and because of practical issues when using invasive methods, near infrared spectroscopy (NIRS) might be a useful and non-invasive optical technique. More specific to provide more information on the oxygenation at muscle level and to gain more insight into the capillary-to-myocyte O_2 exchange and the relationship between convective and diffusive capacity at the level of the myocyte (Poole et al., 2011). The reliability and validity of NIRS as an appropriate technique to measure non-invasively peripheral tissue oxygenation has been established in erector spinae (Kell et al., 2004) and limb skeletal muscles (Mancini et al., 1994) as well as in brain tissue (Mehangoul-Schipper et al., 2002) at rest and during cycle ergometric exercise (Pereira et al., 2007). Also, the reliability has been established in forearm skeletal muscles using an incremental handgrip protocol (Celie et al., 2012). NIRS evaluates the concentration of deoxy[Hb+Mb] and oxy[Hb+Mb] at the level of the microcirculation in exercising skeletal muscle and could be used as an expression of microvascular O_2 extraction (Abe et al., 1997; Chance and Banks, 1995).

Mitochondrial myopathies (MM) are multisystem disorders, clinically characterized by a large variety of signs and symptoms predominantly affecting skeletal muscle tissue (Di Mauro, 2011). These MM patients present one or more genetic defects responsible for an impaired oxidative capacity of the skeletal muscle tissue resulting in a reduced capacity to increase skeletal muscle O₂ extraction. Differences in VO₂ (Dandurand et al., 1995; Dysgaard Jeppesen et al., 2003; Grassi et al., 2009; Siciliano et al., 1999) and VO₂-kinetics (Taivassalo et al., 2003) and blood flow have already been found in MM patients during cycle ergometry protocols in different studies. Also, lower deoxy[Hb+Mb]-values as a marker for reduced O₂ extraction have been reported in MM patients during a cycle ergometer protocol (Grassi et al., 2007). However for some patients cycle ergometric testing is potentially life-threatening as these patients can suffer from cardiomyopathy and might be at risk for arrhythmia during the test. Therefore, handgrip exercise protocols were introduced for measuring O₂ desaturation using venous blood samples. These O₂ desaturation values were significantly lower in MM patients during exercise, presuming a reduced O₂ extraction due to a decreased oxidative capacity in skeletal muscle (Hanisch et al., 2006; Jensen et al., 2002; Taivassalo et al., 2002). To our knowledge there was only one study (Van Beekvelt et al., 2001) that combined optical non-invasive NIRS measurements with a non exhaustive handgrip protocol. These authors found significant lower muscular VO₂ values in MM patients during handgrip exercise measured with NIRS. However some concerns can be raised with the interpretation of the absolute deoxy[Hb+Mb] values and the influence of subcutaneous adipose tissue in this study (Van Beekvelt et al., 2001).

The purpose of the present study was to test whether skeletal muscle oxygenation responses, measured with an optical method as NIRS, would differ between MM patients and age- and gender-matched healthy subjects at submaximal and maximal level during an incremental handgrip exercise. The reliability of the handgrip exercise protocol using this technique was established in a previous study (Celie et al., 2012). It was hypothesized that the increase in deoxy[Hb+Mb] in response to exercise would be lower in MM patients as compared to healthy controls, as a reflection of a limited capacity to increase microvascular O₂ extraction and thus an altered Q_m/VO_{2m}-relationship.

METHODS

Subjects

We studied two groups of subjects. A first group consisted of 11 patients suffering from a mitochondrial myopathy (MM). The second group consisted of 11 age- and gender-matched untrained healthy controls. It was not possible to find matched control subjects for body weight or height as weight loss is a predominant symptom for MM patients. Moreover, short stature is one of the prevalent symptoms in the MELAS genotype (MM) (De Vriese et al., 2006). The patients were recruited from the Neuromuscular Reference Center at the Ghent University Hospital, Belgium. The subjects were fully informed of any risk associated with the experiments before giving their written consent for participation. The study was approved by the ethics committee at the Ghent University Hospital. Procedures were in accordance with the recommendations of the Helsinki Declaration. The patients' (4 women, 7 men) age, height and body weight were respectively 29 ± 12 years, 166.5 ± 11.7 cm and 60.7 ± 16 kg (mean values \pm SD). Control subjects' age, height and body weight were respectively 29 ± 12 years, 177.4 ± 10 cm and 77.2 ± 16.8 kg (mean values \pm SD). All patients were diagnosed as having MM based on clinical presentation and on the results of the biochemical analysis in a skeletal muscle biopsy, which remains the gold standard for diagnosis of mitochondrial diseases. This was done by evaluation of OXPHOS function in skeletal muscle from selected patients by spectrophotometry using standard enzyme assays (Van Coster et al., 2001) and by Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) (Smet et al., 2009). The z score was calculated as the activity ratio for the patient sample minus the mean activity ratio for the control samples divided by the SD for the control samples. When the z-score was lower than -1.96 or higher than +1.96, the result for the patient sample was considered as significantly different ($p < 0.05$) from the result in the control samples (Van Coster et al., 2001). Results with z scores of less than -1.96, indicative of significantly decreased OXPHOS activity in the patient tissue sample, are presented in bold. All diagnostic information of the MM patients is presented in table 1. Patients presenting complex V subcomplexes at BN-PAGE analysis are known to present intramitochondrial translation defects in the analysed tissue (Van Biervliet et al., 2009).

Table 1: Diagnostic data of all Mitochondrial Myopathy (MM) patients

Gender (M/F) Age (years)	Clinical description	OXPHOS analyses						Blue Native PAGE + activity staining	Tissue
		Spectrophotometric investigations (z scores*)							
		I	II	II+III	III	IV			
F/17	Exercise intolerance, fatigability	-3.25	0.69	0.08	2.08	1.54	Normal	Skeletal muscle	
M/13 ²⁶	Mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes (MELAS)	-10.86	1.18	1.38	1.73	-0.98	Complex I ↓↓ Complex IV ↓ Complex V subcomplexes ²⁵	Skeletal muscle	
M/45	Visual failure, ptosis, external ophthalmoplegia, neurosensorial hearing loss, exercise intolerance, muscle weakness, ataxia	-3.95	-1.08	-1.02	ND	-0.42	Complex I ↓ Complex IV ↓ Complex V subcomplexes ²⁵	Skeletal muscle	
F/54 ²⁷	Maternally inherited diabetes and deafness (MIDD)	-0.56	-0.23	0.61	-0.12	0.21	Normal, except for Complex V subcomplexes ²⁵	Skeletal muscle	
M/28	Exercise intolerance	-8.25	0.84	2.78	1.32	0.21	Complex I ↓↓↓	Skeletal muscle	
F/25	Exercise intolerance	-10.17	ND	0.41	0.01	0.26	ND	Skeletal muscle	
M/19	Mental retardation, dystonia, pyramidal signs in lower limbs (Leigh syndrome)	-5.23	-1.82	-0.94	ND	-0.91	ND	Skeletal muscle	
F/21	Exercise intolerance, hypertrophic cardiomyopathy, mild mental retardation	-7.59	0.32	0.15	ND	-0.33	Complex I ↓↓↓	Skeletal muscle	

M/24	Exercise intolerance, hypertrophic cardiomyopathy	-8.26	0.99	0.54	ND	-0.33	Complex I ↓↓	Skeletal muscle
M/36	Exercise intolerance	-7.29	-0.77	0.16	ND	-1.15	Complex I ↓↓	Skeletal muscle
M/32	Exercise intolerance	-3.31	-1.18	-0.44	ND	-1.38	Complex I ↓↓	Skeletal muscle

M: Male; F: Female

↓: moderately reduced, ↓↓: severely reduced

* z score: To control for mitochondrial mass, the ratio of the logarithm of each OXPHOS complex to the logarithm of the citrate synthase activity is calculated. Subtracting the mean activity ratio for the control samples from the activity ratio of the patient samples, divided by the SD for the control samples gives the z score. When the z score was lower than -1.96 or higher than +1.96, the result for the patient sample was considered as significantly different ($p < 0.05$) from the result in the control samples²². Results with z scores of less than -1.96, indicative of significantly decreased OXPHOS activity in the patient tissue sample, are presented in bold.

²⁶ Published in Acta Gastroenterol Belg. 2009 Jul-Sep;72(3):365-8, Van Biervliet et al.

²⁷ Published in Ophthalmic Genet. 2010 Dec;31(4):240-3, Robberecht et al.

²⁵ Published in Electrophoresis. 2009 Oct;30(20):3565-72, Smet et al.

Measurements

During the incremental exercise test, skeletal muscle tissue oxygenation was measured with a near infrared spectroscopy (NIRS) system (Oxiplex TS, ISS, Champaign, Illinois, USA). This system is based on an infrared light absorption method, where the infrared light is emitted at different wavelengths. The NIRS-probe consisted of eight light-emitting diodes operating at wavelengths 750 and 830nm and one detector fibre bundle (source-detector distance = 2.0-3.5cm). The deoxy[Hb+Mb] data were stored at a frequency of 25Hz and afterwards digitally averaged into 1s-values. The probe was positioned longitudinally on the M. flexor carpi ulnaris and M. flexor digitorum superficialis and secured with Velcro straps around the dominant upper arm. Pen marks were made on the skin to detect probe displacement during the exercise.

Study design and protocol

Preceding the test, the maximal voluntary contraction (MVC) (expressed in Newton (N)) force was determined in the dominant arm using a hydraulic handgrip dynamometer (Saehan corporation, Masan, South-Korea) for all subjects (best of three attempts). Between every attempt, a 5 minute resting period was included. After the placement of the NIRS probe on muscles, a pneumatic cuff was placed around the ipsilateral upper arm . The subjects performed the complete exercise protocol in supine position. The protocol started with a forearm arterial occlusion by inflating the cuff at 260mmHg. In the protocol, the duration of the occlusion was not specified as it was executed until a steady state in deoxy[Hb+Mb] was reached. After the occlusion, there was a minimum 5 minute rest period before baseline conditions were reached again and then the incremental cyclic contraction protocol (ICCP) started. The time interval between the end of the arterial occlusion and the beginning of the exercise task, before baseline values were reached, varied among all subjects. This task consisted of 2 minute periods during which incremental cyclic contractions were performed at ½Hz (1 second contraction, 1 second relaxation), at different intensities of maximal voluntary contraction (% MVC). The contraction periods were separated by a 60-s rest period. The work intensity started at 20% MVC and increased by 10% MVC at each step. This protocol was executed until exhaustion was reached and the subjects were no longer able to produce the required force. The duration of this test procedure was about 1h. The reliability of this protocol has been established in a previous study (Celie et al., 2012)

Data analysis

To determine the mean values within this protocol, a smoothing procedure was performed in which the 10-s mean values were determined by means of a moving average (Boone et al., 2010). When skeletal muscle tissue is exposed to an arterial occlusion, acute local hypoxia is induced. The amplitude of the deoxy[Hb+Mb] response during arterial occlusion (i.e. the difference between the highest 30-s average of deoxy[Hb+Mb] during occlusion and the 30-s average of deoxy[Hb+Mb] preceding the occlusion) was used as index for maximal O₂ extraction and was set at 100%. The changes in deoxy[Hb+Mb] during each work step (i.e. the

mean of the highest 10-s value) were expressed relative to this amplitude (Celie et al., 2012) . These calculations were made to reduce possible influence of forearm adipose tissue thickness as values are relatively presented towards their individual maximum value.

Statistical analysis

Statistical analysis for MVC and amplitude of the occlusion steady state was performed using an independent samples t-test. A Repeated Measures ANOVA was used to compare the increase in deoxy[Hb+Mb] (in %) during ICCP between the MM population and the controls. When a significant main effect was found, post-hoc analysis was performed by means of an independent samples t-test. The level of significance was set at $p < 0.05$. Statistical computations were performed using SPSS® software (version 18; SPSS Lead Technologies Inc., Chicago, IL, USA). All data are presented as mean \pm standard deviation (SD).

RESULTS

MM patients had a significantly lower MVC ($p < 0.001$) as compared to control subjects ($\bar{x} = 255.1 \pm 127.5N$ vs. $\bar{x} = 529.7 \pm 137.3N$). Typical deoxy[Hb+Mb] outputs measured during arterial occlusion and the ICCP in one healthy and one diseased subject are shown in Figure 1 (a+b). No significant difference was found ($p = 0.899$) between both populations for the arterial occlusion deoxy[Hb+Mb] amplitude. All patients tolerated the test well as no pain was perceived after the test protocol.

Mean submaximal and maximal deoxy[Hb+Mb] values (expressed as a percentage) during ICCP for MM patients and controls are presented in Figure 2. Statistical analysis revealed that there was a significant main effect for groups with an $F = 36,263$ ($p < 0.001$) indicating that the increase in deoxy[Hb+Mb] showed a significantly different pattern in the two populations. Although deoxy[Hb+Mb] increased significantly during the ICCP in both populations, the increase in deoxy[Hb+Mb] was significantly higher in healthy controls. Post hoc analysis revealed significant percentual deoxy[Hb+Mb] differences between MM patients and healthy controls at all intensities (% MVC). All p- and t-values are included in Figure 2.

Fig. 1a-b.: Typical deoxy[Hb+Mb] outputs as a response to the arterial occlusion and the ICCP in one healthy subject (a) and one diseased(MM) subject (b). (BL 1= Baseline 1; BL 2= Baseline 2; AO max= Arterial Occlusion deoxy[Hb+Mb] maximum value).

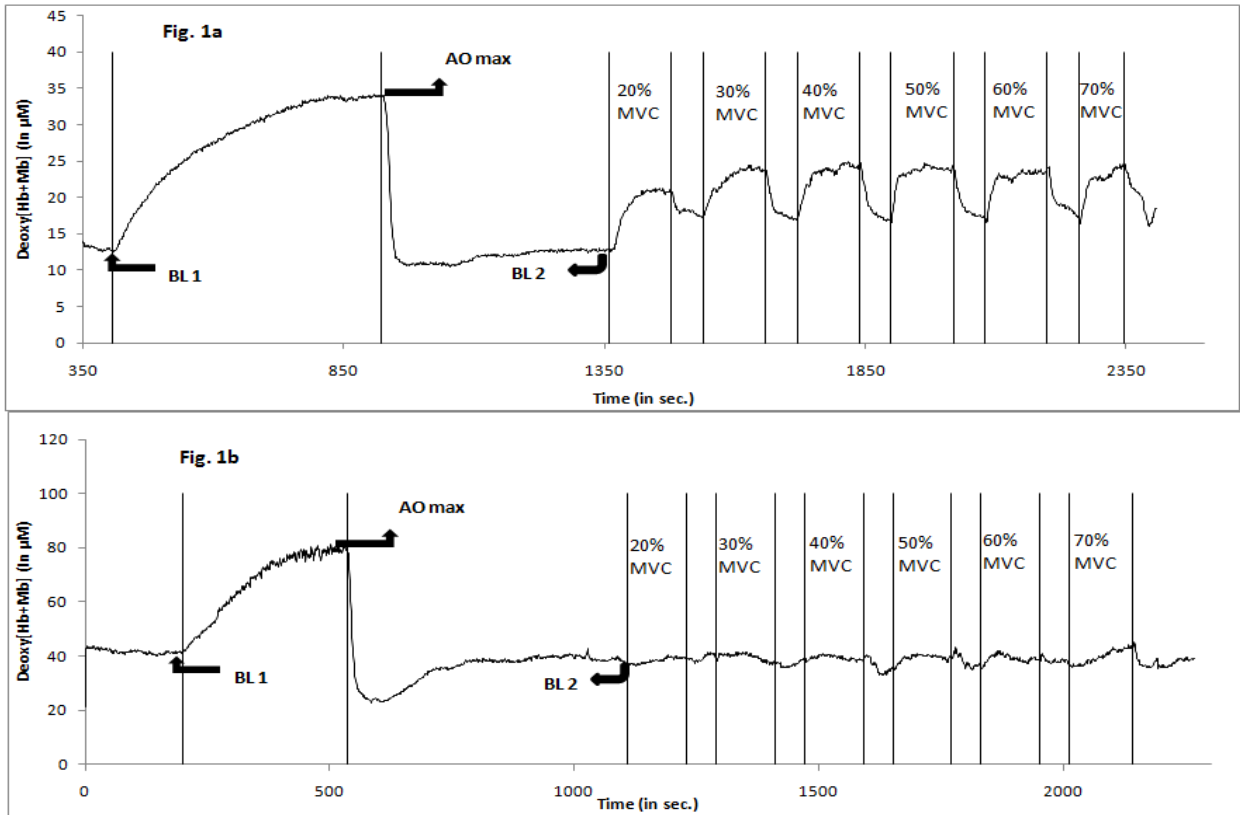
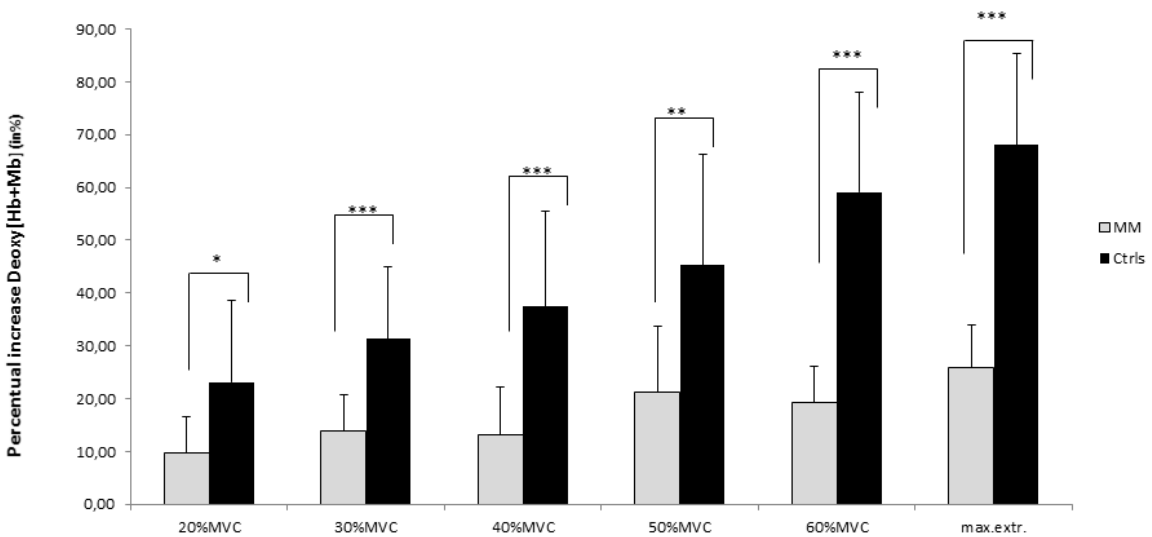


Fig. 2. Mean submaximal and maximal deoxy[Hb+Mb]-values (expressed as a percentage) during ICCP for patients and controls.



