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IRVINE

Application of TR-NIRS in Healthy and Diseased Children during Exercise

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Pharmacology

by

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2017

DEDICATION

To my wife Andrea

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NIRS Investigation of Muscle Oxygenation in Healthy and Diseased Children

PUBLICATIONS

Cook D J, Nguyen C, Chun H N, L Llorente, **Chiu A S**, Machnicki M, Zarembinski T I, Carmichael S T. Hydrogel-delivered brain-derived neurotrophic factor promotes tissue repair and recovery after stroke. Journal of Cerebral Blood Flow and Metabolism 2017;37(3):1030-1045.

Tran B D, **Chiu A,** Tran C, Rogacion D R, Tfaye N, Ganesan G, Galassetti P R. Exercise and Repeated Testing Improves Accuracy of Laser Doppler Assessment of Microvascular Function Following Shortened (1-minute) Blood Flow Occlusion. Microcirculation 2016;23(4):293-300.

Spiegel S, **Chiu A**, James A S, Jentsch J D, Karlsgodt K H. Recognition deficits in mice carrying mutations of genes encoding BLOC-1 subunits pallidin or dysbindin. Genes, brain and behavior 2015;14(8):618-624.

ABSTRACT OF THE DISSERTATION

Application of TR-NIRS in Healthy and Diseased Children during Exercise

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Time Resolved-Near-infrared spectroscopy (TR-NIRS) is a non-invasive, non-harmful tool to assess the oxygenation status of tissue-specific myoglobin and hemoglobin. A series of studies was done to assess differences in muscle oxygenation during exercise between healthy children and adults and in children with sickle cell anemia. The first study demonstrated a novel tissue water correction to improve the accuracy of TR-NIRS-determined hemoglobin/myoglobin of *vastus lateralis muscle* with varying adipose tissue thickness. This correction allows for more accurate comparisons between groups with systematically different adipose tissue thicknesses. This novel correction was incorporated into the second study which compared the NIRS-determined muscle oxygenation patterns of early pubertal boys and girls, late pubertal boys and girls, and young men/women during a ramp-style progressive exercise test on a cycle ergometer. This revealed a different pattern in maintaining oxygen supply in late pubertal boys and young men compared to the other groups. The last study presented pilot data from mid/late

pubertal children with sickle cell anemia during a ramp-style cycle ergometer test. Children with sickle cell anemia did not increase their total muscle hemoglobin/myoglobin during exercise and had drastically lower muscle oxygenation throughout exercise compared to healthy controls. Together, these studies provide compelling evidences for the usefulness of TR-NIRS as a tool to assess muscle oxygenation in healthy and diseased children and to provide a way for quantitative assessment of intervention success and effect.

CHAPTER 1: INTRODUCTION

Exercise is most potent physiological stressor in healthy everyday life. During exercise, skeletal muscles consume oxygen to create ATP to continue to generate power. Oxygen delivery via hemoglobin (Hb) to the muscle requires coordinated circulatory and ventilatory systems to continuously provide oxygen to the muscles to continue work. Although total oxygen uptake has been measured for many years via ventilatory gas exchange, muscle-specific oxygen supply and usage has been difficult to measure. Since exercise is such a potent stressor, muscle-specific oxygenation response can reveal difference in development or disease-states that are not apparent at rest.

This dissertation presents experimental data that uses near infrared spectroscopy (NIRS) to assess the oxygen supply and utilization of skeletal muscles during exercise in early and late pubertal children compared to adults. Studies of the known physiological and biochemical differences between the skeletal muscles of children and adults presented below suggest that there would be differences in muscle-specific oxygen utilization. A brief introduction to the underlying muscle physiology and NIRS method will help frame the potential causes behind the differences in muscle oxygenation.

Muscle Physiology in Children

Due to practical and ethical considerations, traditional methods like muscle biopsies to investigate skeletal muscle physiology has been largely limited to

children with disorders or cadaver studies. Whereas the physical characteristics like muscle fiber type distribution and metabolic substrates of healthy adults has been more thoroughly investigated, the similarities and differences of the physical and biochemical characteristics of skeletal muscles in health children are less well defined. This is especially true of early pubertal children who have been understudied.

Some of the earliest muscle biopsies from the 1970s done in pubertal children (age 11) showed that vastus lateralis muscle contained ~55% type I myosin heavy chain (MHC) fibers[1]. These type I muscle fibers were categorized by histochemical staining for myosin ATPase; with type I having fewer myosin ATPase than their type II (MHC II) counterparts. In contrast, biopsy results from the vastus lateralis of untrained young males (age 24-30) with similar methods showed on average ~36% type I fiber[2]. Since those early studies, investigation into the percentage of type I fibers in healthy adults has been shown to vary from ~20% to ~60% depending on their activity level and type of exercise training[3]. Additionally, a study of biopsies from infants (age 0-2, N=21) also showed a large range in type I fiber ratios (36-75%) despite having significantly higher group means compared to adults [4]. In addition to muscle biopsies, limited number of studies on cadavers from children give additional insight into children's muscle fiber type distribution. Although limited by a small sample size, a cadaver study on vastus lateralis cross-sections of previously healthy children ages 7-18 (N=7) showed an age-related

progressive decrease in percentage of type I fiber compared to adults aged 19-37 (N=15)[5].

Overall, biopsy and cadaver studies on healthy pubertal children have shown either higher [4,5] or similar [6–8] composition of type I muscle fibers compared to adults. Some of the discrepancy appear to come from differences between the ranges of type I fiber ratio in children of varying age groups as well as the training status of healthy young adults. Other differences could be due to the different methodological ways "type I" and "type II" fibers were determined (e.g., enzyme activity, MHC type, fatiguing qualities, mitochondrial density). Because of these conflicting data, whether healthy young children on average have a higher type I/type II fiber ratios than adults remain controversial.

In addition to the potential differences in muscle fiber type ratios, evidence from additional studies suggests that children do not fully utilize their available type II motor unit compared to adults[9]. Although currently there is no way to directly assess neuromotor unit recruitment, several lines of evidence have emerged that support the hypothesis that children do not utilize their type II units to the same as extent of that of adults. One set of experiments assess motor unit activation by comparing the volitional muscle contractile force with that of an externally-evoked maximal muscle contractile force[10]. This method, termed interpolated-twitch techniques, attempts to quantify the degree to which a volitional maximal muscle contraction engages a muscle - an indication of motor unit activation[11]. When this relationship is compared between children and adults,

children showed decreased maximal volitional motor neuron activation compared to adults in multiple studies[12–14]. A potential result of this difference, per Henneman's size principle (that small motor units are recruited before large ones) [15], is that type I fibers would be recruited first with type II fibers recruitment as more force is required – meaning that children would activate a relatively lower number of type II fiber at their corresponding relative work[9].

To address anatomical basis of oxygen supply to muscle in adults- needle biopsies are done and histologically stained for capillaries. Like the ethical dilemma with determining muscle fiber types, differences between capillary densities in children vs. adults is largely unstudied. To my knowledge, there are currently no published studies that directly assess the capillary density of skeletal muscles in healthy children compared to adults. However, indirect evidence would suggest that children would have greater or similar capillary density compared to adults. First, in results from muscle biopsies done in adults show higher capillary density with more type I compared to type II fibers[3,16]. If children do indeed have higher percentage of type I fibers, their capillary density would also be higher. Second, in animal and human studies, smaller fiber diameter of correlated to higher capillary density per measured volume[17,18]. These higher relatively higher capillary densities would allow for more surface area for oxygen diffusion into myocytes[19].

Oxygen Uptake during Exercise in Children

Any introduction on oxygen consumption necessitates a brief discussion on studies of oxygen uptake (VO_2) measured via gas exchange. Cardiopulmonary

exercise testing (CPET) is the gold standard to measure the amount of oxygen consumed by determining the difference between amounts of oxygen breathed in vs. breathed out. It can be used to approximate whole body oxygen metabolism ($\dot{Q}O_2$) by measuring rates of ventilatory oxygen and carbon dioxide uptake ($\dot{V}O_2$ and $\dot{V}CO_2$)[20]. Previous comparisons of $\dot{V}O_2$ between children and adults demonstrate faster rise and a greater oxygen cost of relative work done in children vs adult[21]. This is mirrored by lower inorganic phosphate to phosphocreatine ratio at peak exercise in children vs. adults [22] - showing a greater reliance on aerobic respiration in children compared to adults.

The differences in the physiological characteristics and oxygen uptake discussed above suggest that there would be substantial changes in muscle-specific oxygen utilization from children to adults. Due to technical limitations, these functional differences between children and adults at the muscle are not fully defined. To address these gaps in knowledge, better non-invasive methods are needed to study the functional differences in muscle oxygen delivery. Advances in near infrared spectroscopy (NIRS) provide promising tools to investigate muscle oxygenation during exercise.

Near Infrared Spectroscopy (NIRS) derived Tissue Oxygenation

Light spectroscopy in general refers to the analysis technique used to determine physical properties of a medium which light travels through. The main advantage spectroscopy in the near-infrared spectrum (typically wavelength of ~650 to 1000 nm) for in-vivo biological investigation lies in its relatively low absorption

by water and hemoglobin and therefore provide good penetration of tissue of a few centimeters[23,24].

The ability of NIRS to determine tissue oxygenation is due to differential absorption of NIR light by hemoglobin in its oxygenated vs. deoxygenated states. At ~800 nm, absorption coefficient of oxyhemoglobin and deoxyhemoglobin are about the same (i.e. an isosbestics point). At NIR wavelengths below ~800 nm oxyhemoglobin has a higher absorption coefficient (i.e. more light is absorbed by oxy- than deoxyhemoglobin). At NIR wavelengths greater than 800 nm, deoxyhemoglobin has a higher coefficient. Therefore, the concentrations of oxy- and deoxyhemoglobin can be determined by quantifying light absorption above and below the isosbestics point.

In muscle, in addition to hemoglobin, oxygen can also binds to myoglobin, which contains only one heme group compared to four of hemoglobin. Hemoglobin (Hb) and myoglobin (Mb) NIR absorption spectra are very similar and therefore current NIRS technologies are not able to distinguish between absorption due to one vs. the other. Relative NIRS contributions from myoglobin vs. hemoglobin is debated with some groups reporting high[25] vs. low relative contributions[26]. Regardless of the relative contributions, NIRS-measured changes in hemoglobin and myoglobin concentration and oxygenation during periods of high oxygen demands (like exercise) will give overall accurate accounts of the muscle saturation and oxygen turnover[25].

Time Resolved-Near Infrared Spectroscopy (TR-NIRS)

Some of the earliest clinical investigations into the muscle using NIRS by Jobsis, Chance and others employed the use of continuous wave (CW) systems where NIR light is emitted at a constant intensity[23,24]. One of the limitations lies in its inability to quantify absolute concentrations of hemoglobin. Since the light source from CW systems are constant in amplitude and frequency – traditional, single detector systems are unable to determine the path length of light traveled. Without path length determination, CW systems can only report relative changes in hemoglobin. In order to quantify the absolute concentrations of oxy Hb+Mb and deoxy Hb+Mb tissue, time-resolved NIRS (TR-NIRS) was developed[24,27]. Briefly, this method uses the temporal information from quick picosecond pulses of light to determine the tissue characteristics needed to calculate absolute concentrations of oxy-Hb+Mb and deoxy-Hb+Mb. Figure 1.1 shows the temporal point spread function (TPSF) of a pulse of light sent into (green) the muscle and the temporal delay and widening of the light from the tissue (red).

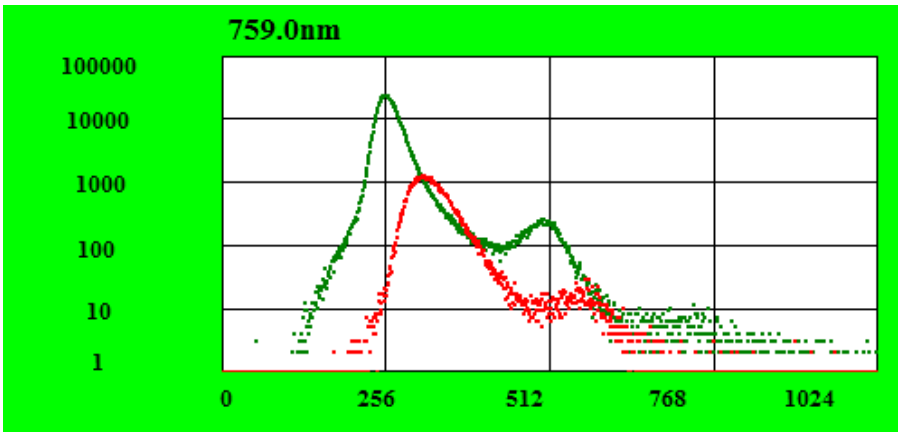

Figure 1.1 Temporal point spread function (TPSF) of the TRS-21. A pico-second scale pulse light (green) sent into tissue with the reflected light (red). Y-axis represent log scale of number of counts detected. X-axis represent an arbitrary unit of time. Image generated from TRS21 (Hamamatsu)

This temporal delay and widening (along with signal attenuation due to absorption) at 2-3 wavelengths around the isosbestics point provides the necessary information needed to obtain absolute [oxy Hb+Mb] and [deoxy Hb+Mb].

While it has been theoretically possible to use TR-NIRS since the 1980s[27] , due to the temporal precision and signal-noise ratio needed, TR-NIRS has been largely unavailable for use during exercise. The TRS-10/20/21 built by Hamamatsu Photonics from Japan is one of a few commercially available TR-NIRS devices capable of determining absolute [Hb+Mb] in muscle during exercise. All NIRS data in the dissertation was collected with the TRS-21 with a 3cm source-detector separation which provides a balance between enough usable signal while penetrating into the superficial muscle (about a 1.5 cm)[28–30].

The overall goal of this dissertation is to apply TR-NIRS technology to investigate differences in muscle oxygenation of children during exercise. Chapter 2 presents a novel strategy to correct for tissue water content. Chapter 3 presents observed differences in patterns of oxygenation and oxygen supply during ramp-type exercise between children and adults. Chapter 4 presents a pilot study for a NIRS investigation of seven children with sickle cell anemia during exercise. These studies and observations are especially important to provide early studies into the use of NIRS to non-invasively quantify microvascular oxygenation in healthy and diseased children. This would provide a tool that can assess the effectiveness of interventions such as exercise training or pharmaceutical interventions for children with diseases that affect oxygen delivery.

CHAPTER 2. Effects of Adipose Tissue Thickness on Water Percentage in TR-NIRS Measurements

I. Rationale

Since we are interested in studying muscle-specific oxygenation during exercise, one thing that needs to be accounted for is the variable thickness of adipose tissue on top of the muscle. Currently, most commercially available NIRS devices assume the tissue investigated is comprised of a single homogenous layer with consistent optical properties[30]. Therefore, the variable layer of skin and adipose tissue can affect optically-derived concentrations of oxy- and deoxyhemoglobin and myoglobin. A well-documented limitation with NIRS-derived measurements is the influence of adipose tissue thickness on the total concentration of hemoglobin and myoglobin[31–34]. Due to a lower concentration of hemoglobin (and no myoglobin) in adipose tissue compared to muscle tissue, as adipose tissue thickness increases, the total concentration in the volume investigated is lower[25]. A few methods have been proposed to appropriately scale the concentrations of total hemoglobin (Hb) and myoglobin (Mb) at various adipose tissue thickness to comparable amounts.

One method proposed has been used by Koga and colleagues in their usage of TR-NIRS [35–37]. Ultrasound-determined adipose tissue thickness is plotted against [total Hb+Mb] at a resting baseline. A linear regression line is determined and each individual's [total Hb+Mb] is scaled relative to the difference at a theoretical ATT = 0 mm. However, this technique has only been published in studies with relatively

small sample sizes and a small distribution of adipose tissue thicknesses in Japanese men. Another method that has been proposed by Niwayama et. al. who derive an equation derived from Monte Carlo simulations of photons' likelihood to travel through adipose or muscle tissue to account for "muscle sensitivity"[38]. The equation then gives a scaling factor for a given ATT. This chapter will assesses the appropriateness and limitations of these two approaches.

Another potential influencing factor of adipose tissue thickness on TR-NIRS measurements lies in the assumptions of the models used to calculate hemoglobin concentrations. Although water absorption in the NIR wavelengths are relatively low, it still needs to be accounted for in the reflected light. Because the earliest NIRS devices were used to investigate cerebral oxygenation[24], the assumption of a relatively fixed water content isn't as problematic as it is when investigating muscle. As the adipose tissue thickness on top of the muscle changes, the water content of the tissue volume survey by NIRS changes as well.

To assess the relationship between adipose tissue thickness and water content - we used another diffuse optical spectroscopic imager that utilizes broadband light rather than NIR developed here at the UCIrvine Beckman Laser Institute. With the additional wavelengths, water content can be assessed in additional to oxy- and deoxy- hemoglobin+myoglobin[39]. These measured tissue water percentages were incorporated into each individual's TR-NIRS measurements to account for the varying water content - which should provide a more accurate model when calculating oxy and deoxy-hemoglobin. Both devices had the same

source-detector separation (3 cm) which should result in similar tissue depth sampled [29]. This chapter will assess the degree to which water correction improves hemoglobin/ myoglobin measurements by NIRS on adipose and muscle tissue.

II. Methods

Subjects

Eighty-three children and eighteen adults (7-18 years old; 21-35 years old) were recruited to the Pediatric Exercise and Genomics Research Center (PERC) human performance laboratory at the Institute for Clinical and Translational Science at UCIrvine under an institutional review board approved protocol. Subjects (and parents, when appropriate) provided informed consent prior to experimental procedures. Subjects did not have serious or chronic health conditions, asthma, and were not obese (BMI% >95% for children and BMI >30 for adults). Children's sexual maturational status was categorized via Tanner stages by survey. Tanner stages 1-2 were grouped as "early pubertal", stages 3 was grouped as "mid-pubertal" and stage 4-5 were grouped as "late pubertal". See Table 2.1 below for group characteristics.