Main effect groups $p < 0.001$

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

DISCUSSION

In the present study the peripheral oxygenation during an incremental dynamic handgrip exercise was studied in patients suffering from mitochondrial myopathy and in healthy subjects. The MM patients showed significantly altered deoxy[Hb+Mb] responses in comparison to matched controls. Whereas in healthy controls the NIRS-derived O₂ extraction gradually increased with incremental intensity, only a small increase was observed in MM patients. The results of the present study are in line with earlier studies using more invasive techniques during handgrip exercise (Hanisch et al., 2006; Jensen et al., 2002; Taivassalo et al., 2002) and/or cycle ergometry tests (Dandurand et al., 1995; Dysgaard Jeppesen et al., 2003; Grassi et al., 2009; Siciliano et al., 1999; Taivassalo et al., 2003). At every intensity during ICCP, patients showed a significantly lower rate of microvascular O₂ extraction in comparison to the healthy matched controls. Deoxy[Hb+Mb] in healthy subjects increased with increasing contraction intensity (%MVC). This suggests that there is a growing imbalance between O₂ supply and O₂ demand, resulting in a progressive increase in microvascular O₂ extraction. This deoxy[Hb+Mb] response as a function of work rate has already been observed during incremental cycle exercise (Grassi et al., 2006, 2007; Ferreira et al., 2005). This pattern has been related to either the Bohr-effect, indicating that a local metabolic acidosis induces a rightward shift of the O₂Hb-dissociation curve or either to differential muscle fiber recruitment, with a progressive recruitment of fast-twitch fibers with a different C(a-v)O₂ pattern in response to exercise (Boone et al., 2014).

The healthy subjects were able to functionally increase the microvascular O₂ extraction during exercise to 72.8 % of the maximal 'forced' O₂ extraction. In the MM patients on the other hand, the increase in deoxy[Hb+Mb] was significantly lower both at submaximal and maximal intensities indicating that O₂ extraction does not increase progressively as observed in healthy control subjects. A possible explanation for this divergent response is the limited ability to produce energy aerobically in the mitochondria. We hypothesize that, because some mitochondria are malfunctioning, myoglobin remains saturated and the intracellular PO₂ will not decrease sufficiently. Consequently, O₂ diffusion will be limited because the PO₂ gradient is not increasing significantly. This limited O₂ diffusion from capillary to myocyte which is

inherent to genetically determined OXPHOS deficiencies leads to a limited capacity to increase microvascular O₂ extraction (Taivassalo et al., 2003). The small increase in deoxy[Hb+Mb] throughout the exercise test should be an expression of this mechanism. It should be noted that the deoxy[Hb+Mb] response in the MM patients was homogeneous as can be estimated from the relatively small standard deviation and thus the small response range (i.e., 17.2- 34.4 % at maximal exercise intensity). To detect MM patients using this method a sensitivity of 91% and a specificity of 91% was found which could be considered as good values. According to these data and compared to other forearm tests where venous blood samples were taken (Taivassalo et al., 2003; Hanisch et al., 2006; Jensen et al., 2002), the NIRS measurements are equally sensitive and specific but less/non-invasive. Since deoxy[Hb+Mb] only increased slightly, it can be suggested that the MM patients compensated the limited O₂ extraction by a hyperdynamic cardiovascular response and thus an emphasized convective O₂ delivery, as observed by Grassi et al. (2006). Therefore, additional use of Doppler ultrasound as a second non-invasive tool to assess muscle blood flow, could be recommended to obtain a more integrative view on the Q_m/VO_{2m} relationship during exercise. Grassi et al. (2009) also observed a less pronounced increase in deoxy[Hb+Mb] (measured with NIRS) during incremental cycle exercise. However, cycle exercise is not recommended in MM patients as these patients can suffer from cardiomyopathy and might be at risk for arrhythmia during the test. To our knowledge, only one study used NIRS during handgrip exercise in MM patients (Van Beekvelt et al., 1999). They found lower VO_{2m}-values, calculated from the changes in deoxy[Hb+Mb] and oxy[Hb+Mb] in response to a 45s arterial occlusion. It should be noted, however, that the authors did not account for possible differences in skinfold thickness at the site of the probe (Van Beekvelt et al., 2001) which might impact the oxygenation responses. Moreover, the arterial occlusion was used to calculate VO_{2m} and was only maintained for 45s (Van Beekvelt et al., 1999). However, arterial occlusion has been used to obtain representative values of maximal capacity for O₂ extraction, so that deoxy[Hb+Mb] values during exercise can be referenced to these maximal values (Celie et al., 2012; Grassi et al., 2007). In this context, it was recently observed that there are temporal differences in the deoxy[Hb+Mb] response to arterial occlusion, i.e. a slower increase in MM patients, but no differences in amplitude in response to arterial occlusion (Boone et al., 2014). Therefore, in the present study, the

occlusion was performed until deoxy[Hb+Mb] showed a clear leveling-off, and was used as reference value for maximal change in deoxy[Hb+Mb].

In summary, it was shown that MM patients had significantly lower deoxy[Hb+Mb] values during this dynamic handgrip test compared to healthy control subjects, indicating that the peripheral oxygenation is altered in this pathological population. The results of the present study suggest that the incremental handgrip test, in combination with non-invasive registration of peripheral oxygenation respectively, could be used as an evaluation tool for detection and/or follow up in interventional pharmacological studies and/or rehabilitation programs.

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STUDY 5

**EVIDENCE FOR MITOCHONDRIAL DYSFUNCTION IN A SUBGROUP OF
CHRONIC FATIGUE SYNDROME (CFS) SCREENED BY NEAR INFRARED
SPECTROSCOPY**

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Manuscript in progress

ABSTRACT

Introduction: Chronic fatigue syndrome (CFS) is characterized by chronic fatigue persisting for at least six months in the absence of any sufficiently explanatory medical condition and in the presence of additional minor criteria, including myalgia and post-exertional malaise. Mitochondrial dysfunction and oxidative damage may underlie the typically experienced exercise intolerance, myalgia and weakness. Previous work showed that Near Infrared Spectroscopy (NIRS) distinguishes patients with mitochondrial myopathy and documented mitochondrial dysfunction from healthy controls by reliably measuring deoxyhaemoglobin and –myoglobin in forearm muscles during an Incremental Cyclic Contractions Protocol (ICCP). This study aimed to screen for mitochondrial dysfunction in CFS.

Methods: Twelve CFS patients and 12 matched healthy controls underwent the ICCP consisting of rhythmic handgrip contractions with increasing intensity. Muscle peripheral oxygenation was measured with NIRS and blood flow with Doppler Ultrasound. Patients with an abnormal profile on NIRS and/or ultrasound were invited for skeletal muscle biopsy, the gold standard for diagnosis of mitochondrial abnormalities.

Results: A significant difference was found in deoxy[Hb+Mb] during ICCP at 50 % and at maximal handgrip force between the patient and control group. This was due to a subset of four CFS patients with limited to absent increases in deoxy[Hb+Mb], similar to the profile observed in documented mitochondrial myopathy. Of these subset, three patients underwent muscle and skin biopsy with cultured fibroblast analyses, indicating patterns of mitochondrial dysfunction.

Conclusions: A subset of 4 out of 12 CFS patients showed significantly altered peripheral oxygen extraction during ICCP. In this subgroup mitochondrial dysfunction was confirmed by skeletal muscle biopsy and cultured fibroblast analysis.

INTRODUCTION

Chronic fatigue syndrome (CFS) is characterized by abnormal fatigue for a duration of at least six months resulting in significant reductions of professional and social activities and associated with a number of minor criteria, without an obvious medical or psychiatric explanation (Fukuda et al., 1994). Hence, the diagnosis of CFS requires careful exclusion of any other explanatory condition. Post-exertional malaise and myalgia are prominent features of CFS and hence have been included into the syndromal definition of CFS (Committee on the Diagnostic Criteria (CDC)).

Previous studies have investigated a potential role of mitochondrial oxidative dysfunction in CFS (Arnold et al., 1984; Barnes et al., 1993; Gibson et al., 1993) but an anatomical or pathophysiological muscular substrate for CFS remains controversial (Fulle et al., 2000). Barnes et al. (1993) already focused on skeletal muscle bioenergetics in CFS using Phosphorous Magnetic Resonance Spectroscopy (³¹P-MRS), but failed to find consistent muscle abnormalities possibly due to heterogeneity in the CFS cohort.

Exercise intolerance, myalgia and muscular weakness are key symptoms in the recognized entity of mitochondrial myopathies (MM). Near infrared spectroscopy (NIRS) has been validated as a tool to assess microvascular O₂ extraction in skeletal muscle through measurement of muscle deoxyhaemoglobin and –myoglobin (deoxy[Hb+Mb]) in forearm muscles during an Incremental Cyclic Contractions Protocol (ICCP) (Celie et al., 2012). By using these non-invasive ICCP NIRS measurements, an unequivocal distinction could be made between healthy control subjects and MM patients suffering from a distinct mitochondrial defect (Celie et al., 2015). One recent study using NIRS in CFS patients demonstrated significantly reduced deoxy[Hb+Mb] increases upon exercise (Miller et al., 2015).

The aim of this study was to screen for mitochondrial dysfunction in a selected group of CFS patients, to compare their results with those obtained in healthy controls and to further investigate through muscle and skin biopsy in order to document histologic and biochemical correlates of noninvasively obtained indications of mitochondrial dysfunction.

METHODS

Subjects

Two samples were compared. A first group consisted of 12 patients diagnosed with chronic fatigue syndrome (CFS). The second group consisted of 12 age and gender-matched untrained healthy controls. CFS patients were recruited from the department of general internal medicine at the Ghent University Hospital, Belgium. All subjects gave written informed consent and the study was approved by the ethics committee at the Ghent University Hospital. Procedures were in accordance with the recommendations of the Helsinki Declaration. The patients' (11 female, 1 male) age, height and body weight were respectively 45 ± 8 years, 165 ± 6 cm and 71 ± 17 kg (mean values \pm SD). Control subjects' age, height and body weight were respectively 46 ± 8 years, 168 ± 7 cm and 70 ± 15 kg (mean values \pm SD).

Diagnostic decision making process CFS

For the assessment of hitherto unexplained and longstanding chronic fatigue we followed a holistic approach based on the biopsychosocial model by Wessely et al. (1999). The integrated diagnostic pathway involved internal medicine assessment, psychodiagnostic screening, rehabilitation assessment and polysomnography combined with a multiple sleep latency test as previously described (Mariman et al., 2013). The internal medicine assessment consisted of comprehensive history taking, also considering any previous medical diagnoses or investigations, and a physical examination, with additional investigations upon indication. A rehabilitation physician evaluated for the presence of any musculoskeletal comorbidity suitable for physiotherapy. Psychodiagnostic screening performed by a clinical psychologist included history taking, the administration of validated questionnaires and psychological tests. Psychiatric consultation was scheduled when the history was suggestive of former or current psychiatric disorder, and whenever hints for the presence of a psychiatric disorder emerged from the psychodiagnostic evaluation or from the multidisciplinary discussion. The outcome of the multidisciplinary discussion was a diagnostic categorization into (i) unequivocal CFS, (ii) CFS with comorbidity, or (iii) a condition that excludes CFS. In the

unequivocal CFS patients that were included in this study, no coexisting sleep or psychiatric disorders could be documented.

Measurements and data analysis

Near Infrared Spectroscopy (NIRS)

During the incremental exercise test, skeletal muscle tissue oxygenation was measured with a near infrared spectroscopy (NIRS) system (Oxiplex TS, ISS, Champaign, Illinois, USA). This system is based on an infrared light absorption method, where infrared light is emitted at different wavelengths. The NIRS-probe consisted of eight light-emitting diodes operating at wavelengths 690 and 830nm and one detector fibre bundle (source-detector distance = 2.0-3.5cm). The changes in deoxy[Hb+Mb] during each work step (i.e. the mean of the highest 10-s value) were expressed relative to the arterial occlusion amplitude (Celie et al., 2012; 2015). These calculations were made to reduce possible influence of forearm adipose tissue thickness as relative values are presented in relation to their individual maximum value.

Doppler US

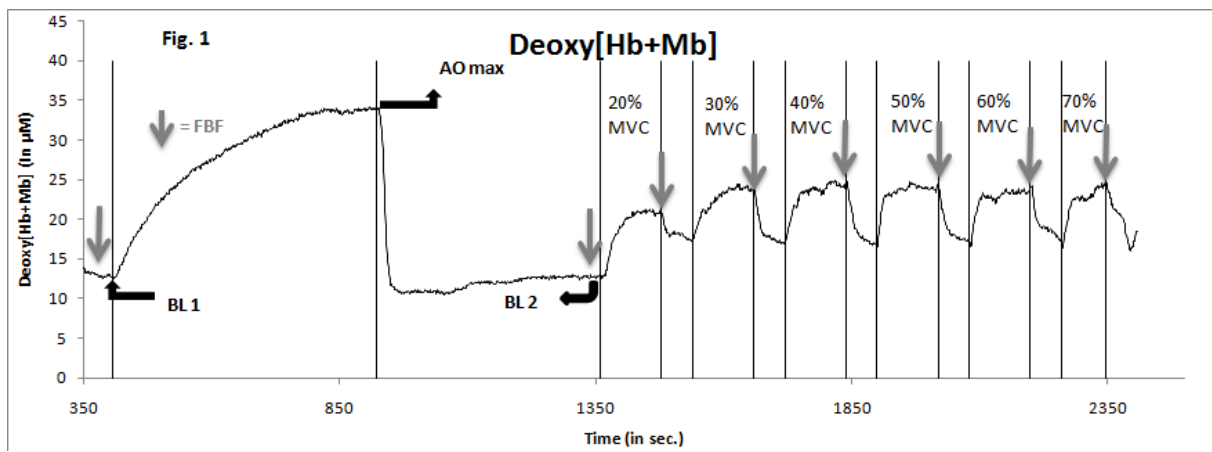
Ultrasound Doppler (Vivid 7, GE Vingmed Ultrasound, Horten, Norway) was used to measure forearm blood flow (FBF) which is a method preferred to plethysmography during exercise (Radegran, 1999). FBF was measured preceding ICCP and after every incremental step (% MVC) in the protocol. Also, for normalization, all FBF values (expressed in ml min⁻¹) during ICCP were expressed as increments above baseline values.

ICCP

The ICCP consisted of rhythmic handgrip contractions with increasing intensity, as described in previous studies (Celie et al., 2012; 2015). This test was performed in supine position using a hydraulic handgrip dynamometer (Saehan corporation, Masan, South-Korea). The NIRS probe was placed on the M. flexor carpi ulnaris and the M. flexor digitorum superficialis to

measure microvascular O₂ extraction. This protocol started with a forearm arterial occlusion by inflating the cuff to 260mmHg, of unpredetermined duration until a steady state in deoxy[Hb+Mb] was reached. The ICCP was executed until exhaustion was reached. A typical NIRS output of a healthy subject during ICCP is presented in figure 1. In order to confirm the results of the first test in the CFS population, a NIRS retest was performed in all CFS subjects three months later, in combination with brachial artery Doppler US measurements of FBF.

Fig. 1: Example of a typical deoxy[Hb+Mb] output of one healthy subject during ICCP with a schematic presentation of all measurement points (FBF and MVC). (ICCP= Incremental Cyclic Contraction Protocol; BL= Baseline; AO= Arterial Occlusion; MVC= Maximal Voluntary Contraction; FBF= Forearm Blood Flow).



Oxidative Phosphorylation (OXPHOS) investigations

Patients with abnormal deoxy[Hb+Mb] values (in %) during ICCP or a hyperkinetic limb blood flow (Taivassalo et al., 2003), were invited to undergo a skeletal muscle biopsy in order to histochemically document mitochondrial dysfunction. The biopsy samples were analyzed by light microscopic (LM) and electron microscopic (EM) examination and by biochemical analysis. Skeletal muscle specimen was analysed using standard histological, histochemical, histoenzymatic and immunohistochemical techniques for light microscopy (Raducu et al., 2015) and was further analysed by EM after fixation in 4% glutaraldehyde. After postfixation in 2% osmium tetroxide, blocks were embedded in araldite for conventional electron microscopy. Ultrathin sections contrasted with uranyl acetate and lead citrate were examined using a CM10 transmission electron microscope (FEI Europe, Eindhoven, The Netherlands) at 60 kV. Spectrophotometric analysis was used for measurement of the catalytic activities of the OXPHOS complexes (complexes I, II, II+III, III and IV) and of citrate synthase. Analyses were performed according to previously described methods (Ajit-Bolar et al., 2013). The OXPHOS function in skeletal muscle was also evaluated by Blue Native Polyacrylamide Gel-Electrophoresis (BN-PAGE) (Ajit-Bolar et al., 2013). The latter was used to separate and visualise the OXPHOS complexes in mitochondria isolated from skeletal muscle. Solubilisation of the complexes, BN-PAGE, and staining of the catalytic activities in the gel were performed as reported earlier (Van Coster et al., 2001). Patient and control samples were loaded in duplicate using equal amounts of mitochondrial protein (50 µg). The Z-score was calculated as the activity ratio for the patient sample minus the mean activity ratio for the control samples divided by the SD for the control samples. When the Z-score was lower than -1.96 or higher than +1.96, the result for the patient sample was considered as significantly different ($p < 0.05$) from the control sample.

Immuno- and fluorescent staining in cultured skin fibroblasts

The mitochondrial membrane potential was evaluated by measuring the red over green fluorescence ratio of the cationic dye 5,5', 6,6'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Invitrogen), with or without 30 ng/ml of the complex I inhibitor rotenone, following a

published procedure (De Paepe et al., 2012). To visualize mitochondria, the fluorescent Mitotracker Red CMXRos dye (Invitrogen) was used according to the manufacturer's instructions. With monoclonal antibodies, the oxidative phosphorylation complexes I (subunit NDUFA9), II (subunit IP), and IV (subunit I) (Abcam, Cambridge, MA) were immunostained and visualized using the labeled streptavidin biotin system (Dako, Glostrup, Denmark).

Statistical analysis

Statistical analysis for MVC and baseline FBF values was performed using an independent samples t-test. A Repeated Measures ANOVA was used to compare the increase in deoxy[Hb+Mb] and FBF (in %) during ICCP between the CFS population and the controls. Because test retest deoxy[Hb+Mb] results were obtained for all CFS subjects, this Repeated Measures ANOVA was first performed by using the test deoxy[Hb+Mb] values and secondly by using retest deoxy[Hb+Mb] values for comparison with healthy control subjects. When a significant main effect was found, post-hoc analysis was performed by means of an independent samples t-test. The level of significance was set at $p < 0.05$. Statistical computations were performed using SPSS[®] software (version 18; SPSS Lead Technologies Inc., Chicago, IL, USA). All data are presented as mean \pm standard deviation (SD).

RESULTS

General results

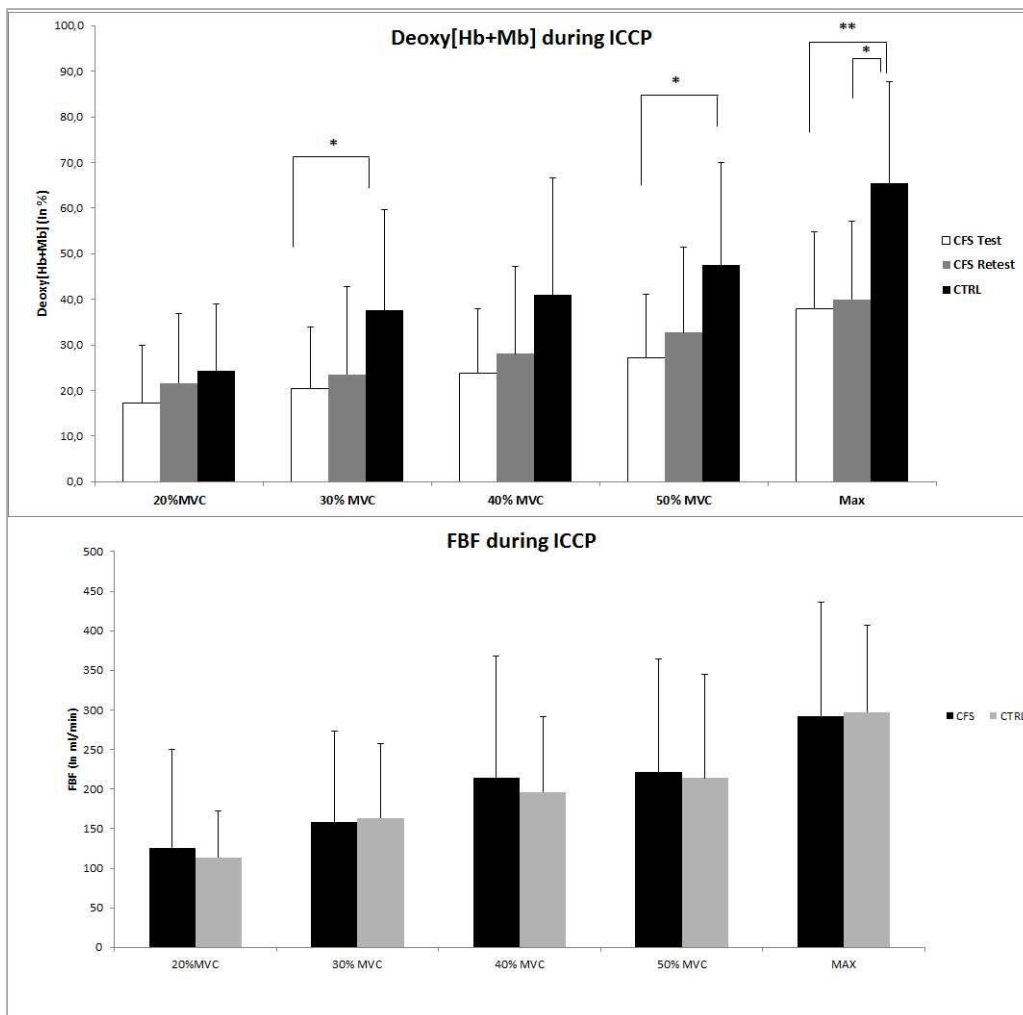
No significant difference in MVC was found between CFS patients ($\mu = 318 \pm 89N$) and healthy control subjects ($\mu = 365 \pm 67N$) ($p = 0.174$). Also, baseline FBF values did not differ significantly between both groups ($p = 0.841$). All patients tolerated the test well.

Mean submaximal and maximal deoxy[Hb+Mb] values (expressed as %) during test-retest ICCP for CFS patients and controls are presented in Figure 2a. A significant difference in deoxy[Hb+Mb] was found between CFS patients and healthy control subjects during ICCP at

both the first test ($p=0.008$) and retest ($p=0.042$). Post hoc analysis results are presented in Figure 2a.

Mean submaximal and maximal FBF values (expressed as increments to baseline in ml/min) during ICCP for CFS patients and controls are presented in Figure 2b. Statistical analysis revealed no significant interaction effect ($p=0.304$) or main effect for groups ($p=0.7$) indicating that the increase in FBF was not significantly different between both groups.

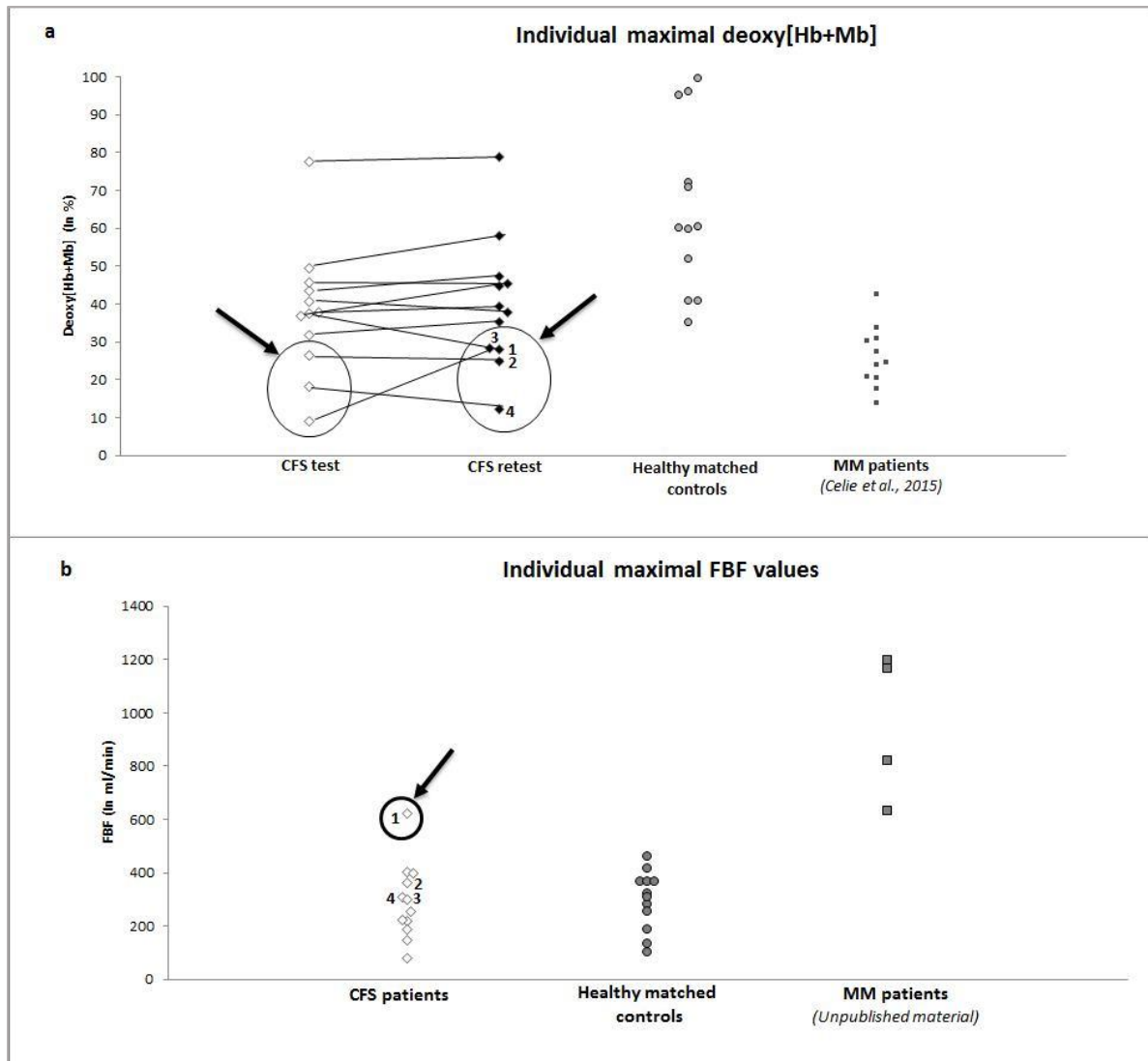
Fig. 2a&b. Mean submaximal and maximal deoxy[Hb+Mb]- test and retest values (expressed as %) (a) and FBF values (b) during ICCP for patients and control subjects. (* $p \leq 0.05$, ** $p \leq 0.01$).



Individual results

As the CFS population consisted of a small number of patients, the mean deoxy[Hb+Mb] values were significantly influenced by four CFS patients without significant increases in deoxy[Hb+Mb] at the test-retest. Moreover, after statistical analysis, one of these CFS patients had extremely high FBF values during every step of the ICCP. These FBF values were higher than mean FBF+ 2SD at every intensity. Individual maximal deoxy[Hb+Mb] and FBF values for all subjects are presented in Figure 3a and b. These findings were similar to previous observations in mitochondrial myopathy patients (Celie et al., 2015; Taivassalo et al., 2003). Three out of the four patients agreed to undergo skeletal muscle and skin biopsy for microscopic and biochemical analyses.

Fig. 3a&b. Individual maximal deoxy[Hb+Mb] (Fig 3a) and FBF (Fig 3b) values during ICCP for all CFS patients and their age- and gender matched healthy control subjects. Additionally, the individual maximal deoxy[Hb+Mb] values from MM patients⁸ in a previous study are presented. The subgroup with lower deoxy[Hb+Mb] rates is marked. (Patient 1, 2, 3 and 4 are respectively marked with 1, 2, 3 and 4)

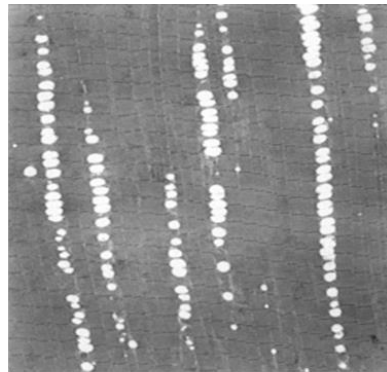


OXPHOS and immuno- and fluorescent staining in cultured skin fibroblasts analyses

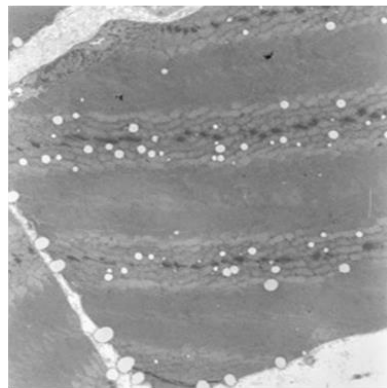
The three patients in whom a biopsy was performed, showed abnormalities in either skeletal muscle tissue or cultured skin fibroblasts. The type of anomaly however, differed between patients. In patient 1, numerous lipid droplets were observed in skeletal muscle on LM and on EM examination (Figure 4). Moreover, cultured skin fibroblasts from this patient, showed a mosaic staining pattern after addition of JC1 (plus rotenone), which is aberrant from control samples. Cells containing mitochondria with a low mitochondrial membrane potential (only green color) were detected among other cells enclosing a mixture of mitochondria with high and low mitochondrial membrane potential (red and green color) (See Figure 5). While the overall activities of the respiratory chain complexes were normal when analyzing skin fibroblast homogenate, this mosaic staining pattern was confirmed by immunostaining of cultured skin fibroblasts with an antibody against a subunit in complex IV. Biochemical analysis in a skeletal muscle homogenate revealed a slight decrease of the activity of complex IV, but only when expressed as ratio over the activity of complex II (see Table 1). Compared to patient 1, only a slight increase of small lipid droplets was seen in patient 2. Notwithstanding, in cultured skin fibroblasts, a mosaic staining pattern was seen after addition of JC1 (plus rotenone). The amount of cells that contained only mitochondria with low mitochondrial membrane potential was, however, less than in patient 1. Catalytic activities of the respiratory chain complexes were normal in the homogenates of skeletal muscle and cultured skin fibroblasts. While microscopic examination did not reveal any abnormality in skeletal muscle tissue from patient 3, biochemical analysis revealed a significant decrease of complex I and complex II+III activity. In contrast to both previous patients, no mosaic staining pattern in cultured skin fibroblasts was observed after staining with JC1 (plus rotenone). However, immunostaining with an antibody against a subunit of complex I, and with an antibody against a subunit of complex IV demonstrated significantly lower Cross Reacting Material (CRM) than control samples. Complex IV activity was significantly decreased in a cultured skin fibroblast homogenate (complex I was not measured).

Fig. 4: LM and EM investigation of skeletal muscle tissue in the three CFS patients with abnormal ICCP deoxy (Hb+Mb) profile.