	Early Pubertal Children (N=32)	Mid Pubertal Children (N=7)	Late Pubertal Children (N=44)	Young Adult (N=18)
Female % (N)	53% (17)	57% (4)	56% (24)	55% (10)
Age (years old)	9.9±1.7	13.0±2.3	16.1±1.7	27.5±3.9
Height (meters)	1.38±0.12	1.59±0.15	1.67±0.09	1.69±0.11
Weight (kg)	33.4±10.1	50.9±15.3	59.0±10.7	67.1±12.9

Table 2.1 Average age, height, and weight of maturational groups studied. Numbers shown are mean±SD

Anthropometric and Body Composition

Standard calibrated scales and stadiometers were used to determine body mass and height. Body composition, including lean body mass, fat mass, and percent body fat was determined by dual-energy x-ray absorptiometry (DXA) using a Hologic QDR 4500 densitiometer (Hologic Inc., Bedford, MA). Superficial v*astus lateralis*-specific water and fat percentage was determined by broadband light spectroscopy using a 3-cm source-detector separation (mDOSI, UCIrvine Beckman Laser Institute, Irvine, CA). Skin and adipose tissue thickness (ATT) above *vastus lateralis* was obtained by ultrasonography (Lumify, Philips Healthcare, Neatherlands) while subjects were seated with quadriceps relaxed, resting parallel to the ground. ATT was measured from the surface of the skin to the top of the muscle via software-provided digital calipers (fig 2.1).

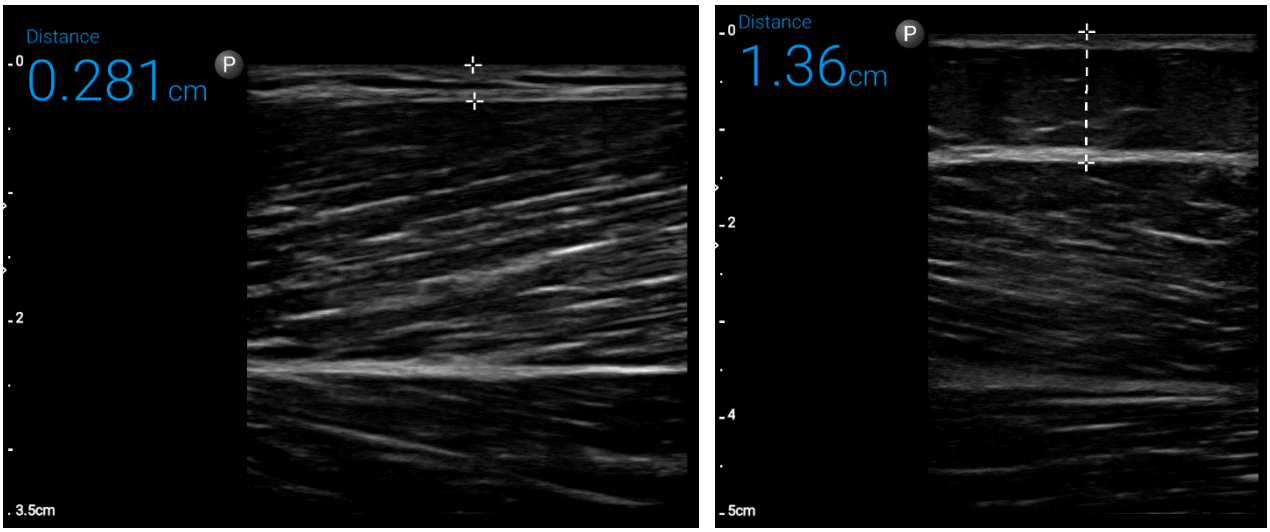

Figure 2.1 Sample ultrasound ATT measurements. ATT above *vastus lateralis* was measured from the top of the skin to the top of the muscle. Left panel is from a late pubertal boy with a low ATT. Right panel from an adult woman with high ATT.

Muscle Hemoglobin/Myoglobin Baseline Measurements

After an initial instrument warm-up, the TRS-21 instrument (Hamamatsu Photonics, Japan) was calibrated using fixed-distance cylinder with a neutral density filter to measure instrument function. With subjects seated on the cycle ergometer, quadriceps resting parallel to the ground, the laser probe with a 3-cm source detector separation was affixed onto the distal third of the *vastus lateralis* at the same location of ATT determination by ultrasound. Probe and leg were wrapped loosely with an elastic bandage to protect photodetector from ambient light. Optical measurements were acquired over three seconds for two minutes of resting baseline. After data collection, each measurement's [oxy-Hb+Mb] and [deoxy-Hb+Mb] was determined via provided software at either at 60% water (default) or the individual's water percentage obtained from mDOSI (water corrected). Data from the 2 minutes were averaged for the baseline measurements. [Total Hb+Mb] was derived from [oxy-Hb+Mb]+[deoxy-Hb+Mb]. Tissue saturation (StO_2) was derived from [oxy-Hb+Mb]/[total Hb+Mb].

Proposed Correction for ATT on [total Hb +Mb]

1. As proposed by Bowen et. al., linear regression were formed by plotting [total Hb+Mb] vs. ATT. The below equation were used where $y = mx + b$ is the linear regression generated where y is the theoretical [Hb+Mb] at a measured ATT "x" [37]. Scaling Factor $= \frac{b}{mx+b}$

2. As proposed by Niwayama et. al., for a given S-D separation of 3 cm, the following equation gives a "muscle sensitivity index" that [Hb+Mb] be scaled by at a measured ATT "x"[38] $\text{Scaling Factor} = \exp\left[-(\frac{x}{8})^2\right]$

Data Analysis and Curve Fitting

Group differences in adipose tissue thickness were tested by analysis of variance (one-way ANOVA) with Sidak's multiple comparisons for differences between the sexes. Significance was set at $p<0.05$. Linear and non-linear regression for adipose tissue thickness vs. NIRS measurements were fit and compared via biostatistics software. Linearity was tested by Wald runs test for randomness which evaluated random distribution of residuals for a linear relationship. (GraphPad Prism 6, GraphPad Software, La Jolla, CA)

III. Results

Effect of Adipose Tissue Thickness on DOSI Measured Water Content

Adipose tissue thickness varied between maturational and sex groups (*Fig. 2.2*).

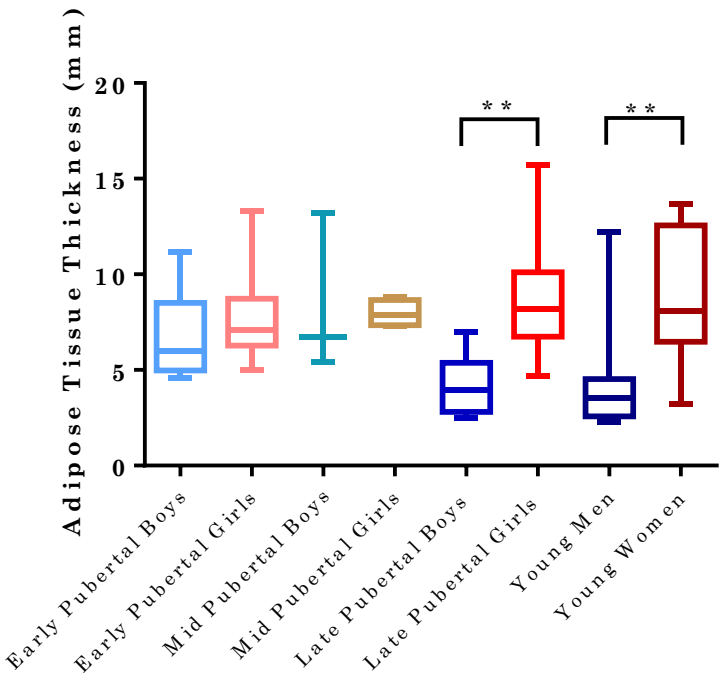

Figure 2.2 Adipose tissue thickness differences by sex and maturational groups. Box and whiskers plot with max-min. ** p<0.001

One-way ANOVA revealed a significant main effect of group F (7, 90)= 7.524, p<0.0001. Sidak post-hoc comparisons between the sexes revealed significant differences (p<0.001) between late pubertal and young adult men vs. women but not early and mid-pubertal boys vs. girls.

As expected, water content in tissue measured was lower with increased adipose tissue thickness (fig 2.3).

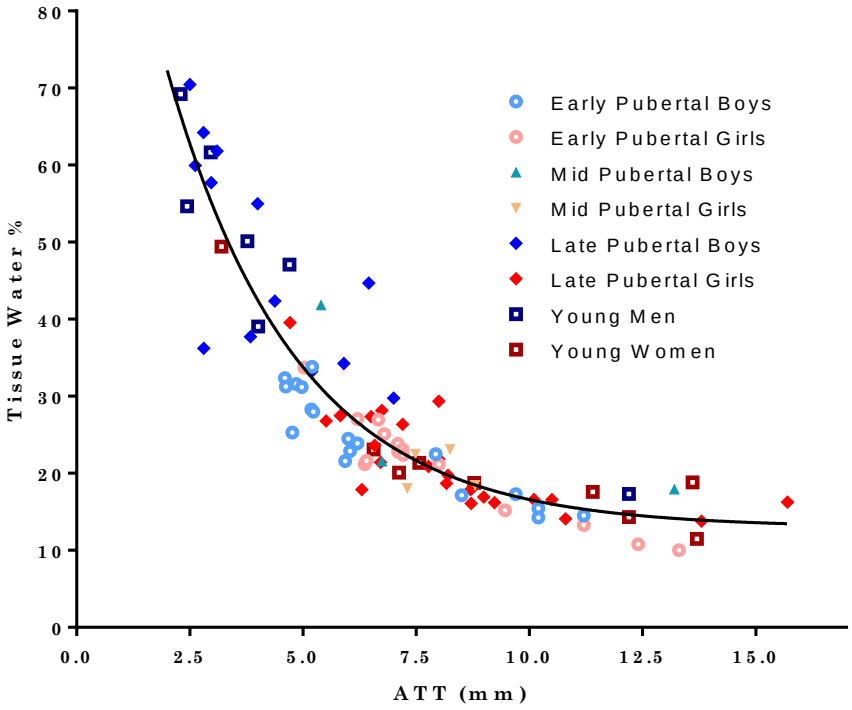

Figure 2.3 mDOSI-derived tissue water content vs. adipose tissue thickness. All sex and maturational groups plotted with an exponential decay fit line. Adjusted R squared = 0.86

There was sharp decrease in tissue water percentage from ATT of 1 to 6 mm with a leveling off after ~6 mm. Maximum tissue water content measured was 70.5% from a late pubertal boy with an ATT of 2.5mm. Minimum tissue water content measured was 10.0% from an early pubertal girl with an ATT of 13.3mm. When all groups with fit with bi-linear, log-log, logarithmic, or single-phase exponential decay functions, single-phase exponential decay yielded the best fit with an adjusted R squared of 0.86. The equation for the exponential decay function was:

$$y = 119.3e^{-0.349x} + 13$$

Effects of Water Correction on Resting TR-NIRS Measurements

After adjusting for the water content, the water corrected [total Hb+Mb], [oxy-Hb+Mb], [deoxy-Hb+Mb], and StO_2 were compared to the default uncorrected values. Figure 2.4a-d display the concentrations "recovered" from what was previously misattributed to water absorption. The changes in [Hb+Mb] mirror the inverse of the water vs. ATT function.

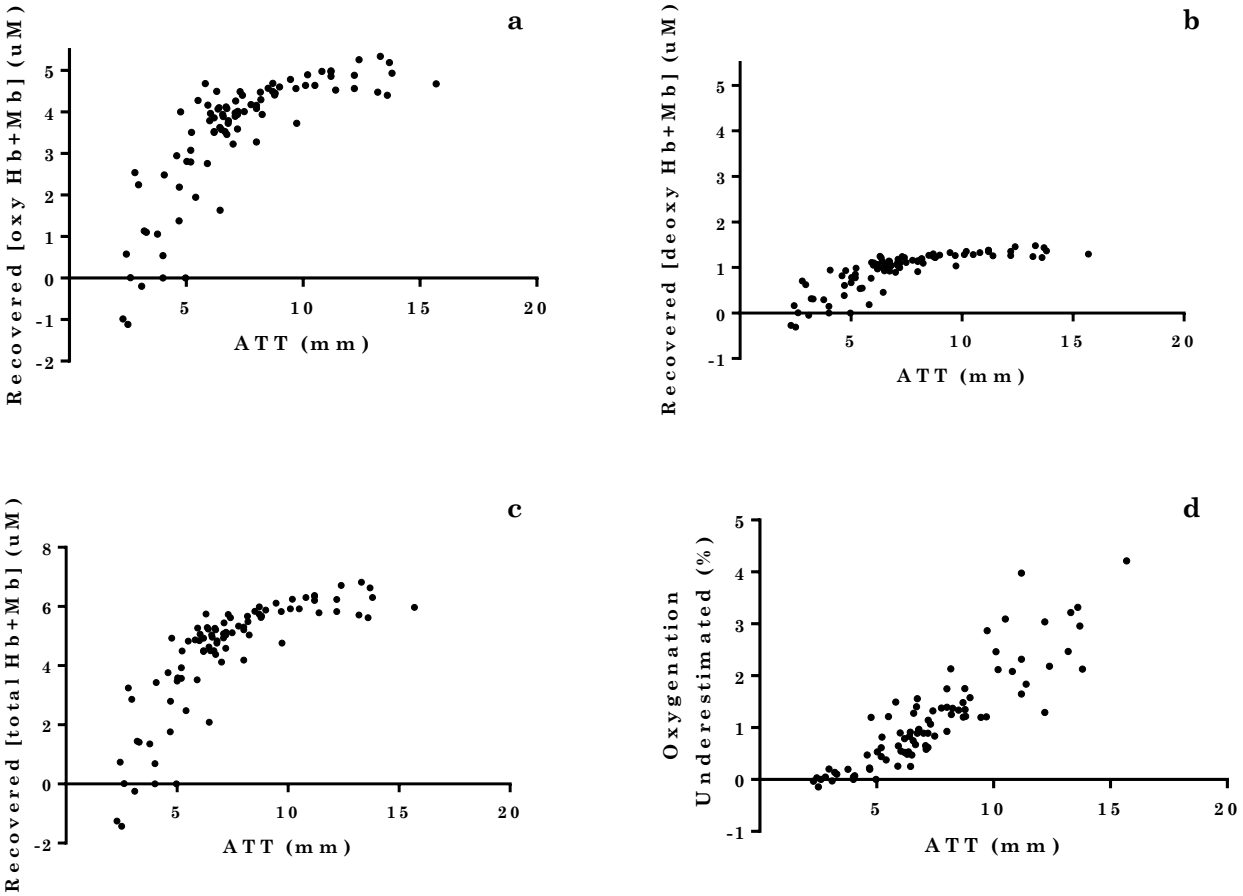

Figure 2.4a-d [Oxy Hb+Mb] (a), [deoxy Hb+Mb] (b), [total Hb+Mb] (c), and StO_2(d) incorrectly attributed to water if not water corrected. Water absorption has a greater effect on oxy-Hb+Mb signal compared to deoxy-Hb+Mb signal. The rate of recovery is proportional to the inverse of the tissue water % vs. ATT function.

The percent changes in [Hb+Mb] recovered after water correction are plotted below against adipose tissue thickness (figure 2.5a-c)

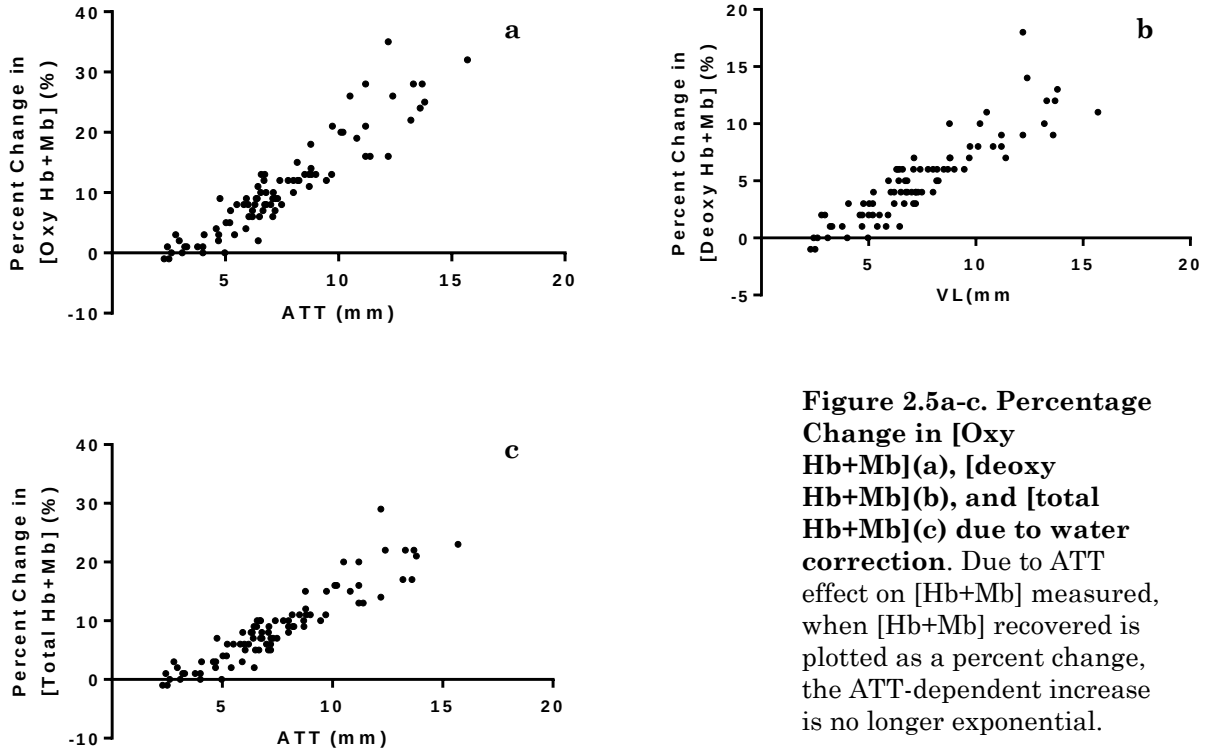

Figure 2.5a-c. Percentage Change in [Oxy Hb+Mb](a), [deoxy Hb+Mb](b), and [total Hb+Mb](c) due to water correction. Due to ATT effect on [Hb+Mb] measured, when [Hb+Mb] recovered is plotted as a percent change, the ATT-dependent increase is no longer exponential.