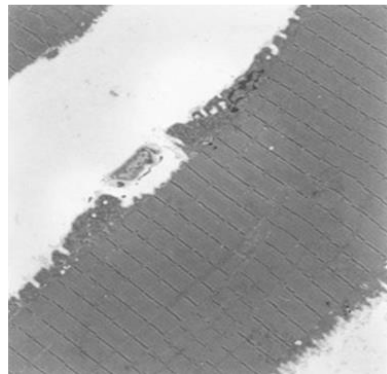
Patient 1



Patient 2

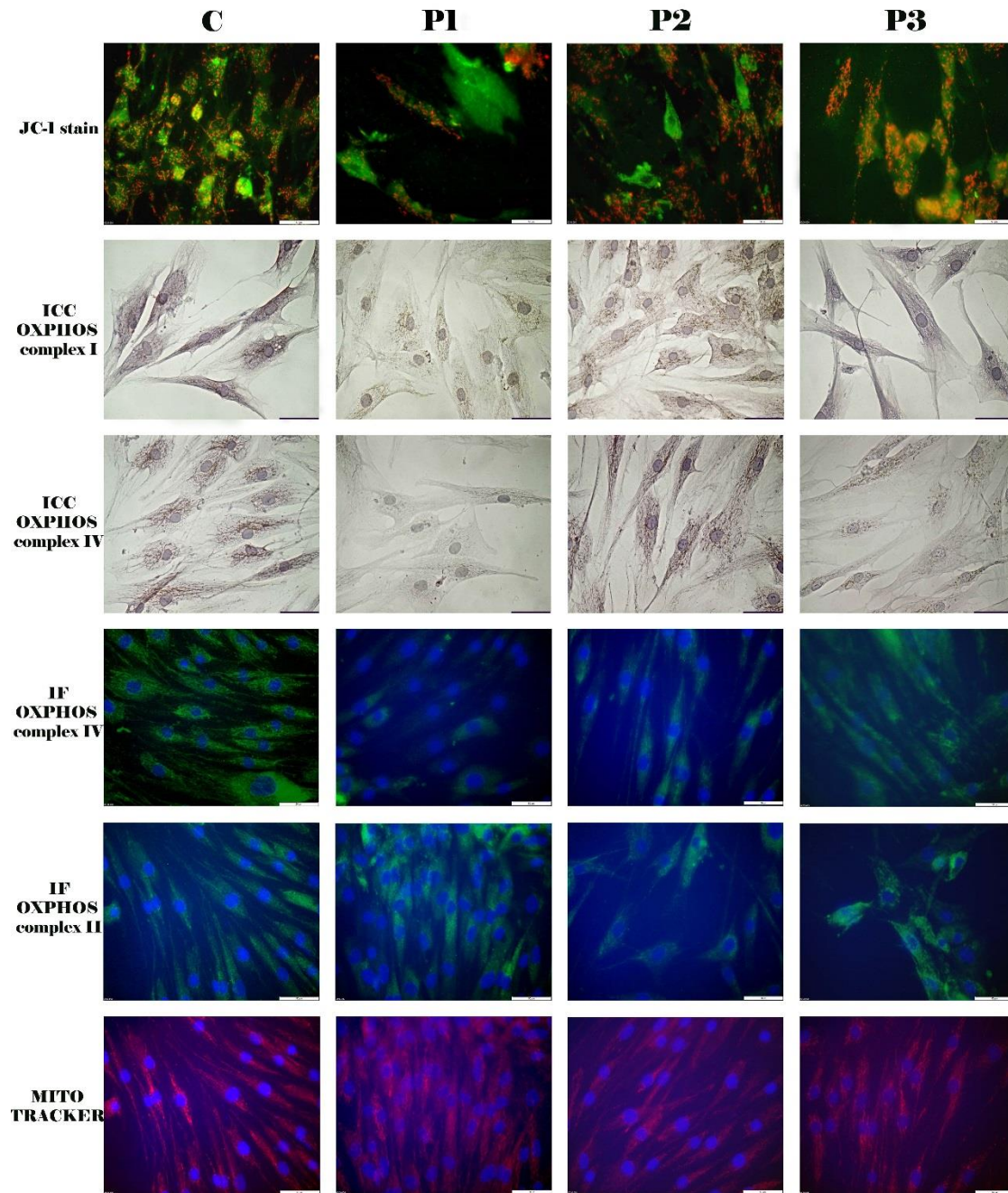


Patient 3



- *Pt 1: Parallel arranged intermyofibrillar rows of multiple lipid droplets are illustrated in a longitudinal cut muscle fibre*
- *Pt 2: A transversely oriented muscle fibre shows a relative abundance of lipid droplets with variable sizes under the sarcolemma and between the myofibrils.*
- *Pt 3: normal amounts of smaller lipid droplets appear in the biopsy such as seen in a longitudinal muscle fibre*

Fig 5: Immuno- and fluorescent staining in cultured skin fibroblasts with JC1 (plus rotenone) in the three CFS patients (P1-3) with abnormal ICCP deoxy (Hb+Mb) profile as compared to control (C).



- JC-1 fluorescent staining after rotenone treatment shows equal distribution of active (red) and inactive (green) mitochondria in C and P3; P1 and P2 display a mosaic staining pattern with a subset of cells containing only inactive (green) mitochondria.
- Immunocytochemical staining (ICC) for oxidative phosphorylation (OXPHOS) complex I subunit 39kd and complex IV subunit I (DAB, brown), nuclei counterstained with hematoxylin (blue), shows reduced staining for complex IV in P1, and complex I and complex IV in P3.
- Immunofluorescent staining (IF) for complex IV subunit I and complex II subunit IP (AlexaFluor488, green), nuclei counterstained with dapi (blue), shows a mosaic staining pattern for complex IV in P1.
- Normal MitoTracker CMXRos staining (red) is present in all cell lines, nuclei counterstained with dapi (blue)

Table 1: Spectrophotometric analysis of the activities of the OXPHOS complexes in skeletal muscle and cultured fibroblast tissue homogenate for the three CFS patients. Abnormalities are presented in bold. (* Results expressed as the logarithm of OXPHOS activity divided by the logarithm of citrate synthase (CS) activity. Control sample ratios are given as mean \pm SD. Deficient activities are considered when the Z-score is $<\pm 1.96$.)

Tissue homogenate	Patient	Complex I I/CS I/II	Complex II II/CS II/II	Complex II+III/ II+III/CSII+III/II	Complex III III/CS III/CS	Complex IV IV/CS IV/II	Citrate synthase*
Skeletal muscle	P1	0.70 0.90	0.78 1.00	0.77 0.99	0.90 1.16	0.96 1.23	181
	P2	0.61 <i>0.80</i>	0.76 1.00	0.76 1.00	0.93 1.21	1.00 1.32	166
	P3	0.46 0.74	0.62 1.00	0.48 0.79	0.90 1.47	1.04 1.69	71
	Controls (n=30)	0.64 \pm 0.06 0.95 \pm 0.10	0.68 \pm 0.04 1.00 \pm 0.00	0.68 \pm 0.04 1.01 \pm 0.06	0.89 \pm 0.07 1.32 \pm 0.13	1.00 \pm 0.06 1.48 \pm 0.10	174 \pm 70
Cultured skin fibroblasts	P1	ND	0.67 1.00	0.63 0.94	0.88 1.31	1.05 1.57	75
	P2	ND	0.66 1.00	0.70 1.06	0.97 1.47	0.95 1.44	104
	P3	ND	0.68 1.00	0.66 0.97	0.74 1.10	0.77 1.14	76
	Controls (n=30)	ND	0.61 \pm 0.05 1.00 \pm 0.00	0.66 \pm 0.07 1.07 \pm 0.11	0.87 \pm 0.07 1.42 \pm 0.14	0.96 \pm 0.05 1.57 \pm 0.14	82 \pm 15

DISCUSSION

This study is the first to determine biochemical and histological correlates of mitochondrial abnormalities in relation to NIRS and Doppler US non-invasive assessment of muscle oxidative metabolism in CFS patients. A subgroup of CFS patients was identified with significantly decreased oxygen extraction using an ICCP standardized exercise protocol. The deoxy[Hb+Mb] pattern of these patients was similar to the NIRS pattern observed in patients with documented mitochondrial myopathy (Celie et al., 2015). The results of the present study are in line with the study of Miller et al. (2015), who were the first to demonstrate a significantly altered oxygenation pattern in CFS patients, although without blood flow measurements. In contrast to the study of Miller et al. (2015), mitochondrial dysfunction was determined in a subgroup of three CFS patients, with blood flow measurement and histologic and biochemical correlates. In the present study mitochondrial dysfunction was documented in the subgroup with decreased oxygen extraction in skeletal muscle and cultured skin fibroblasts. In patient 1, a clear increase of the amount of fat droplets was observed in skeletal muscle whereas in cultured skin fibroblasts, a mosaic pattern was seen following staining of the mitochondria with JC1 (plus rotenone). The increased amount of fat droplets, observed in skeletal muscle tissue and the mosaic pattern, observed in cultured skin fibroblasts, were however less pronounced in patient 2. Notwithstanding the divergent degree of anomalies, an equal pattern could be recognized in these two patients. A mosaic staining pattern is usually observed in patients suffering from mitochondrial DNA abnormalities (e.g. point mutation, deletion or depletion of the mitochondrial DNA). Hence, the results of patient 1 and 2 are indicative for the presence of such a mitochondrial abnormality. In patient 3 however, a different pattern could be recognized as no skeletal muscle microscopic abnormalities could be detected. On the other hand, complex I deficiency was established biochemically, and, more surprisingly, a deficient complex IV activity in cultured skin fibroblasts (no complex I measurements were performed in fibroblasts). Complex I and complex IV contain the largest number of mitochondrial encoded subunits. Deficiencies of either complex I, or complex IV, or a combined deficiency of complex I and IV, are usually caused by a disturbed intramitochondrial synthesis of the mitochondrial encoded subunits. The significantly decreased activity of complex II + III (succinate cytochrome 3 reductase), in combination with

a normal activity of the individual complexes II and III, is rather suggestive for a decreased amount of functionally active co-enzyme Q.

The heterogeneity in NIRS response and the possible existence of different subgroups among CFS patients may explain previously reported contrasting results in physiologic responses including reduced muscle strength (Fulcher and White, 2000; Wagemakers et al., 1988), maximal oxygen consumption (De Becker et al., 2000; Sargent et al., 2002), muscle metabolism (Fukuda et al., 1994; Arnold et al., 1984; Barnes et al., 1993; Gibson et al., 1993; Fulle et al., 2000) and blood flow (McCully et al., 1996; 2003; 2004; McCully and Natelson, 1999). A typical example of these discrepancies in the existing literature are conflicting reports on blood flow in CFS, ranging from reduced blood flow (McCully et al., 1996; 2004; McCully et al., 1999), attributed to autonomic dysregulation or reduced capillary density (Lindh et al., 1995) to normal blood flow as compared to healthy subjects (McCully et al., 2003). In the present study FBF was either normal or in one patient significantly higher, potentially indicating a compensatory hyperkinetic muscle circulation during exercise (Taivassalo et al., 2003), as observed in MM with increased capillary areas (Taivassalo et al., 2012). CFS represents a mere syndromal description lacking validated pathophysiology and diagnostic tests. The particular experimental approach in the present study seems to allow the identification of a subgroup of CFS patients with mitochondrial abnormalities.

A possible confounder may be identified in the concomitant use of metformin in 2 out of the three CFS patients with the observed histologic and biochemical abnormalities suggestive of mitochondrial dysfunction. This biguanide has been shown to inhibit complex I activity of the mitochondrial respiratory chain (El Mir et al., 2000; Owen et al., 2000). However, exercise intolerance and muscular pain preceded the initiation of metformin in these patients. Clearing this potential issue of adverse effects is important, in view of the widespread advocacy for early treatment and documented beneficial effects of metformin in type 2 diabetes mellitus and even in the stage of peripheral insulin resistance (Tran et al., 2015). These promising findings need to be confirmed in a randomised prospective assessment of a larger sample of CFS patients with and without metformin, prior to and during metformin treatment.

In conclusion, NIRS and Doppler US measurements during a non-exhaustive ICCP, can identify a subgroup of CFS patients with potential mitochondrial abnormalities.

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PART III

GENERAL DISCUSSION

The purpose of this research was to develop a non-invasive and non-exhaustive screening protocol for malfunctioning peripheral oxygenation in Chronic Fatigue Syndrome (CFS) and Mitochondrial Myopathy (MM) populations, complaining about muscle weakness and exercise intolerance. Therefore, non-invasive measurements on forearm muscles during non-exhaustive handgrip exercise were proposed. As sustained dynamic cyclic contractions are preferable when aiming to challenge the oxidative metabolism, an Incremental Cyclic Contraction Protocol (ICCP) was developed in different steps and tested in pathological populations. These steps will consequently be discussed in this part to assess the applicability and quality of this research.

1. METHODOLOGICAL ISSUES OF NEAR INFRARED SPECTROSCOPY (NIRS)

As the main purpose of this research was to use an exercise protocol in clinical populations, we aimed to develop a non-exhaustive test with non-invasive measurements to gain more insight into their peripheral oxygenation mechanisms. Therefore, NIRS technology was used to estimate O_2 extraction at muscle level during exercise. Because NIRS technique is not the gold standard method to measure O_2 extraction, there were some methodological barriers requiring further investigation before using this instrument in patient populations. This is of particular importance because reliability and validity of NIRS measurements have not been shown on forearm muscles when performing handgrip exercise. Of course, handgrip exercise was an important item in this study as exhaustive exercise should be avoided in MM and CFS patients, due to the possible comorbidity of cardiomyopathy in MM patients and the fact that disuse of leg muscles is an important issue in MM and CFS patients. Such a chronic physical inactivity can be a contributing or disturbing factor of the distinctive response to whole body exercise in MM and CFS patients. Also, complaints of upper limb muscle pain and exercise intolerance are a common feature in CFS patients (Ickmans et al., 2014). Therefore, it was very interesting to apply NIRS technology on upper limb muscles during exercise. Moreover, it is important to highlight the role of using relative deoxyhaemoglobin and myoglobin (deoxy[Hb+Mb]) values during ICCP as reliability of these measurements have, to our knowledge, never been established in the past. These relative NIRS values are the result of a physiological calibration of the NIRS measurements which was carried out by applying a cuff,

inflated above the systolic blood pressure, on the upper arm. As a consequence, absolute oxyhaemoglobin and myoglobin (oxy[Hb+Mb]) and O₂ saturation (SmO₂) will decrease and deoxy[Hb+Mb] will increase until a steady state is reached. The NIRS response during arterial occlusion was calculated as the difference between maximal (deoxy[Hb+Mb])/minimal (oxy[Hb+Mb] and SmO₂) and resting values and was set as the 100% value. Every response to exercise (i.e. difference between maximal /minimum and baseline value) was set relative to the arterial occlusion maximal/minimal values. This latter technique was already used in some studies (Mancini et al., 1994; Wilson et al., 1989; Esaki et al., 2005; Grassi et al., 2007) and could offer a possible solution to eliminate other influences like subcutaneous Adipose Tissue Thickness (ATT) and blood flow (O₂ delivery). However, to our knowledge, this was the first study investigating the reliability of these relative deoxy[Hb+Mb] values on forearm muscles during exercise. Moreover, the impact of using these relative deoxy[Hb+Mb] values to eliminate the influence of subcutaneous ATT and FBF was, to our knowledge, never studied before.

1.1 Reliability of NIRS parameters during this ICCP

When aiming to study pathological populations, using NIRS as a technique to measure microvascular O₂ extraction, it is thoroughly important to investigate reliability and possible disturbing influences on this NIRS signal. The reliability and validity of this technique has already been established in previous studies, although in most cases on brain tissue or in large muscle groups (Kell et al., 2004; Pereira et al., 2007). Additionally, these studies did not execute an arterial occlusion before the exercise protocol to physiologically calibrate NIRS parameters. Therefore, it was of major importance to investigate the reliability of these relative deoxy[Hb+Mb] values towards the arterial occlusion maximum in forearm muscles during the ICCP. In agreement with most of the NIRS reliability studies (Kell et al., 2004; Pereira et al., 2007), it was shown in study 1 that these relative deoxy[Hb+Mb] values as well as the SmO₂ values were reliable parameters during the ICCP. However, it is important to notice that reliability at the lower intensities were absent or small, probably due to the heterogeneous deoxy[Hb+Mb] response. Moreover, the lower intensities were also used for familiarisation of the protocol and this could be another possible explanation for the low ICC values. At the

higher and maximal relative deoxy[Hb+Mb] intensities, there was a good reliability ICC value. Because the ICC values are not sufficiently to show agreement between the test and retest, Bland Altman plots for every intensity are presented in Figure 1, 2, 3, 4, 5 and 6. In general, some outliers can be recognized in these plots. Hence, as a first step in this research it was shown that these relative deoxy[Hb+Mb] values, measured during ICCP, could be reliably used to gain more insight into peripheral O₂ extraction mechanisms.