Effectiveness of Mathematical ATT Corrections on total [Hb+Mb]

As adipose tissue thickness increased, [total Hb+Mb] did not follow the linear trend proposed by Bowen et. al. (fig 2.6).

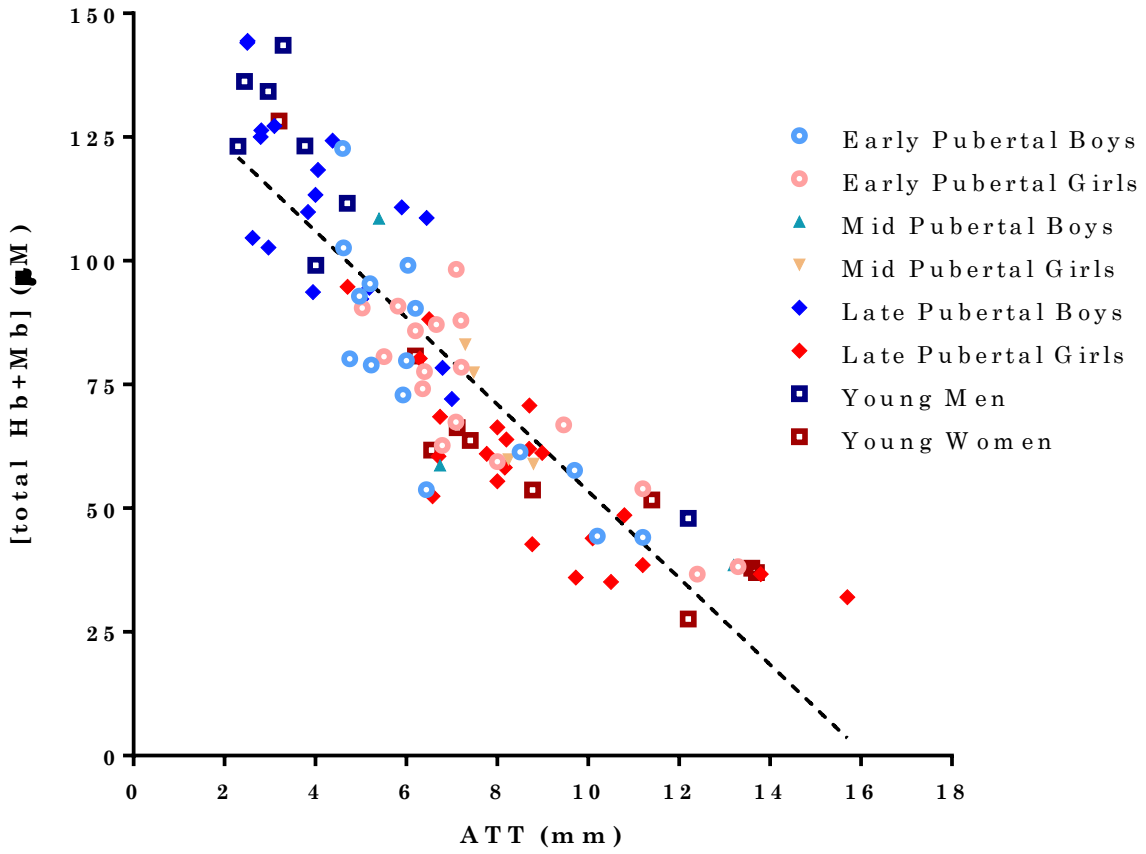

Figure 2.6 [Total Hb+Mb] decreased non-linearly vs. adipose tissue thickness. ATT above ~8 mm had a slower rate of decrease. ATT above 13 mm had lower variability and higher [total Hb+Mb] than predicted by the model.

Statistical test for linearity revealed a non-random distribution for residuals – indicating a systematic shift from linearity ($p<0.01$). Similarly, using the method proposed by Niwayama et. al. derived from a Monte Carlo simulation, [total Hb+Mb] ATT correction yielded a slow increase in [total Hb+Mb] until -7 mm where there was an exponential increase in the theoretical muscle [Hb+Mb]. The Subject with an ATT of 15.6 would have a [Hb+Mb] of > 1000 μM (figure 2.7)

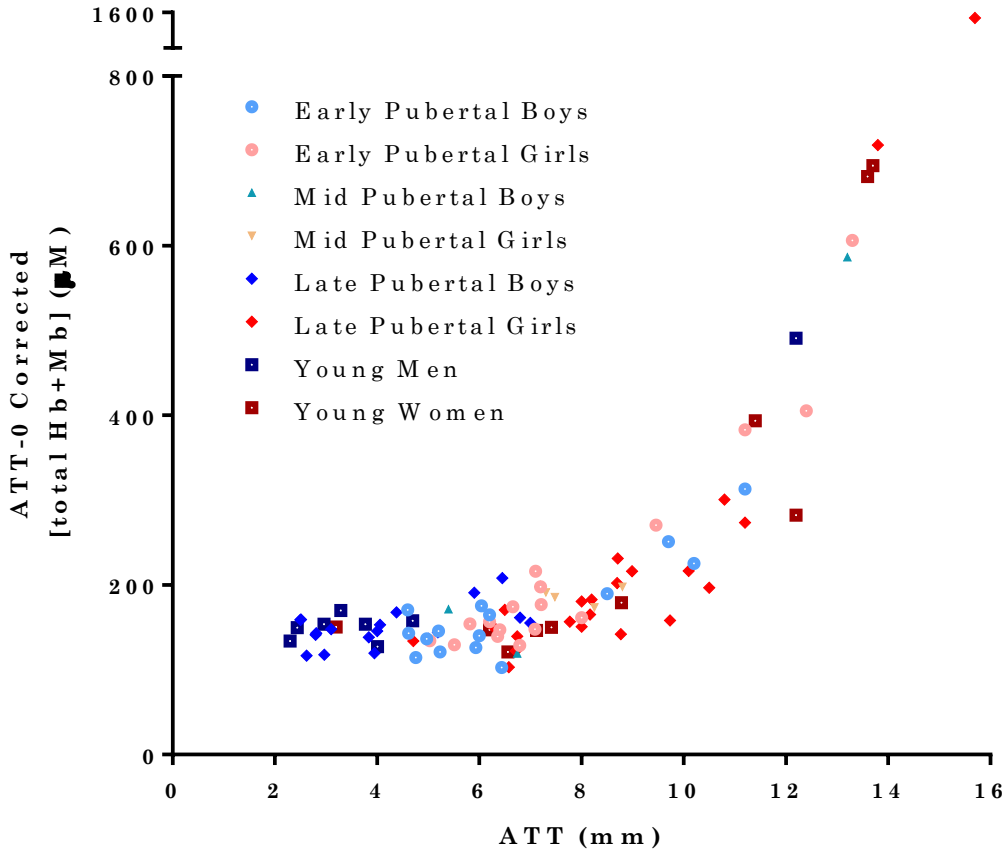

Figure 2.7 Adipose tissue thickness correction from Monte-Carlo simulations for muscle sensitivity. The model greatly underestimates muscle signal at ATT >7mm. Theoretically, [total Hb+Mb] should be relatively constant across ATT following correction.

IV. Discussion

Adipose and Muscle Tissue Water Content

Tissue water content dropped off quickly from a high of 70.5% water as adipose tissue thickness increased. For example, even a relatively low ATT of 5 mm, our results show that tissue water would be expected to be less than 35%. Our NIRS-derived water content is in good agreement from tissue water percentage estimated from other methods: muscle water content measured via dehydration have estimated water content in skeletal muscles to be about 68-77% water[40–42]. In

adipose tissue water content was on average 9-15% [43,44]. This suggests that that adipose and muscle tissue water content can be portably and accurately measured in-vivo via broadband DOS.

Extent of Improvement in NIRS-derived [Hb+Mb] After Water Correction

Following the inverse of the shape of the water curve, error in NIRS-derived [Hb+Mb] increased quickly and level off. However, due to the ATT attenuation of the [total Hb+Mb] measured, the percent error increased in roughly a linear fashion as ATT increases. Because of water's higher absorption at 759 nm compared to 834 nm, when water is overestimated (default), more signal from oxy-Hb+Mb compared to deoxy-Hb+Mb is incorrectly attributed to water. In absolute μM concentrations, this translate to a relatively low [deoxy-Hb+Mb] error of <2 μM across our range of ATT. A systematic underestimation of 1-2 μM for [deoxy-Hb+Mb] is probably within individual measurement variability. For [total Hb+Mb], water correction has a greater effect. For subjects with ATT greater than our average of 7.5 mm, not correcting for water would lead to a >10% underestimation of [total Hb+Mb].

Water correction is probably most important for measured tissue oxygenation. Although water-correction only accounted for a maximum of ~4% oxygenation difference, these differences could influence data interpretation. Tissue oxygenation appears to be tightly controlled and oxygenation drops less than 10% on average even during peak exercise. If adipose tissue thickness were similar across a cohort of investigation or in within-subjects comparison like a training study - this systematic underestimation of tissue saturation of a few percentage

points might not affect data interpretation. However, water correction would allow for more appropriate comparison of [total Hb+Mb] and tissue oxygen saturation between groups with systematically different adipose tissue thicknesses (e.g., men vs. women, children vs. men, trained vs. untrained). This would be especially important when comparing muscle oxygenation in healthy vs. diseased populations which often have systematically thicker adipose tissue thickness.

Despite the benefits of water correction, water measurements via mDOSI to supplement TR-NIRS measurements would probably be cost prohibitive. However, with the presented fit equation for ATT vs water content above- one could use that to estimate the water content. While an individual's measured water content would obviously be more accurate, using this equation as a reference to estimate water content would improve the assumptions behind Hb+Mb determination and allow for better comparison across groups.

Adipose Tissue Thickness Correction for [total Hb+Mb]

The two proposed methods tested to correct for adipose tissue thickness both underestimated signal contribution from muscle as adipose tissue thickness increased above ~7-8 mm. Despite the proposed usefulness of both methods - when applied on a wide range of ATT, both approaches failed to predict [total Hb+Mb] signal at higher ATT. Error is large enough where both methods are inappropriate to compare cohorts with a larger ATT differences. Similar results were obtained in a previous experiment that used a blood phantom and intralipid to simulate different adipose tissue thicknesses[45]. It appears that in-vivo muscle TR-NIRS has good

sensitivity for the muscle at even thicker ATT (i.e.,. 1-1.5 cm). The ATT correction methods tested here would not enable meaningful comparisons between late pubertal boys vs. girls and men vs. women who systematically vary in ATT. However, with the absolute [Hb+Mb] given by TR-NIRS - comparisons of percent increase/fold change from baseline would still be meaningful even without mathematically correcting for ATT. Even if there were less muscle volume sampled in an individual, percentage increase would reflect comparable corresponding changes in [Hb+Mb] in response to exercise[25].

Towards a Two-Layer Model

These data highlight some of the limitations that adipose tissue thickness has on obtaining useful and comparable muscle oxygenation measurements. Ideally, future NIRS devices would incorporate a two layer model to account for the differences between signal from adipose-tissue and the muscle underneath. This could be done using a priori information (a set of scattering and absorption values for adipose tissue with an adipose tissue thickness input from the user), additional wavelengths to directly calculate fat and water percentage (similar to the broadband DOS measurements made here), or even to use [total Hb+Mb] to back estimate adipose tissue thickness. This would allow for more direct comparison between different populations and to increase usability towards a tool that can be used in clinic to determine muscle oxygenation and changes. In its current state, muscle NIRS is a powerful tool that's still limited to research purposes requiring expertise in analysis and interpretation.

CHAPTER 3. Patterns of Muscle Oxygenation in Children and Adults during Ramp-type Progressive Exercise

I. Rationale

Most exercise and NIRS studies conducted have been on healthy and diseased young men with a large percentage of studies focusing on trained men. While these studies have been useful in validating the use of NIRS, the applicability of these findings to women and children are less clear. Furthermore, the uses of muscle NIRS during exercise in diseased population warrants additional emphasis. Though studies involving trained and untrained children and women are emerging[46–48], none have made direct comparisons of the sex and maturational differences between children and adults. These gaps in knowledge bring into question the extent to which one can apply findings from men to women and children. This is especially important to properly frame the potential differences in muscle microvascular in children with diseases. For example, if there were increases in oxygen supply after training in diseased children - what magnitude might be expected from normal maturation vs. effect of the intervention? What are the differences in pattern and magnitude of changes (if any) in tissue oxygenation during a progressive exercise test? The experiment in this chapter aimed to clarify the differences in healthy, recreationally active but untrained early pubertal and late pubertal children with young adults.

Due to differences in size (men and women are larger than young boys and girls) making meaningful comparisons between adults and children requires the

correct normalization to test for size-independent effects. It would not be as beneficial to identify differences between children and adults that are only the result of adults having larger muscles. This problem was addressed in two ways. First, comparisons across groups were made at the same relative work (i.e., same percent peak work rate). This comparison is a functional one - since a subject's peak work rate is their maximal power output, comparisons of [Hb+Mb] changes at comparable submaximal percentages would scale to the power produced. Second, comparisons across groups were made at the same lean body mass adjusted oxygen uptake (VO_2/LBM). Our group and others have shown that oxygen uptake scales well as a function of lean body mass(LBM)[49]. This comparison is a biological one - since VO_2/LBM is a direct measurement of oxygen consumed by unit muscle, comparisons at various VO_2/LBM would eliminate potential confounding effects of muscle efficiency, mechanical efficiency, or VO2 kinetic differences between the groups. Using LBM is especially important when comparing between men, women, and children since women and children tend to have higher percent body fat than men. Normalizing VO_2 by the relatively inactive fat mass would put women and children at a disadvantage.

Lastly, due to cost and availability, most muscle NIRS studies have been conducted with continuous-wave (CW) NIRS devices that only track individual's relative changes[50,51] or spatially resolved (SRS) NIRS devices that exhibit problems with variable adipose tissue thickness[31,52]. CW-NIRS studies can only track timing changes relative to an individual's peak deoxygenation and not absolute changes

inhemoglobin/myoglobin concentrations. TR-NIRS allows for direct comparisons of actual changes in [Hb+Mb] observed in tissue volume. These measurements are scarce in young men vs women comparisons and with even fewer measured in children[53].

II. Methods

Subjects

The early pubertal, late pubertal children, and young adults from chapter 2 were analyzed for the changes to hemoglobin/myoglobin oxygenation patterns during progressive ramp test.

Cardio-Pulmonary Exercise Testing (CPET)

Subjects were seated on an electronically-braked adult or pediatric cycle ergometer (Excalibur Sport or Corival Pediatric, Lode BV, Netherlands) and seat height adjusted to maintain a ~10 degree bend at the knee during full extension while cycling. There was a 2 minute period of seated rest to assess baseline gas-exchange and NIRS measurements followed by a 1 minute unloaded pedaling before the ramp. The ramp-type progressive test increased continually at a predetermined rate (7-30 watt per minute) until voluntary exhaustion or subjects were unable to maintain pedal rate of 60 revolutions per minute (RPM). The work rate increase was determined by height, weight, and activity level to normalize the length of ramp test to around 10 minutes. The end of the test was followed by 2 minutes of unloaded pedaling at 30 RPM.

Measurement of Peak and Submaximal Oxygen Uptake

After calibration by standard oxygen concentrations, Gas exchange was measured breathe by breathe using Vmax metabolic cart (Carefusion, San Diego, CA). Peak VO_2 was calculated as the maximum of 20-second rolling average over the last 2 minutes of exercise.

Hemoglobin/Myoglobin Measurements

TR-NIRS measurements were recorded on the *vastus lasteralis* every 3 seconds for the duration of the exercise tests per methods in Chapter 2. Measurements were corrected by each individual's tissue water content. Water-corrected hemoglobin myoglobin concentrations were averaged into ten second bins for the duration of the exercise tests (R project, R Foundation for Statistical Computing, Austria). Comparison of oxygenation patterns between the sex/maturational groups were made in two ways:

1) Comparing between relative work rates

Thirty second averages of total, oxy-, deoxy-hemoglobin + myoglobin, and tissue saturation were compared at individual's 0, 20, 40, 60, 80, 100% peak work rate. Maturational and sex group means were analyzed.

2) Comparing between relative oxygen uptakes per lean body mass.

Ramp time at each individual's 20, 30, 40, 50, 60 ml/min/kg-lean body mass was determined by 30 second averages of time-interpolated VO_2/LBM and matched for corresponding NIRS-measurements.

Statistical Analysis

For each outcome, a mixed model was performed with the repeated measurement of stage (relative work rate or VO_2/LBM) specified as a discrete values 0%, 20%, 40%, 60%, 80% peak or 20, 30, 40, 50, 60 ml/min/kg respectively. In addition, sex and puberty were included as fixed effects. The full model evaluated main effects of stage, sex and puberty; two way interactions of sex x stage, puberty x stage; and the three way interaction of sex x puberty x time with adipose tissue thickness as a co-variate. Within-subject correlation was accounted for in the model. Least Square Means were generated for each of the main and interaction effects. Differences in least squared means were evaluated where a significant effect was found. The significance of the difference between LS Means was judged based on an adjusted p-values. (SAS Software, SAS Institute Inc, Cary, NC)

III. Results

Percent changes in oxy, deoxy, total [Hb+Mb], StO2 from baseline were compared between sex and maturational groups at relative work rates (fig 3.1).