Fig. 1: Test-retest Bland Altman plot for 20% MVC

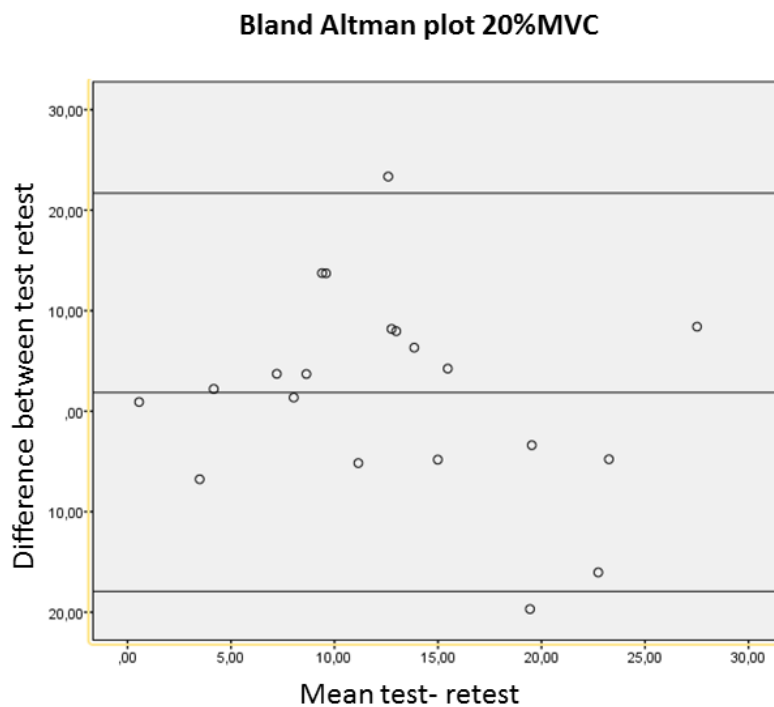


Fig. 2: Test-retest Bland Altman plot for 30% MVC

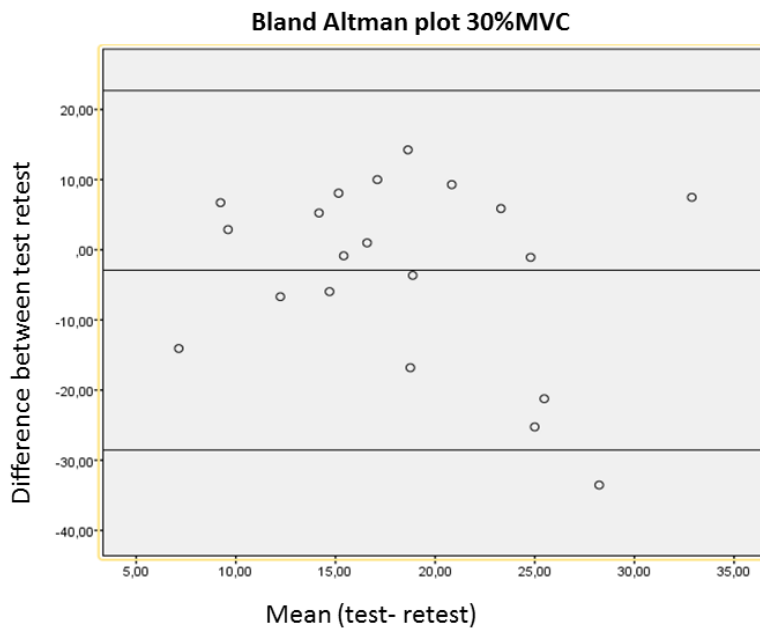


Fig. 3: Test-retest Bland Altman plot for 40% MVC

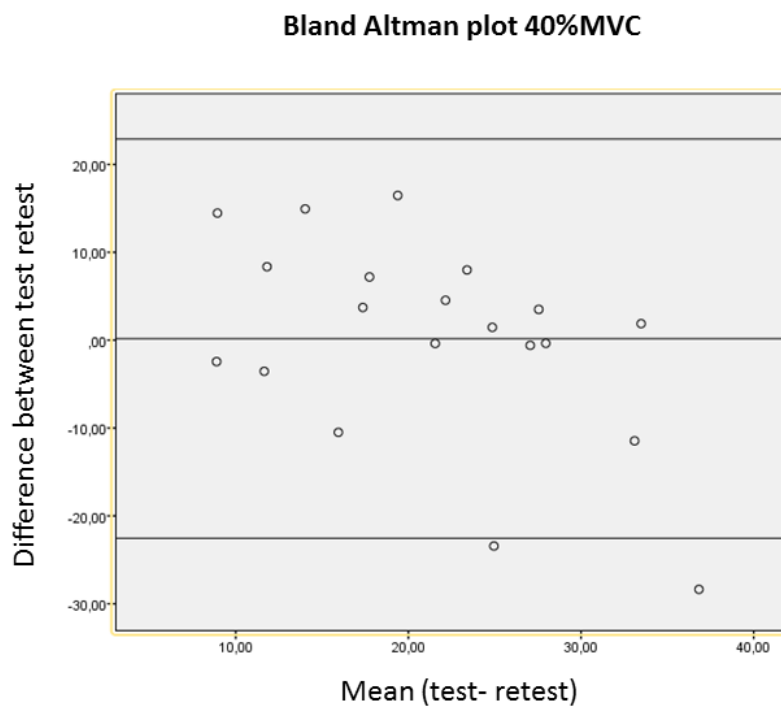


Fig. 4: Test-retest Bland Altman plot for 50% MVC

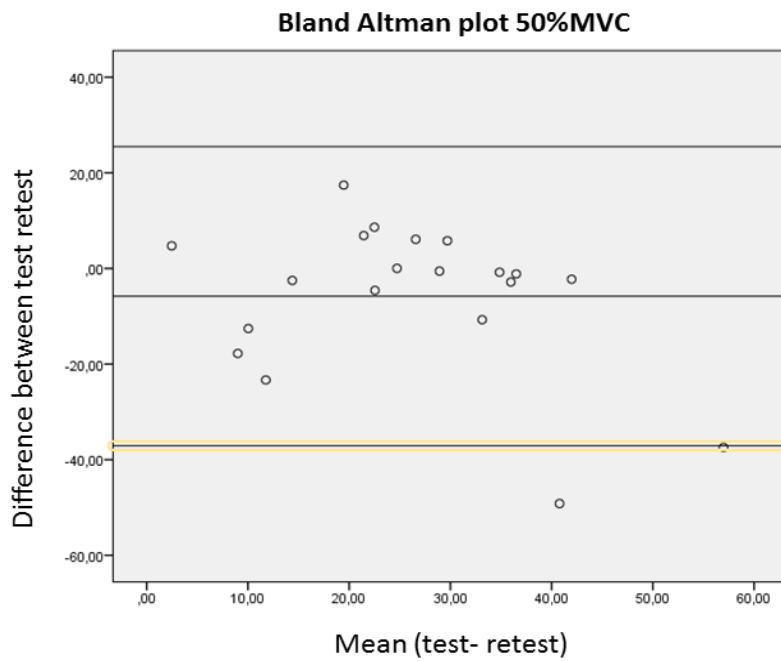


Fig. 5: Test-retest Bland Altman plot for 60% MVC

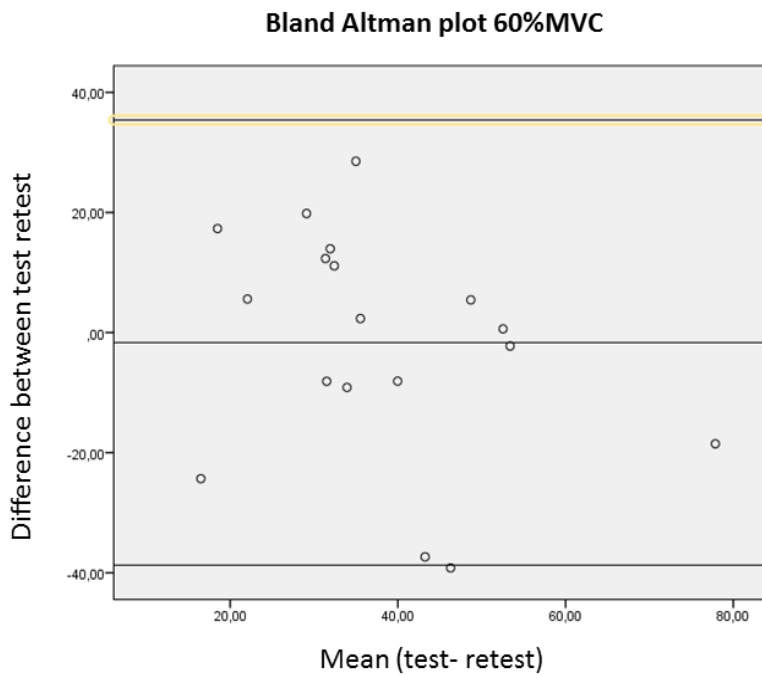
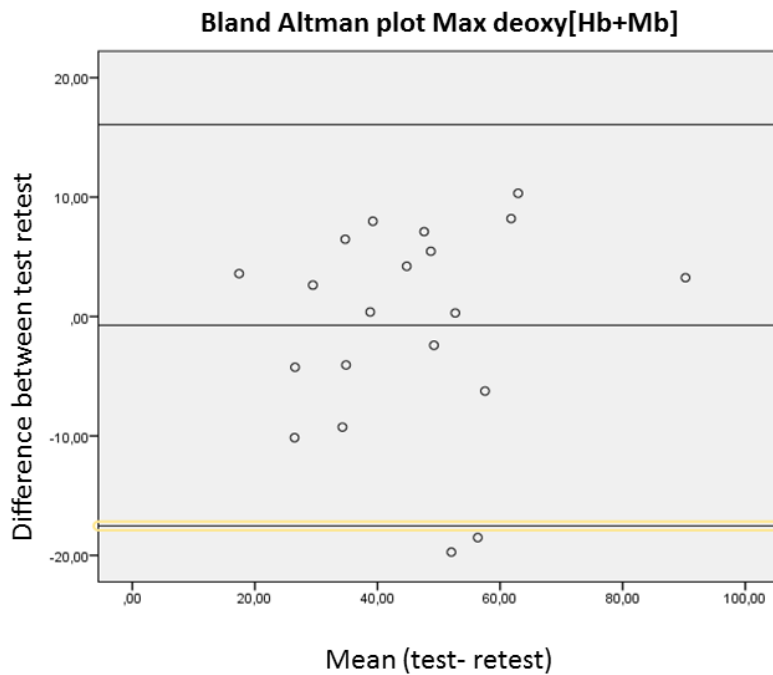


Fig. 6: Test-retest Bland Altman plot for maximal deoxy[Hb+Mb]

1.2 Possible influence of subcutaneous ATT and blood flow on NIRS measurements

In study 2 of this research, it was observed that all absolute NIRS parameters (in μM) except tHb were influenced by subcutaneous ATT indicating that ATT might blunt the NIRS responses to exercise. However, when using the relative deoxy[Hb+Mb] values, the impact of subcutaneous ATT was no longer present. Hence, the statement that it is impossible to use NIRS in clinical populations as a consequence of ATT influence (Ferrari et al., 2011) should be refined, in particular because this physiological calibration and non-exhaustive handgrip exercise offer an increased applicability of the NIRS technique in patients. A plausible explanation for this influence on absolute parameters could be the fact that, when ATT at the measurement site was between 6mm and 14 mm, the NIRS absorption coefficient (μa) quickly decreased (Wolf et al., 2003). In this study, mean subcutaneous ATT ($\pm\text{SD}$) for all subjects was 6.5 ± 3.4 mm, indicating that μa was decreased in the studied population, thereby possibly blunting the NIRS signal. Of course, it is very important to be aware of the depth sensitivity of the NIRS device, because there are a lot of different NIR technologies. Generally taken, penetration depth of the infrared signal is half of the interoptode distance of the NIRS probe (Ryan et al., 2012). As mentioned in the methods part, our NIRS device has source detector distances

ranging from 2.0 – 3.5 cm, which means that depth sensitivity ranges from 1.0 – 1.75 cm. As a second step in this research, it was of major importance to investigate whether it was possible to exclude the influence of subcutaneous ATT by performing an arterial occlusion as physiological calibration. Otherwise, it would not have been correct to execute this ICCP in subjects with a large skinfold (>6mm) at the site of the NIRS probe. As was shown in this study, it is possible to exclude subcutaneous ATT influence by using relative oxy[Hb+Mb] or deoxy[Hb+Mb] values.

It has also been shown in study 2 that FBF influences all absolute NIRS parameters, except the relative oxy- and deoxy[Hb+Mb] values. A significant relation was observable as well, between absolute deoxy[Hb+Mb] and tHb on the one hand, and the maximal force at exhaustion on the other hand. Moreover, these maximal force values correlated significantly positive with maximal FBF values. First of all, it is crucial to emphasize that the possible influence of FBF on NIRS parameters requires a different approach than the possible influence of subcutaneous ATT. It was the aim of this study to investigate the possible relationship between FBF on the one hand, and absolute and relative NIRS parameters on the other hand, specifically in an explorative way. This was in particular because the relation between these measurements was carried out in only one preceding study (Fadel et al., 2004). A higher maximal force at exhaustion implicates an improved functional capacity of the muscle to contract at a higher load, especially because a higher maximal force while performing cyclic contractions increasingly elicits the aerobic energy delivery system. The higher maximal FBF value represents an improved functional capacity at muscle level as well, probably due to an increased capillary density and modifications in dilator function related to endothelial change (Delp and Laughlin, 1997; Sinoway et al., 1986; Smolander, 1994; Snell et al., 1987). As this ICCP is based on rhythmic dynamic contractions to predominantly challenge the oxidative metabolism, the relation between both values (i.e. FBF and maximal force) is obvious. Whereas MVC -and thus incremental load- during ICCP is higher, FBF values will be increased too in these individuals, probably to have an expanded passage of O₂, free fatty acids and glucose. The hypothetical question of the repercussions of these divergences in maximal force and FBF on NIRS parameters, remains unanswered. Possibly, individuals with a higher MVC and FBF may have a higher amount of capillaries that influences the NIR penetration at muscle

level. In these individuals, capillary blood flow will be increased at the site where deoxy[Hb+Mb] is measured. Moreover, it has been shown that haemoglobin flux at capillary level increases during exercise up to two- or threefold (Kindig et al., 2002; Copp et al., 2009). This abundantly higher capillary O₂ supply at the site of the NIRS probe means that microvascular haemoglobin flux will be increased at that location. As such, larger absolute deoxy[Hb+Mb] increases will be possible during exercise compared to subjects with a lower haemoglobin flux at the measurement site. Consistent with this explanation, absolute deoxy[Hb+Mb] measurements will depend on intensity and capillary haemoglobin flux. It is however questionable, whether it is favourable to measure this higher capillary haemoglobin flux, due to improved functional capacity rather than clinical related peripheral problems in some pathologic populations. Therefore, it is interesting to observe that the relationship between intensity (MVC) and O₂ delivery on the one hand, and NIRS parameters on the other hand, disappears by using relative values. In our opinion, the use of absolute or relative oxy[Hb+Mb] and deoxy[Hb+Mb] depends on the experimental purpose of the study.

When aiming to test clinical populations for detection of certain muscle abnormalities, one should definitely use relative values as it excludes possible (de)conditioning effects in muscle oxygenation due to an improved or impaired functional capacity of the muscle. It is of major importance to investigate these issues, especially when aiming to execute these measurements during ICCP in patient populations, where (de)condition effects have been used in the past to explain possible differences as a secondary cause. Hence, to exclude these possible (de)conditioning effects, it could be strongly advised to use the relative deoxy[Hb+Mb] parameters in patient populations.

In the first part of this research, the appropriate use of the NIRS measurements during ICCP were analysed. It was shown that SmO₂ as well as relative deoxy[Hb+Mb] were reliable measurements during this protocol. However, SmO₂ values during ICCP were significantly blunted with increasing ATT values and could therefore, not be used in further analyses of subjects. The relative deoxy[Hb+Mb] values during ICCP were reliable measurements to assess microvascular O₂ extraction in diverse populations with minimal influence of ATT, deconditioning effects (maximal force at exhaustion) and limb blood flow. Consequently,

these ICCP relative deoxy[Hb+Mb] measurements could be reliably used to analyse healthy and clinical populations, thereby eliminating the blunting ATT effect on the NIRS signal and blood flow (O_2 delivery) influence.

2. RELATIVE DEOXY[Hb+Mb] IN MM PATIENTS

The conclusions, drawn in this previous part, allowed us to start using ICCP relative deoxy[Hb+Mb] measurements in patient populations. Before using this experimental set up as a screening tool for mitochondrial abnormalities in cohorts complaining about muscle pain and weakness, post-exertional malaise and exercise intolerance, it was necessary to understand arterial occlusion responses of a Mitochondrial Myopathy (MM) population and to investigate whether severe MM could be identified using ICCP measurements.

2.1 Response during and following arterial occlusion

In the second part of this study, significant differences were found in the deoxy[Hb+Mb] signal during and after arterial occlusion, as well as during ICCP, in patients suffering from severe MM with clear mitochondrial dysfunction at muscle level. As such, the kinetics of MM patients were significantly slower compared to healthy subjects during arterial occlusion. A possible explanation for these divergent kinetics could be the heteroplasmic nature of this mitochondrial disorder, whereby a mixture of pathogenic mtDNA and wild type mtDNA is present at muscle level (Durham et al., 2007; DiMAuro, 2010). During brachial arterial occlusion, the circulation of red blood cells in forearm muscles will be interrupted. As a consequence of this blood pooling, O_2 will be transported to the few functioning mitochondria, where O_2 will be consumed. Hence, this process will take significantly more time in MM patients compared to healthy control subjects, as this clinical population has a reduced amount of functioning mitochondria. This may explain the slower kinetics of deoxy[Hb+Mb] values during arterial occlusion in MM patients. In Figure 7 and Figure 8, this explanation was presented schematically.