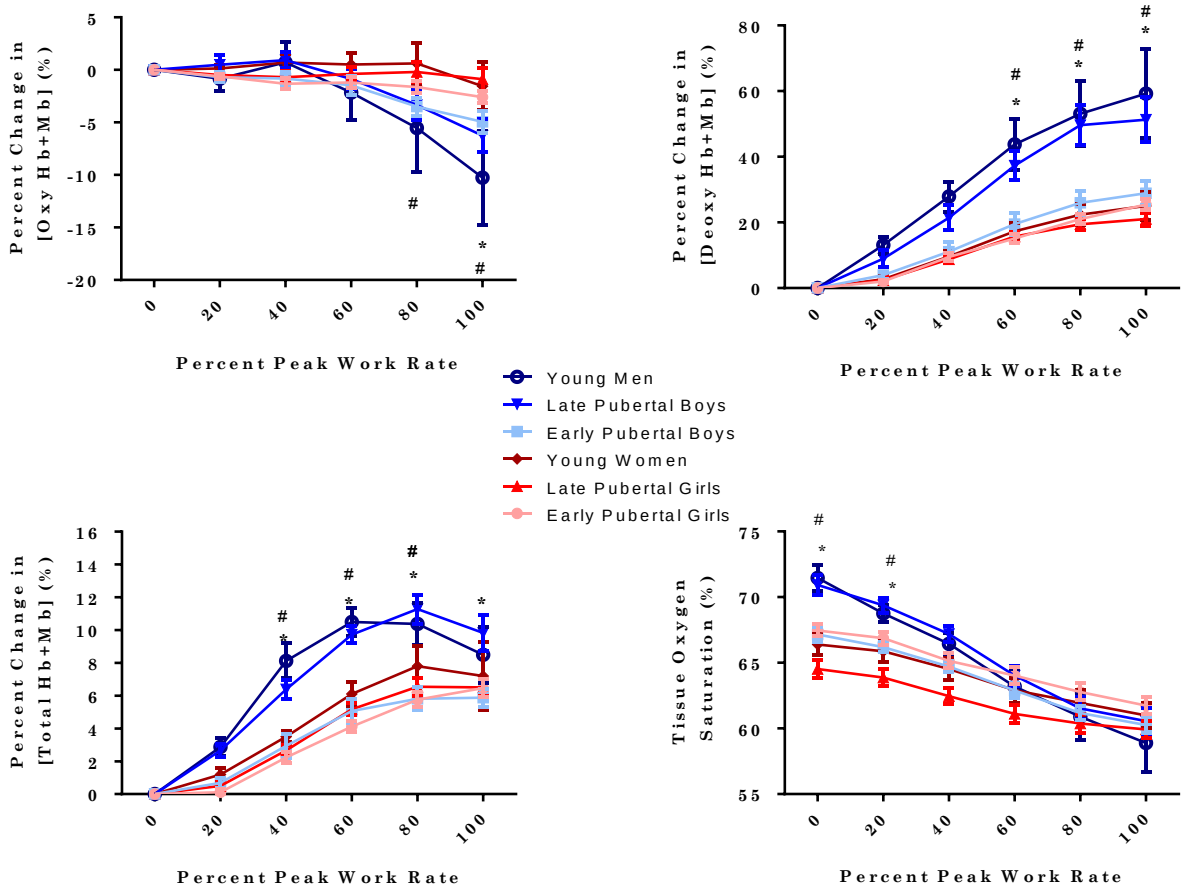

Figure 3.1 Greater changes in [oxy Hb+Mb], [deoxy Hb+Mb], [total Hb+Mb], and tissue oxygenation in late pubertal boys and young men compared to early pubertal children and young women when compared at same relative work rates. All variables had significant sex * group * percent peak work rate interaction. Indicating that post pubertal males responded to the exercise test differently than early pubertal children and post pubertal females. # = Young men significantly different from early pubertal children, late pubertal girls, and young women ($p<0.05$). * = Late pubertal boys significantly different from early pubertal children, late pubertal girls, and young women ($p<0.05$).

For oxy Hb+Mb, with a mixed model, there was a significant three way interaction of sex*group*percent work rate $F(10,420) = 2.78$ ($p<0.0025$). Post hoc comparisons showed that young men had significantly greater percent decrease of [oxy Hb+Mb] compared to females and early pubertal boys at 80 and 100% peak work ($p<0.05$). Late pubertal boys also had a greater decrease in [oxy Hb+Mb] than women and children at peak work rate. For percent change in [deoxy Hb+Mb], there was a

significant three way interaction of sex*group*percent work rate $F(10,420) = 4.82$, $p<0.0001$. Post hoc comparisons found late pubertal boys and young men having significantly percent increase in [deoxy-Hb+Mb] compared to early pubertal children and women at 60-100% peak work rate. Percent change in [total Hb+Mb] also had a significant sex * group * percent work rate interaction $F(10,420) = 4.82$, $p<0.0001$ with late pubertal boys having greater increase in [total Hb+Mb] from 40-100% and young men with greater increases in [total Hb+Mb] from 40-80% peak work (but not 100% peak).

StO_2 during progressive ramp test also had a significant three way interaction of sex * group * percent work rate: $F(10,420)= 4.27$, $p<0.0001$ with late pubertal boys and young men having higher tissue oxygenation at baseline and 20% peak work.

When oxygenation measurements were compared across lean body mass normalized oxygen uptake (VO_2/LBM), results were similar (figure 3.2)

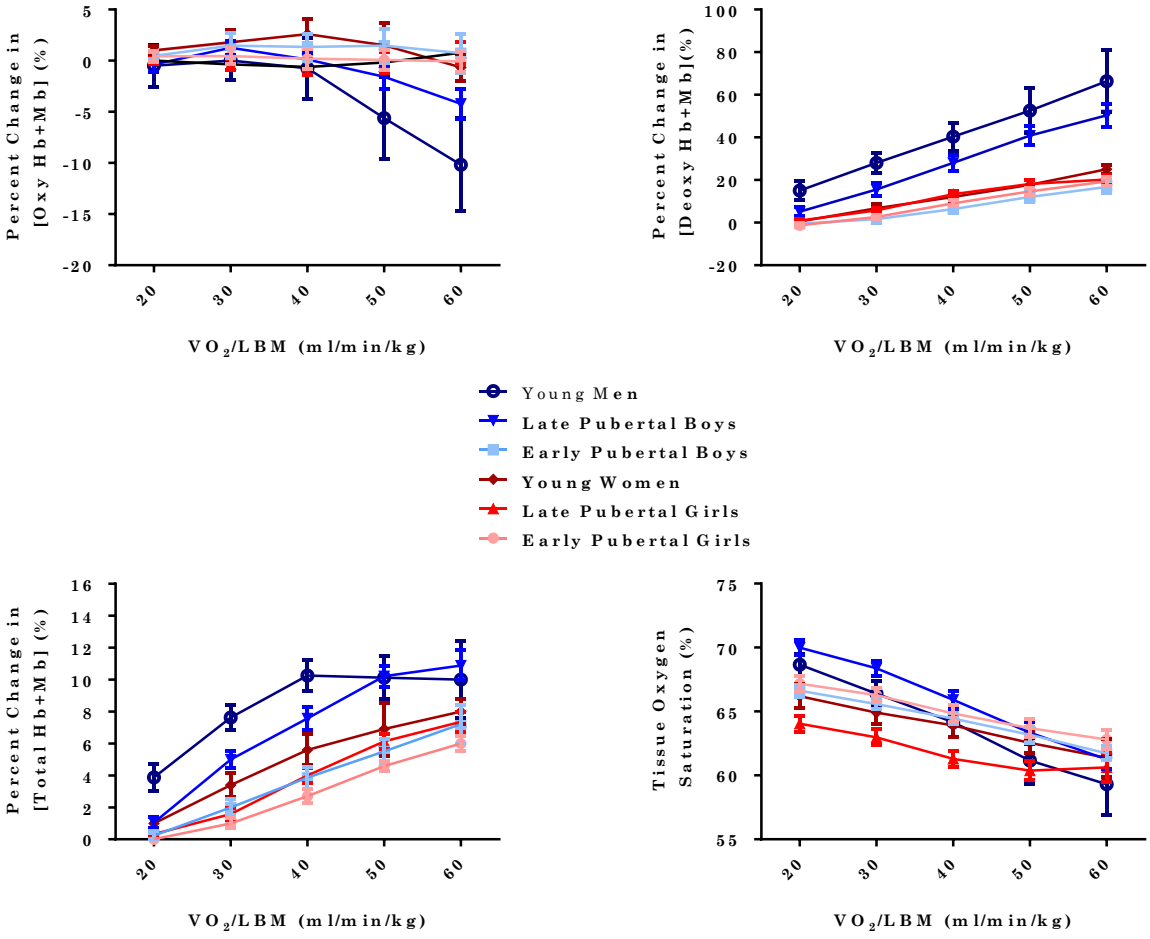

Figure 3.2 Late pubertal boys and young men still display altered fractional extraction and tissue oxygenation compared to early pubertal children and late pubertal girls/women when compared at the same relative oxygen uptake

Mixed model analysis on VO_2/LBM normalized [Hb+Mb] showed three way interaction of sex * maturational * oxygen uptake in [oxy Hb+Mb] $F(8,315) = 3.08$, $p = 0.0023$; [deoxy Hb+Mb] $F(8,315) = 5.65$, $p<0.0001$; StO2% $F(8,315) = 3.32$, $p=0.0012$. [total Hb+Mb] no longer had a significant three way interaction when compared across VO_2/LBM. There were significant two way interactions of

sex*group, sex*stage, and group*stage F (2,315) = 4.18, p=0.02; F (4,315) = 2.51, p=0.0419; F (8,315) = 3.06, p=0.0025.