Fig. 7: Schematic presentation of the O_2 cascade at skeletal muscle level during arterial occlusion while measuring NIRS in healthy subjects (PO_2 =oxygen partial pressure; $oxy[Hb]$ = oxyhaemoglobin; $deoxy[Hb]$ = deoxyhaemoglobin; $oxy[Mb]$ =oxymyoglobin; $deoxy[Mb]$ =deoxymyoglobin; mt = mitochondria).

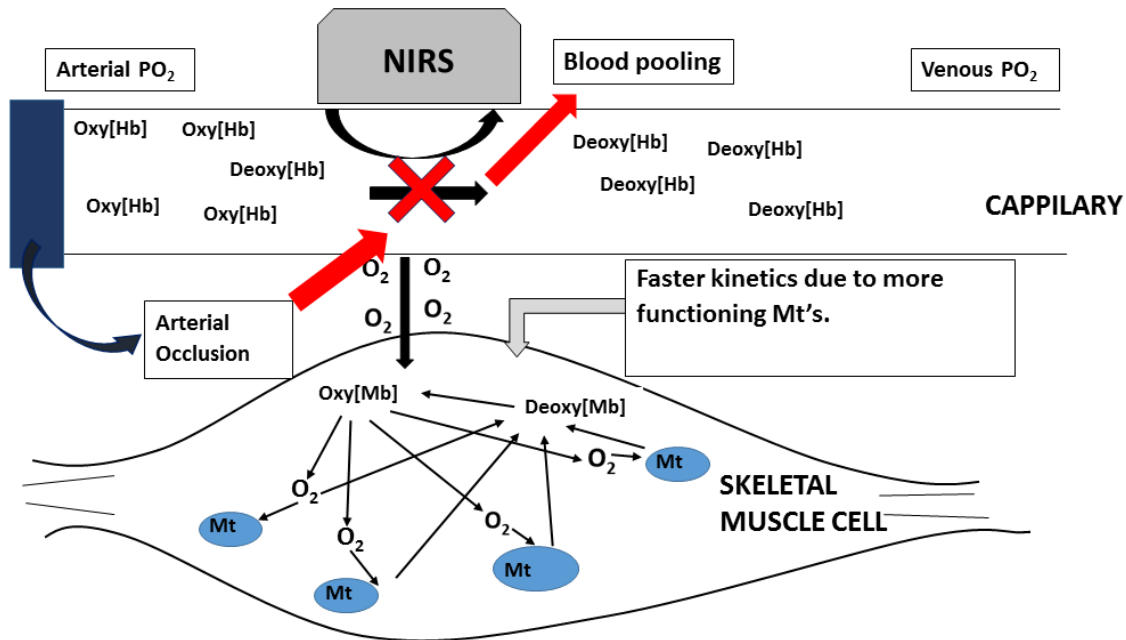
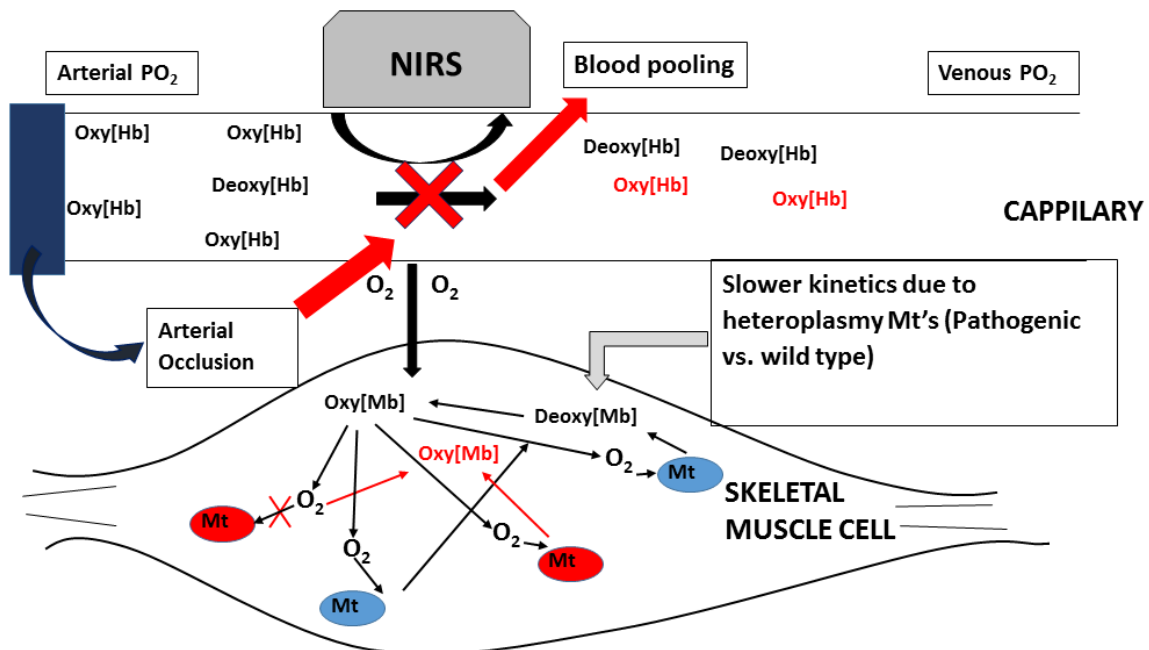


Fig. 8: Schematic presentation of the O_2 cascade at skeletal muscle level during arterial occlusion while measuring NIRS in MM patients (PO_2 =oxygen partial pressure; $oxy[Hb]$ = oxyhaemoglobin; $deoxy[Hb]$ = deoxyhaemoglobin; $oxy[Mb]$ =oxymyoglobin; $deoxy[Mb]$ =deoxymyoglobin; mt = mitochondria).



A second finding in study 3 were the faster post-occlusive oxy[Hb+Mb] and deoxy[Hb+Mb] responses in the MM population compared to healthy controls. This means that post-occlusion reoxygenation took place earlier in the MM cohort, thereby demonstrating a faster O₂ delivery following cuff release. A faster reoxygenation is an indication for a decreased skeletal muscle oxidative capacity (Kime et al., 2003; Callewaert et al., 2012) due to mitochondrial dysfunction in the MM patient population. By performing an arterial occlusion in MM patients in study 3, two determinants could be identified as a reflection of the decreased muscle oxidative capacity, i.e. slower deoxy[Hb+Mb] kinetics during arterial occlusion and a faster reoxygenation following cuff release.

2.2 During ICCP

In study 4, it has been shown that MM patients had a significantly altered pattern of relative deoxy[Hb+Mb] during ICCP, compared to healthy control subjects. These results were in line with all other studies comparing exercise responses between MM patients and healthy control subjects during whole body (Dandurand et al., 1995; Dysgaard Jeppesen et al., 2003; Grassi et al., 2009; Siciliano et al., 1999; Taivassalo et al., 2003) or handgrip (Hanisch et al., 2006; Jensen et al., 2002; Taivassalo et al., 2002; Van Beekvelt et al., 2001) exercise. From these latter studies, only two of them carried out exercise tests with non-invasive NIRS measurements (Grassi et al., 2007,2009; Van Beekvelt et al., 1999). To our knowledge, Van Beekvelt et al. (1999) was the only past study carrying out a handgrip exercise test analysing NIRS measurements on forearm muscles. However, some methodological concerns could be raised when considering possible influences on the NIRS signal, i.e. ATT and O₂ delivery. In a second study, these authors reported that they did not account for possible differences in subcutaneous ATT among the subjects (Van Beekvelt et al., 2001). With a sensitivity and specificity of both 91% in study 4, it could be stated that equal values were found using these measurements, compared to invasive methods for detection of decreased oxidative capacity. Also, relative deoxy[Hb+Mb] response was quite homogeneous in our study as the MM population had a relatively small response range (17.2% to 34.4%) at maximal intensity. Hence, in this study, a contribution was made in order to solve two major issues concerning

this research area, i.e. the need of non-invasive and not-intensive screening tools and the need for reliable measurements with sufficient sensitivity and specificity.

In an attempt to explain these divergences between both populations, it is important to refer to the first part of this dissertation on peripheral oxygenation. As elucidated, scientific evidence suggests that skeletal muscles possess the property to create an “O₂ depleted region” during exercise, thereby improving O₂ passive diffusion capacity (Gayeski and Honig, 1985, 1987; Honig et al., 1997; Voter and Gayeski, 1995; Richardsson et al., 1995a,b, 1998). A crucial element in this theory is the O₂ desaturation of intramuscular myoglobin to create this O₂ depletion during exercise. Myoglobin is an important intramuscular protein to transport O₂ to the mitochondrial reticulum. This mitochondrial reticulum comprises the entity of cytosolic mitochondria, forming a dynamic partially interconnected reticular network which spreads over the whole cytosolic volume excluding the nucleus (Sukhorukov et al., 2012). During exercise, O₂ delivery to the mitochondrial reticulum will be increased and intramuscular myoglobin desaturation will take place, thereby creating an “O₂ depleted region”. It should be questioned however, how this theory of a “depleted O₂ region” could be applied to a MM population with severe mitochondrial dysfunction. It could be hypothesized that the intramuscular myoglobin O₂ desaturation does not completely occur in MM patients as a consequence of clear mitochondrial malfunctioning. Consequently, O₂ passive diffusion from haemoglobin to the myocyte will be significantly restricted and microvascular O₂ extraction will be reduced as well. Interestingly, this inability of passive O₂ diffusion to the muscle can be detected when measuring relative deoxy[Hb+Mb] values during ICCP. In Figure 9 and Figure 10, divergences in capillary-to-myocyte O₂ transport are schematically presented for both healthy control subjects and MM patients.

Fig. 9: Schematic presentation of the O_2 cascade at skeletal muscle level during ICCP while measuring NIRS in healthy subjects (PO_2 =oxygen partial pressure; $oxy[Hb]$ = oxyhaemoglobin; $deoxy[Hb]$ = deoxyhaemoglobin; $oxy[Mb]$ =oxymyoglobin; $deoxy[Mb]$ =deoxymyoglobin; mt = mitochondria).

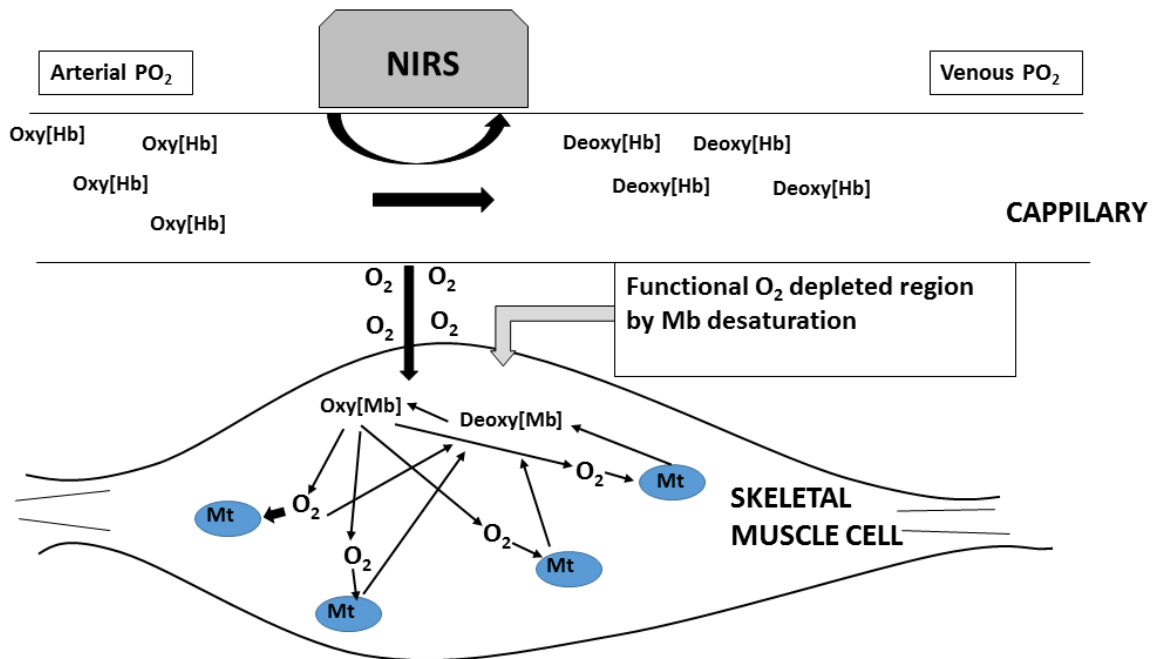
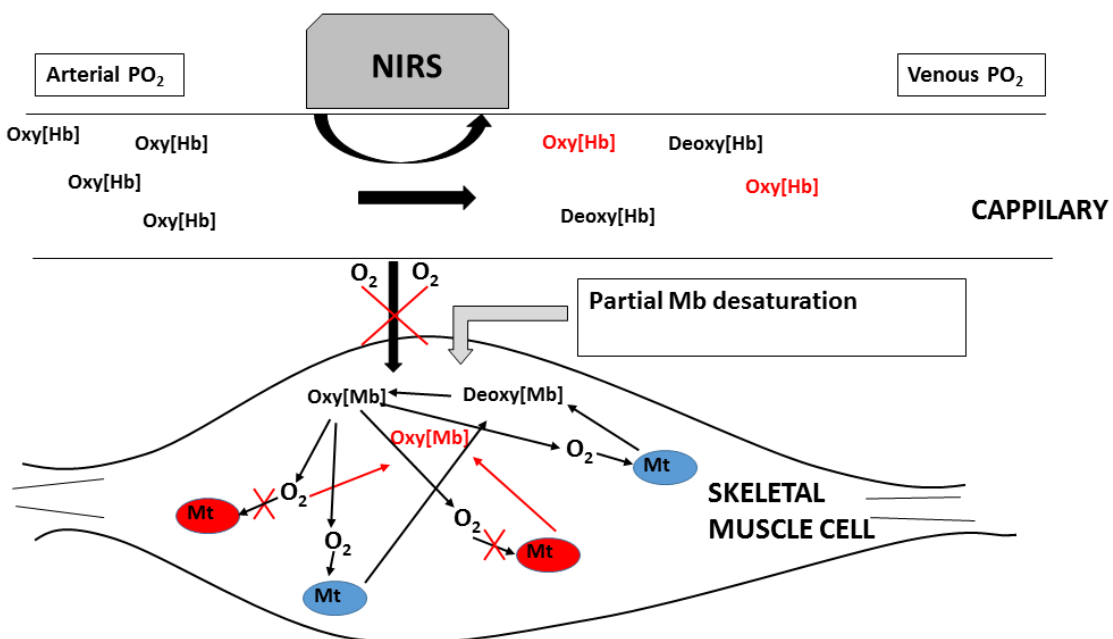


Fig. 10: Schematic presentation of the O_2 cascade at skeletal muscle level during ICCP measuring NIRS in MM patients (PO_2 =oxygen partial pressure; $oxy[Hb]$ = oxyhaemoglobin; $deoxy[Hb]$ = deoxyhaemoglobin; $oxy[Mb]$ =oxymyoglobin; $deoxy[Mb]$ =deoxymyoglobin; mt = mitochondria). Pathologic response (malfunctioning mitochondria) are presented in red.



In this second part, a clear distinction was shown in relative deoxy[Hb+Mb] during ICCP between healthy control subjects and MM patients with a severe mitochondrial dysfunction. It is essential to emphasize that this was a crucial step in this research to be able to make a clear contrast in peripheral oxygenation mechanisms between this pathologic population and healthy control subjects. In general, three deoxy[Hb+Mb] abnormalities could be identified as a marker for reduced muscle oxidative capacity in this MM population. (i) Slower deoxy[Hb+Mb] kinetics during arterial occlusion. (ii) A faster decrease in deoxy[Hb+Mb] after cuff release, indicative for an earlier reoxygenation. (iii) A significant altered relative deoxy[Hb+Mb] pattern during ICCP. Hence, by measuring relative deoxy[Hb+Mb], it is possible to accurately and reliably measure peripheral oxygenation abnormalities without possible influence of subcutaneous ATT and capillary (de)conditioning.

3. RELATIVE DEOXY[Hb+Mb] AND FBF RESPONSES IN CFS PATIENTS

To our knowledge, no previous studies have investigated oxidative metabolism in CFS patients with non-invasive assessment of microvascular O₂ extraction and limb blood flow by using NIRS and Doppler Ultrasound. The only preceding study using NIRS to establish altered oxygenation responses to exercise in CFS patients, did not measure blood flow (Miller et al., 2015). Additionally, no corrections were made for subcutaneous ATT in this latter study, whereby mean ATT values were higher than 6 mm (Miller et al., 2015). As was mentioned before, caution is advised when analysing NIRS values without ATT correction methodology. Moreover, Wolf et al. (2003) has shown that the NIRS absorption coefficient (μ_a) is significantly influenced when subcutaneous ATT is higher than 6mm. Based on clear observations of an excessive O₂ delivery during and after exercise in MM patients, brachial artery blood flow measurements were added in this study (Jeppesen et al., 2012; Taivassalo et al., 2003; Taivassalo et al., 2012). Considering the relative deoxy[Hb+Mb] values, significant differences were found at different steps during ICCP. Notwithstanding the lack of overall, significant differences regarding relative deoxy[Hb+Mb]- and FBF values between CFS patients and their healthy matched control subjects, a subgroup of four CFS patients was identified with abnormal peripheral oxygenation responses. Similarities could be identified in relative deoxy[Hb+Mb] pattern between these four CFS patients and the MM patients of previous

observations. Hence, based on abnormal ICCP responses in four of these patients, skeletal muscle biopsy collection was proposed. Biochemical and histological analysis on these biopsy samples revealed mitochondrial abnormalities in 3 out of 3 of these patients (one did not agree for skeletal muscle biopsy collection). In general, two patterns of aberrant skeletal muscle biopsy and fibroblast results could be recognized. In patient 1 and two, a clear increase of the amount of fat droplets was seen in skeletal muscle whereas in cultured skin fibroblasts, a mosaic pattern was seen following staining of the mitochondria with JC1 (plus rotenone). The increased amount of fat droplets, observed in skeletal muscle tissue and the mosaic pattern, observed in cultured skin fibroblasts, were however less pronounced in patient 2. A mosaic staining pattern is usually observed in patients suffering from mitochondrial DNA abnormalities (e.g. point mutation, deletion or depletion of the mitochondrial DNA). In patient 3 however, a different pattern could be recognized as skeletal muscle microscopic abnormalities could not be detected. On the other hand, complex I deficiency was biochemically determined, and, more surprisingly, a deficient complex IV activity in cultured skin fibroblasts (no complex I measurements in fibroblasts). Complex I and complex IV contain the largest number of mitochondrial encoded subunits. Deficiencies of either complex I, or complex IV, or a combined deficiency of complex I and IV, are usually caused by a disturbed intramitochondrial synthesis of the mitochondrial encoded subunits. The significantly decreased activity of complex II+III, in combination with a normal activity of the individual complexes II and III, is rather suggestive for a decreased amount of functionally active co-enzyme Q.

The heterogeneity in NIRS response within this CFS population is in agreement with multiple previous mentioned studies, investigating physiological responses to exercise in this CFS population (Fulcher and White, 2000; Wagemakers et al., 1988; De Becker et al., 2000; Sargent et al., 2002; Fukuda et al., 1994; Arnold et al., 1984; Barnes et al., 1993; Gibson et al., 1993; Fulle et al., 2000). Additionally, discrepancies in CFS patients' blood flow response were in agreement with existing literature (McCully et al., 1996; McCully et al., 1999; McCully et al., 2003; McCully et al., 2004). In this study, blood flow was either normal, but in one patient significantly higher during ICCP. As indicated previously, increased limb blood flow values have been shown in MM patients as a representation of the compensatory hyperkinetic muscle

perfusion (Taivassalo et al., 2003). In contrast to these findings in MM patients, it has been accepted that CFS patients have a reduced blood flow, probably due to autonomic dysregulation or reduced capillary density (Lindh et al., 1995). The results of the present study show the possible existence of subgroups within the CFS population as one patient showed an abundantly higher muscle perfusion while the other patients showed no divergent FBF responses compared to healthy control subjects.

The novelty of this research, in our opinion, is the approach towards CFS patients complaining about exercise intolerance, post-exertional malaise, muscle pain and weakness. These CFS patients were included in study 5, as these complaints are primordial features in the syndromal description of the disease. While a lot of preceding studies considered CFS patients as one group, often with heterogeneous responses to exercise, it was the purpose of this study to detect an eventual subgroup with abnormal peripheral oxygenation responses during ICCP. Hence, these measurements during ICCP were developed exactly for this purpose, i.e. reliable detection for disturbed oxidative metabolism by analysing non-invasively peripheral oxygenation mechanisms. It is of major importance to emphasize the word “screening” as it is impossible to establish a clear diagnosis based on these measurements. On the other hand it is almost impossible and cost ineffective to carry out skeletal muscle biopsy in every patient complaining about exercise intolerance, post-exertional malaise and muscle weakness. In our opinion, this ICCP measurements could offer an optimum balance between invasive skeletal muscle biopsy analysis on the one hand and cost effectiveness and applicability on large samples on the other hand. Of course, when patients should be declared “positive” after this screening, skeletal muscle biopsy analysis remains crucial for diagnosis.

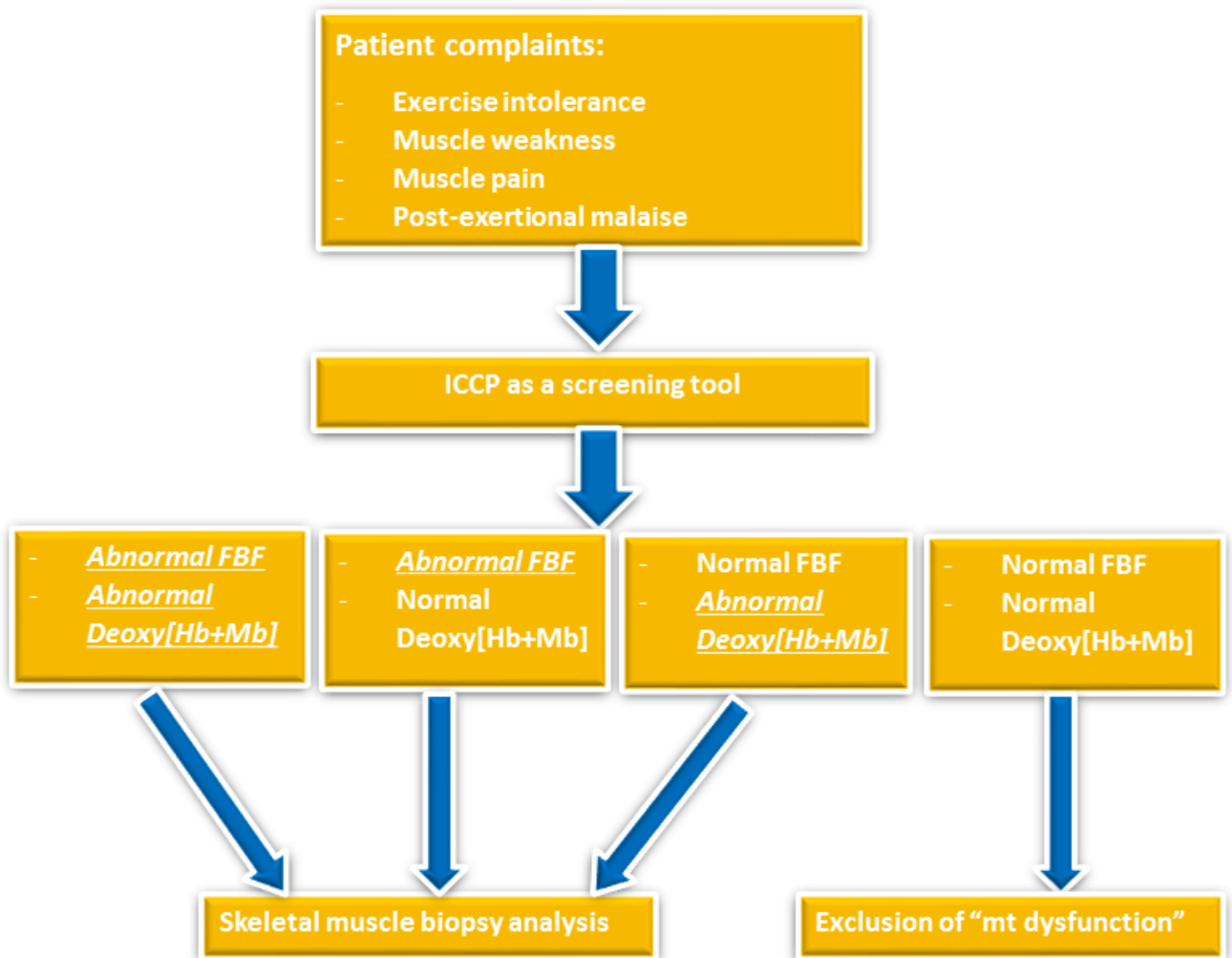
Besides these “peripheral” muscle complaints in CFS patients, “central” problems are reported as well in the literature. As mentioned in the introduction, these problems are mainly related to autonomic dysfunction or central sensitisation (exaggerated pain perception). The relationship between both central and peripheral problems, in particular in combination with mitochondrial dysfunction, remains however questionable. Recently, Meeus et al. (2013) related these variables to each other, stating that mitochondrial dysfunction in muscle and/or central nervous system (CNS) cells could have an important influence on the central

sensitisation related problems in these patients. A possible mechanism could be the fact that mitochondrial dysfunction is associated with an increased oxidative and nitrosative stress (ROS and NOS). Consequently, there will be an increased amount of tissue injury as well as an increased inflammatory response. Another contribution could be made by ATP depletion (as a consequence of reduced aerobic energy delivery) in the CNS cells, thereby inducing hypersensitivity of the N-Methyl-D-Aspartic acid (NMDA)- receptors. An increased sensitivity of these nociceptive receptors is responsible for an elevated pain perception (Park et al., 2000). According to this hypothesis, it is clear that mitochondrial dysfunction could have a major impact on the centrally located symptomatology of these CFS patients as well. As was stated before, however, there is only moderate evidence for histochemically determined mitochondrial dysfunction in CFS patients.

A possible explanation for mitochondrial dysfunction in CFS patients is the type of medication intake. All patients with histologic abnormalities of mitochondrial dysfunction, were taking metformin when ICCP took place. Previous work revealed however, that this biguanide inhibits complex I activity of the respiratory chain in the mitochondria (El-Mir et al, 2000; Owen et al., 2000).

Based on this research, we propose a screening framework for CFS and MM patients suffering from exercise intolerance, post-exertional malaise and muscle weakness or pain to detect eventual peripheral oxygenation abnormalities (See figure 11). Hence, Doppler Ultrasound and relative NIRS measurements during ICCP can be properly used as a first line screening tool in large cohorts to detect eventual microvascular or muscle abnormalities. Despite the fact that significant differences were found in deoxy[Hb+Mb] kinetics during and after arterial occlusion between MM patients and healthy control subjects, it is important to study relative deoxy[Hb+Mb] during exercise. This is due to the fact that there is a large overlap between both MM and control populations when only using the arterial occlusion set up. Patients with abnormal muscle perfusion (Q_m) and/or microvascular O_2 extraction, should be further investigated by performing skeletal muscle biopsy analysis, which is still the gold standard to establish muscle oxidative dysfunction.

Fig. 11: Framework proposition for CFS patients complaining about exercise intolerance, muscle pain or weakness and post-exertional malaise



4. LIMITATIONS AND FUTURE DIRECTIONS

In this dissertation, a very broad area of scientific research was covered, ranging from profound fundamental research on peripheral oxygenation and muscle diseases, to the implementation of applicable tests with non-invasive measurements. Of course, there are some limitations connected to this research, requiring further and more profound research in this area. A first limitation of this research is the fact that the validity of NIRS measurements relative to venous PO₂ measurements during ICCP has not been investigated. This is of course important matter as conflicting validity results have been reported in the past when using this technique. In general, one should be extremely careful when using this technique as there is still a lot of scientific controversy about NIRS measurements. Therefore, it would be appropriate to perform a validation study where the relation between relative deoxy[Hb+Mb] values and venous PO₂ measurements during cyclic dynamic exercise is investigated. Notwithstanding the lack of validation studies concerning NIRS, we were aware of certain problems when using this technique and an attempt to avoid possible disturbing influences was made in this dissertation (study 2). Our main interest of course, was the fact that the blunting effect of subcutaneous ATT in overweighted subjects was neutralized when using relative NIRS values. Also, the possible influence of blood flow was investigated whereas relative values are uninfluenced by O₂ delivery. A major issue, which was not treated in this research, was the question whether these measurements during ICCP are reliable to carry out in patient populations. Also, the reliability study solely focused on the reliability of the relative deoxy[Hb+Mb] values but not on FBF measurements during ICCP. Consequently, despite the practical difficulties to test these patient populations, reliability of FBF and relative deoxy[Hb+Mb] measurements during ICCP should be investigated in these patient populations.

A second limitation requiring future research was the lack of a larger MM and CFS patient sample size in study 3, 4 and 5. Considering the MM population, this was due to a limited prevalence of patients suffering from a severe mitochondrial myopathy in Belgium. Consequently, there was no ability to include more patients in the investigation of the

deoxy[Hb+Mb] pattern during arterial occlusion and ICCP. To accentuate the importance of this framework and eliminate possible influences of medication, this ICCP should be carried out in much larger cohorts of CFS patients and other patients complaining about muscle weakness and pain, post-exertional malaise and exercise intolerance. Consequently, it could be analysed whether aberrant FBF and relative deoxy[Hb+Mb] results of these patients represent indeed muscle oxidative dysfunction, as was found in study 5.

A third limitation towards the use of NIRS and FBF measurements, especially in a CFS population, was the fact that there was no registration of haematocrit and blood pressure before and during the ICCP. This was in particular because hypotension, probably due to autonomic and vasomotor dysregulation, is a common feature in CFS patients. Therefore, blood pressure should be included in future studies on larger CFS cohorts as it possibly influences peripheral oxygenation and in particular limb blood flow. In the introduction, we have listed some studies stating that blood pressure and haematocrit (amount of RBC) may have an important influence on peripheral oxygenation. Therefore, despite the fact that no variances are supposed in these patient populations it is a limitation in this research that these parameters were not included to assess their possible influence on the relative deoxy[Hb+Mb] values during ICCP.

A fourth limitation is the fact that these “peripheral” issues (e.g. mitochondrial dysfunction) in CFS patients were never related to eventual “central” problems ranging from autonomic dysregulation to hypersensitivity of peripheral stimuli (central sensitisation). In the discussion part, a hypothetical explanation was provided about the possible mechanisms of mitochondrial dysfunction influencing these central issues. It could be interesting to investigate the possible relationship between CFS patients’ peripheral oxygenation and mitochondrial functioning results with pain perception on the one hand. On the other hand it would be interesting to look for eventual associations between histochemically determined mitochondrial abnormalities and oxidative damage (ROS) as this could be a possible mediator in this process.

If the aim of future studies would be to use this tool in larger sample sizes, it could be very useful to reduce the length of this procedure by performing an arterial occlusion and a subsequent single bout of high intensity dynamic handgrip exercise. As such, eventual conveniences and differences between ICCP and a short part of the ICCP NIRS and FBF responses should be further elaborated. This is in particular because the higher intensities possess higher reliability coefficients and relative deoxy[Hb+Mb] differences between patients and healthy control subjects were more pronounced in these higher intensities. Also, when future studies on large sample sizes would be able to establish the reliability and quality of this screening tool, a possibility would be to use it as an assessment in intervention studies. Consequently, this tool could be used to detect eventual improvements or deteriorations in peripheral O₂ extraction and/or limb blood flow after certain pharmacological, nutritional or training interventions in patients suffering from abnormal peripheral oxygenation and reporting significant improvement in validated health questionnaires, such as MOS-SF36. Of course, there are other patient populations with reported microvascular and mitochondrial problems that could be investigated using this tool.

5. CONCLUSIONS

In this dissertation we aimed to present an overall image of scientific evolution in a very broad area of peripheral oxygenation at muscle level and pathological conditions limiting these oxygenation mechanisms. In the last century, the 'classical view' of Auguste Krogh concerning peripheral oxygenation has been challenged by investigations using modern technologies. Moreover, in the late 1980's, a whole new research area started to unfold the pathogenesis of Mitochondrial Myopathies (MM). Notwithstanding the significant evolutions that have been made in this area of severe MM diseases, there is a lack of information on patients suffering from moderate types of this illness. Contrary to MM diseases, Chronic Fatigue Syndrome (CFS) doesn't own a clear aetiology and is based on the exclusion of any other disease. The similarities between both diseases are the unique symptomatology of exercise intolerance, post-exertional malaise, muscle pain and weakness. Despite the heterogeneity in scientific reports about CFS patients, commonalities have been identified between both populations when focussing on oxidative metabolism and muscle abnormalities. In this research we have developed an exercise protocol (ICCP) with reliable and relative deoxy[Hb+Mb] measurements. Hence, it has been shown that it is possible to overcome methodological issues influencing the NIRS signal when performing this ICCP with relative deoxy[Hb+Mb] values. A clear distinction could be made when assessing relative deoxy[Hb+Mb] values between healthy control subjects and a MM population with severe mitochondrial malfunctioning. Although larger sample sizes are needed to assess the applicability of this research, 4 out of 12 CFS patients showed peripheral oxygenation abnormalities when performing this ICCP. Further investigation revealed mild mitochondrial abnormalities in skeletal muscle biopsy and cultured fibroblast samples in 3 out of 3 patients. To further elaborate the screening quality of this research, larger cohorts should be tested. The preliminary data of this research applied to patients complaining about exercise intolerance, post-exertional malaise, muscle pain and weakness, are however, promising.

SUMMARY - SAMENVATTING

Background

In one century of scientific evolution, the classical view of Auguste Krogh and Adolphe Eugene Fick concerning peripheral oxygenation has progressed. These evolutions influence of course the point of view of certain diseases suffering from muscle or microvascular abnormalities. Two diseases where such abnormalities have already been shown are Mitochondrial Myopathy (MM) and Chronic Fatigue Syndrome (CFS). The aetiology of MM is a mutation or deletion in critical mitochondrial or nuclear DNA regions, responsible for the construction of mitochondrial (sub)complexes and enzymes. Due to this clear mitochondrial malfunctioning, peripheral oxygenation will be impaired in this population, with a decreased O₂ extraction and a hyperdynamic muscle perfusion during exercise. Contrary to MM, there is still a lot of controversy about peripheral oxygenation abnormalities in CFS patients. CFS is a disease that is based on exclusion of any other disease and can be recognized by a long lasting fatigue which endures for at least six months. Inconsistent findings have been reported in this patient populations concerning muscle strength, aerobic capacity, mitochondrial functioning and skeletal muscle blood flow. These divergences have often been explained by the large heterogeneity in responses to exercise within this CFS population.

Near Infrared Spectroscopy (NIRS) has been recognized as a valid and reliable technique to measure microvascular O₂ extraction in brain tissue and large muscle groups at rest and during exercise. In small muscle groups (e.g. forearm muscles) however, few data are available on the reliability of this technique. Moreover, some problems like the influence of subcutaneous Adipose Tissue Thickness (ATT) or O₂ delivery (blood flow) on NIRS signalling have been recognized when using this technique during exercise. Until now, no gold standard has been established to measure skeletal muscle blood flow. The most optimal technique described so far, for limb blood flow assessment, during exercise, is Doppler Ultrasound.

The above mentioned techniques, i.e. NIRS and Doppler Ultrasound, could be used as an indirect marker to estimate peripheral oxygenation mechanisms in healthy and patient populations. A major advantage when using these techniques is the non-invasive character and applicability during exercise. However, when using this NIRS technique, there are still some methodological issues to overcome before using this device on forearm muscles in patient populations. From the MM or CFS patient's perspective, there is a need for non-

invasive and non-exhaustive exercise protocols to screen for eventual microvascular or muscle abnormalities.

Aims

In the construction of a screening tool for microvascular or muscle abnormalities, our first aim was to study the reliability of relative NIRS measurements in small forearm muscles during an Incremental Cyclic Contraction Protocol (ICCP) (**study 1**). Moreover, to overcome some methodological barriers, we aimed to study the influence of ATT and O₂ delivery on relative deoxyhaemoglobin and –myoglobin (deoxy[Hb+Mb]) parameters (**study 2**). In **study 3 and 4** we aimed to study eventual differences in deoxy[Hb+Mb] during and after arterial occlusion and during ICCP between healthy control subjects and MM patients, suffering from severe mitochondrial malfunctioning. The last step of this research was to identify CFS patients who may suffer from muscle or microvascular abnormalities as well (**study 5**).

Research and conclusions

Figure A below provides a schematic overview of the 5 studies carried out in this research

Fig A: Overview of the studied population, used techniques and experimental aims in the different scientific reports. (Healthy Subjects= HS; Mitochondrial Myopathy patients= MM; Chronic Fatigue Syndrome patients= CFS; Deoxyhaemoglobin and –myoglobin= deoxy[Hb+Mb]; Incremental Cyclic Contraction Protocol= ICCP; Adipose Tissue Thickness= ATT; Near Infrared Spectroscopy= NIRS).

	Study 1	Study 2	Study 3	Study 4	Study 5
Population	HS		MM / HS		CFS / HS
Methods	NIRS	NIRS Doppler US	NIRS	NIRS	NIRS Doppler US
Experimental aims	Investigate reliability of relative deoxy[Hb+Mb] during ICCP	Assess possible influence of ATT and BF on absolute and relative NIRS values	Compare post occlusive hyperemic deoxy[Hb+Mb] response	Compare relative deoxy[Hb+Mb] values during ICCP	Compare relative deoxy[Hb+Mb] values and FBF during ICCP

Conclusion 1: By measuring relative deoxy[Hb+Mb] values (in %) during ICCP, it was possible to reliably estimate peripheral O₂ extraction and to exclude possible influences of subcutaneous ATT and O₂ delivery.

Research: In **study 1**, the reliability of the NIRS parameters during ICCP has been investigated in healthy control subjects. In this study, it has been shown that Intraclass Correlation Coefficients (ICC's) were sufficiently high to establish reliability of relative deoxy[Hb+Mb] values and O₂ saturation (SmO₂) during this handgrip exercise protocol.

To investigate the possible influence of subcutaneous ATT and blood flow (O₂ delivery) on absolute and relative NIRS parameters, we studied the relationship between these parameters (**study 2**). During this ICCP, all absolute maximal NIRS parameters except total

haemoglobin (tot[Hb]) were influenced by ATT, whereas all absolute maximal NIRS parameters were affected by O₂ delivery. When using relative deoxy[Hb+Mb] values (towards the arterial occlusion maximal value) however, no relationship was found with limb blood flow and subcutaneous ATT.

Conclusion 2: By using this experimental set up, it has been shown that a clear distinction could be made between healthy subjects and MM patients, suffering from severe mitochondrial malfunctioning.

Research: Deoxy[Hb+Mb] values during and after arterial occlusion, as well as during ICCP, were compared between healthy control subjects and MM patients (**study 3 and 4**). In general, three deoxy[Hb+Mb] abnormalities could be identified as a marker for reduced muscle oxidative capacity in this MM population. (i) Slower deoxy[Hb+Mb] kinetics during arterial occlusion. (ii) A faster decrease in deoxy[Hb+Mb] after cuff release, indicative for an earlier reoxygenation. (iii) A significant altered relative deoxy[Hb+Mb] pattern during ICCP.

Conclusion 3: By using this experimental set up, a subgroup within the CFS population could be identified, suffering from mitochondrial abnormalities. These mitochondrial abnormalities were confirmed after analysis on skeletal muscle and skin biopsy.

Research: This ICCP was applied to CFS patients, frequently complaining about muscle weakness or pain, exercise intolerance and post-exertional malaise. Significant differences in relative deoxy[Hb+Mb] values were found between CFS patients and healthy subjects, but the most crucial finding was the identification of 4 subjects with an abnormal relative deoxy[Hb+Mb] response (**study 5**). Skeletal muscle and skin biopsy analysis revealed mitochondrial abnormalities in 3 out of 3 CFS patients who agreed for skeletal muscle and skin biopsy collection.

To summarize, we propose the above mentioned non-invasive measurements during a non-exhaustive ICCP as a tool to screen for muscle and microvascular abnormalities in patients complaining about muscle weakness or pain, exercise intolerance and post-exertional malaise. A second step in this “screening framework” is the analysis of skeletal muscle and skin biopsy samples in patients with abnormal relative deoxy[Hb+Mb] or limb blood flow responses during

ICCP. As such, the quality of this research will be investigated while testing larger cohorts with these complaints.

Achtergrond

In een eeuw vol wetenschappelijke evolutie, is de klassieke visie op perifere oxygenatie, van Auguste Krogh en Adolphe Eugene Fick, geëvolueerd. Deze evoluties hebben natuurlijk een weerslag op de manier waarop verschillende ziektes, waarvan spier- en microvasculaire abnormaliteiten primordiale kenmerken zijn, bestudeerd worden. Twee ziektebeelden waarbij dergelijke abnormaliteiten reeds zijn aangetoond, zijn Mitochondriale Myopathie (MM) en Chronische Vermoeidheid Syndroom (CVS). De etiologie van deze eerder vernoemde MM, zijn mutaties of deleties in enkele cruciale mitochondriale of nucleaire DNA regio's, die verantwoordelijk zijn voor de synthese van de mitochondriale (sub)complexen en enzymen. Door deze duidelijke mitochondriale dysfunctie, zal de perifere oxygenatie gehypothekeerd worden bij dergelijke patiënten, met een verminderde O₂ extractie en een hyperdynamische spierperfusie tijdens inspanning. In tegenstelling tot MM, is er nog steeds veel controverse omtrent perifere oxygenatie abnormaliteiten bij CVS patiënten. CVS is een ziekte die gebaseerd is op exclusie van enig ander ziektebeeld en wordt gekenmerkt door een vermoeidheid die standhoudt voor langer dan zes maanden. Er werden reeds veel inconsistente bevindingen gerapporteerd bij CVS patiënten met betrekking tot spierkracht, aerobe capaciteit, functioneren van de mitochondria en spierdoorbloeding. Deze tegenstrijdigheden werden reeds voor een groot deel verklaard door de grote heterogeniteit binnen deze CVS populatie.

Near Infrared Spectroscopy (NIRS) is reeds een valide en betrouwbare techniek gebleken om microvasculaire O₂ extractie te meten in de hersenen en grote spiergroepen in rust en tijdens inspanning. In kleine spiergroepen (bvb. voorarmspieren) daarentegen, werden tot hiertoe weinig studies uitgevoerd om de betrouwbaarheid van deze techniek na te gaan. Bovendien werden nog enkele variabelen zoals onderhuids vet en O₂ levering geïdentificeerd die een storende invloed uitoefenen op het NIRS signaal. Tot op heden is er nog geen gouden standaard gekend voor het meten van spierdoorbloeding tijdens inspanning. De meest

optimale techniek om een accurate inschatting te maken van bloeddorstroming naar een lidmaat, specifiek tijdens inspanning, is gebruik van een Doppler Ultrasound.

De hierboven vermelde technieken, nl. NIRS en Doppler Ultrasound, kunnen gebruikt worden als een indirecte marker voor het inschatten van perifere oxygenatie bij gezonde en patiëntenpopulatie 's. Het grote voordeel bij het gebruik van deze technieken is het non-invasieve karakter en de toepasbaarheid ervan. Desondanks zijn er nog enkele methodologische problemen die opgelost moeten worden alvorens deze NIRS techniek te gebruiken op voorarmspieren bij patiënten. Vanuit het klinisch perspectief van de MM en CVS patiënten, is er een urgente nood aan non-invasieve en niet intensieve inspanningstests om zo patiënten te controleren op eventuele microvasculaire en spier abnormaliteiten.

Doelstellingen

In de opbouw van een nieuwe screeningsmethode voor microvasculaire en spier abnormaliteiten, was het nagaan van de betrouwbaarheid van deze relatieve NIRS metingen op de voorarmspier tijdens een Cyclische Inspanningstest met Toenemende Belasting (CITB) ons eerste doel (**studie 1**). Bovendien werd een poging gedaan om enkele methodologische problemen, op te lossen door het gebruik van de relatieve deoxyhaemoglobine en myoglobine (deoxy[Hb+Mb]) parameters (**studie 2**). In **studie 3 en 4** was het doel van de studie om eventuele verschillen in deoxy[Hb+Mb] tussen MM patiënten en gezonde subjecten na te gaan. Ten laatste was het de bedoeling van dit onderzoek (**studie 5**) om een subgroep CVS patiënten te identificeren met eventuele microvasculaire en/of spierproblemen.

Onderzoek en conclusies

De onderstaande figuur A geeft een schematische weergave van de 5 studies weer binnen het kader van dit onderzoek.

Fig A: Overzicht van de bestudeerde populaties, de gebruikte technieken en de experimentele doelstellingen bij de verschillende studies. (Gezonde Subjecten= GS; Mitochondriale Myopathie patiënten= MM; Chronisch Vermoeidheidsyndroom= CVS; Deoxyhaemoglobine en -myoglobine = deoxy[Hb+Mb]; Spier Doorbloeding= SD; Cyclische Inspanningstest met Toenemende Belasting= CITB; Near Infrared Spectroscopy= NIRS)

	Studie 1	Studie 2	Studie 3	Studie 4	Studie 5
Populatie	GS		MM / GS		CVS / GS
Methodes	NIRS	NIRS Doppler US	NIRS	NIRS	NIRS Doppler US
Doelstellingen	De betrouwbaarheid van de relatieve deoxy[Hb+Mb] waarden nagaan tijdens CITB	De mogelijke invloed van subcutaan vet en SD bestuderen op absolute en relatieve NIRS waarden	Deoxy[Hb+Mb] respons vergelijken tijdens en na arteriële occlusie	Relatieve deoxy[Hb+Mb] respons vergelijken tijdens CITB	Relatieve deoxy[Hb+Mb] en SD respons vergelijken tijdens CITB

Conclusie 1: Door het meten van de relatieve deoxy[Hb+Mb] waarden (in %) tijdens CITB was het mogelijk om de perifere O₂ extractie op een betrouwbare manier in te schatten en om de mogelijke invloed van subcutaan vet en O₂ levering uit te sluiten.

Onderzoek: In **studie 1** werd de betrouwbaarheid van bepaalde NIRS parameters nagegaan tijdens CITB bij gezonde subjecten. Op die manier werd de betrouwbaarheid aangetoond van de relatieve deoxy[Hb+Mb] waarden en de O₂ saturatie (SmO₂) gedurende handknijpinspanning.

Om de mogelijke invloed van subcutaan vet en O₂ levering na te gaan op absolute en relatieve NIRS parameters, werd de relatie tussen deze parameters bestudeerd (**studie 2**). Er werd gevonden dat alle absoluut uitgedrukte NIRS parameters behalve totaal haemoglobine (tHb) beïnvloed worden door subcutaan vet. Bovendien werd deze invloed eveneens vastgesteld tussen de absolute weergaven van de NIRS parameters en O₂ levering. Wanneer de relatieve deoxy[Hb+Mb] (t.o.v. de arteriële occlusie maximale waarde) waarden echter werden gebruikt, vonden we geen correlatie met spierdoorbloeding en subcutaan vet.

Conclusie 2: Mits toepassing van dit experimenteel protocol, was het mogelijk om een duidelijk onderscheid te maken tussen gezonde subjecten en MM patiënten, waarbij ernstige mitochondriale defecten aanwezig waren.

Onderzoek: Deoxy[Hb+Mb] waarden gedurende en tijdens de arteriële occlusie alsook tijdens het CITB werden vergeleken tussen gezonde proefpersonen en MM patiënten (**studie 3 en 4**). Over het algemeen werden er drie duidelijke abnormaliteiten in deoxy[Hb+Mb] signaal vastgesteld als marker voor een gereduceerde oxidatieve capaciteit van de skeletspier binnen deze MM populatie: (i) Een tragere deoxy[Hb+Mb] kinetiek tijdens arteriële occlusie; (ii) Een snellere afname van deoxy[Hb+Mb] direct na het loslaten van de manchet; (iii) Een significant ander patroon in relatieve deoxy[Hb+Mb] gedurende CITB.

Conclusie 3: Mits toepassing van dit experimenteel protocol, was het mogelijk om een subgroep te identificeren in de volledige CVS populatie, waarbij mitochondriale abnormaliteiten werden vastgesteld na analyse op hun spier- en huidbiopsie.

Onderzoek: Het CITB werd toegepast op CVS patiënten, waar veelvuldig klachten voorkomen van spierzwakte en –pijn, inspanningsintolerantie, en malaise na inspanning in (**studie 5**). Er werden significante verschillen in relatieve deoxy[Hb+Mb] gevonden tussen CVS patiënten en gezonde controleproefpersonen. De meest belangrijke bevinding was dat er 4 patiënten geïdentificeerd werden met een abnormale deoxy[Hb+Mb] en SD respons, die het gemiddelde op die manier vertekenden. Bovendien toonden analyses op spier- en huidbiopsie monsters, mitochondriale abnormaliteiten aan bij 3 van de 3 CVS patiënten die toezegden voor afname van een spier- en huidbiopsie.

Samenvattend kunnen we stellen dat deze non-invasieve metingen tijdens een niet intensief CITB gebruikt kunnen worden om te screenen voor microvasculaire en/of spier abnormaliteiten bij patiënten met als meest voorkomende klachten spierpijn en/of –zwakte, inspanningsintolerantie en malaise na inspanning. Een tweede stap in dit “screeningskader” is de analyse op een monster na afname van een spier- en huidbiopsie bij patiënten met een abnormale relatieve deoxy[Hb+Mb] of spierdoorbloeding tijdens CITB. Op die manier zal de kwaliteit van dit onderzoek geëvalueerd kunnen worden door grotere cohorten te testen met dezelfde klachten.

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C1-C3:

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19th annual congress of the European College of Sport Science (July 2-5, 2014: Amsterdam, Netherlands): “Sport science around the canals”.

Boone J, **Celie B**, Bourgois J. The Impact of Aerobic Fitness Status on the Physiological Responses to Incremental Ramp Exercise. 2014 (Oral Presentation).

LIST OF PUBLICATIONS

19th Annual meeting of Vereniging voor Kinesiologie (VK) (december 12, 2014, Antwerp, Belgium):

Verbelen B, **Celie B**, Boone J, Bourgois J. The impact of a 3 weeks detraining period on forearm muscle perfusion and oxygenation in male and female rowers during incremental handgrip exercise. 2014; WINNER VTS PRIZE (Best poster presentation).

20th annual congress of the European College of Sport Science (June 24-27, 2015: Malmö, Sweden): "Sustainable Sport".

Bourgois J, Dumortier J, Boone J, De Backer T, **Celie B**. Forearm muscle blood flow and deoxygenation during dynamic handgrip exercise in limb specific trained athletes. 2015 (Oral presentation).

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