IV. Discussion

Late pubertal boys and young adult men display different tissue oxygenation patterns during an incremental ramp test compared to early puberty boys and females. In late/post pubertal male, total muscle hemoglobin/myoglobin had greater increases at a lower relative work rate compared to other groups. Early pubertal children, late pubertal girls, and young women appear to have better matching of O_2 delivery to utilization compared with late pubertal boys and young men, who had a greater increases in [deoxy Hb+Mb] above 40% peak power. Despite having higher resting tissue oxygenation, post pubertal male muscle oxygenation dropped to similar levels as the other groups at peak exercise. These differences were maintained when compared at similar LBM-normalized VO_2 suggesting these differences were not fitness or work dependent. Together these data demonstrate sex and maturational tissue oxygenation responses to similar relative work rates on progressive ramp exercises.

Increased Oxygen Diffusive Capacity in Late Pubertal Boys and Men

Late pubertal boys and young men had greater increases in [total Hb+Mb] at lower percentage peak power compared to early pubertal children, and late pubertal girls and young women. This would indicate a greater concentration of red blood cells (RBC) in tissue since [myoglobin] is expected to be unchanged. With a higher concentration of RBC/hemoglobin, there is more surface area available for blood-

myocyte O2 flux - increasing the potential for oxygen diffusion independent of blood flow. This increased RBC concentration could occur with either decreased spacing between individual RBCs (termed "capillary longitudinal recruitment") or "de novo" recruitment of previously unused/underused capillaries. While the filling of previously dormant or unused capillaries is a possibility, experiments in humans and animal models have demonstrated that, contrary to observations made by August Krogh in the early 1910s[54], most muscle capillaries are flowing at rest[55].

Davis and Barstow estimated that a <10% increase in [total hemoglobin] during exercise could be fully explained by capillary longitudinal recruitment that brings muscle hematocrit up closer to that of systemic arterial hematocrit[25]. If this mechanism is dependent on systemic hematocrit, greater increases in [total Hb] at 80 -100% peak work rate in late pubertal boys/men would be expected with a 10-15% greater venous hemoglobin concentration compared to women and children[56]. Additionally, since we didn't control for stages in female's menstrual cycles - some women could have lowered blood hemoglobin concentration during their menstrual period.

However, the differences in [total Hb] increases at relative submaximal work were unexpected. When compared at similar VO_2/LBM, this relationship is not as clear; statistical results indicate that there was no three way interaction between sex x maturation x stages when VO_2/LBM was used to determine stage. This suggests that some of the differences might be due to maturational differences in hemodynamic response at submaximal work unique to a progressive ramp-test.

Different types of exercise at submaximal intensities should be tested to further explore sex and maturational differences in total hemoglobin concentration in a non-progressive setting.

Higher Fractional Extraction in Late Pubertal Boys and Men

In muscle, [deoxy-Hb+Mb] reflects the balance between oxygen supply and metabolic demand[57]. Late pubertal boys and young men had greater increases in [deoxy-Hb+Mb] compared to the other groups at both relative percent peak work rate and similar VO_2/LBM. Greater increases in [deoxy-Hb+Mb] signals an oxygen supply/demand mismatch and results in greater fractional oxygen extraction in late pubertal boys/ young men. Greater fractional extraction would be predicted by greater type II muscle fibers in men vs. women. Studies on rat muscles with different fiber type distributions show that fractional extraction is greater in muscles with faster twitch glycolytic fibers at rest and following muscle stimulation[58,59]. Greater fractional extraction would also result in smaller increases in blood flow relative to demand. Greater blood flow has been reported in women compared to men during same relative leg extension exercises[60]. Given similar [deoxy Hb+Mb] increases in early pubertal children - these data would predict similar matching of blood flow to demand at similar submaximal work rates.

Moreover, this lower blood flow in older boys/men isn't fully compensated by the increased [total hemoglobin]; as evidenced by greater drop in tissue oxygenation (stO2) across ramp exercise. Since tissue oxygenation reflects supply via perfusion and diffusive capacity (hemoglobin concentration) with metabolism - the greater

drop in tissue oxygenation further illustrate greater fractional oxygen extraction in men during exercise.

This conclusion of greater fractional extraction in men is different from what was previously reported by Murias et. al[61]. Their observations were most clear in un-normalized VO_2 (L/min) and un-normalized work rate. However, when compared across percent peak power as in the current study, these sex-difference was abolished. These conclusions were drawn from CW-NIRS measurements that detected an earlier [deoxy Hb+Mb] percent increase relative to peak in women compared to men. When a similar analysis was done on our TR-NIRS data set, no sex difference between men and women was found. One potential difference might be that earlier studies used CW-NIRS which incorrectly assumes constant light scattering at peak exercise and incorrectly derives [deoxy Hb+Mb] - leading to a different peak and different relative timing profile observed[62]. The authors also noted that given the blood flow and fiber type differences - they expected higher fractional extraction in men vs. women.

Men's Different Tissue Oxygenation during Exercise

The greater tissue oxygenation at rest in late pubertal boys and men observed could be explained by a lower resting oxygen metabolism (due to increased type II fibers[58,59]) or lower beta-oxidation/oxidative phosphorylation ratio[63,64], higher hematocrit[56], and/or greater capillary density[65] compared to female and early pubertal boys. With this data set there's no way to determine the cause but a variety of these factors could explain higher resting StO_2.

Our NIRS-derived tissue oxygen saturation reached a common low of 60.4±3.4% on average in all groups at peak exercise. Assuming resting values of 37ºC and a pH of 7.4, a tissue oxygenation of 60% would give an estimated microvascular pO2 of ~31 mmHg from a normal hemoglobin dissociation curve[66]. At peak exercise, a microvascular pO2 of 31 mmHg is almost certainly an underestimation due to the Bohr Effect (drop in pH), increased CO2, increased temperature, or greater 2-3-bisphosphoglycerate concentrations reducing the hemoglobin oxygen affinity. Furthermore, these are relatively superficial muscle measurements and Koga et. al. has demonstrated increased saturation in deeper vastus lateralis during exercise[29]. Still, these results are in relatively good agreement with H-NMR derived peak exercise mean capillary PO2 of 38±2 mmHg estimated from trained men[67]. With additional measurements or reference estimates, NIRS could provide accurate and non-invasive muscle microvascular pO2 measurements during exercise in various healthy and diseased populations.

Limitations

The NIRS measurements on this study was done only on a single muscle (*vastus lateralis)* during cycle ergometer test. Although it has been used due to its representative activity during cycling, region specific changes cannot be discounted. Also, these muscle oxygenation measurements were relatively superficial with mean penetration of ~1.5 cm below skin. Koga et. al showed with a high-powered NIRS device depth-related oxygenation heterogeneity in the *vastus lateralis*[29]. The results from deeper muscle might differ between the sex and maturational groups.

Also, comparisons of blood flow between groups was not measured; these assumptions should be compared in children vs. adults during leg exercises. In general, the subjects were not trained but were relatively active - studies have shown that regular exercise training alters the oxygenation patterns. To be able to apply these sex and maturational differences more broadly, studies on sedentary and overweight/obese groups would be beneficial. It would be interesting to see the greater fractional extraction in men observed would be evident in sedentary men as well.

CHAPTER 4. Muscle Oxygenation in Children with Sickle Cell Anemia during Incremental Ramp Exercise: A Pilot Study.

I. Rationale

Sickle cell disease (SCD) affects millions worldwide and is the most common inherited blood disorder in the United States[68]. Individuals with 2 copies of the abnormal ß globin gene produce sickle hemoglobin (Hb-S). Red blood cells that contain Hb-S have shortened survival - leading to chronic anemia for individuals with sickle cell anemia (SCA). In addition, Hb-S have reduced oxygen carrying capacity characterized by a "right-shift" in oxygen dissociation curve (i.e., Hb-S have lower hemoglobin saturation % than healthy Hb at a given pO2)[69,70]. One of the hallmarks of the disease is the polymerization of Hb-S leading to "sickling" of RBC that has been associated with decreased oxygen tension[69]. These sickled RBC leads to vaso-occlusive pain crises and leads to tissue damage[71].

Functionally, children and adults with SCA have significant reductions in cardiopulmonary fitness with lowered VO_2peaks. Currently there is no consensus on whether moderate or high intensity exercise is harmful to individuals with SCA. Acute exercise challenges have been shown to be well tolerated by children with SCA[72] - however, the long term effects of exercise training are unknown. Given the cardiovascular health benefits of regular exercise in healthy population, whether exercise training would be beneficial in children with SCA should be explored. To properly assess the effectiveness and safety of various exercise prescriptions -

quantitative tools and measurements will allow for empirical evidence behind the possibility of an exercise prescription in SCA children. Since Hb-S has similar NIRS absorption characteristics as healthy hemoglobin[73]. NIRS technologies could be a useful tool to investigate muscle-specific oxygenation changes in children with SCA during exercise - giving insight into microvascular hemoglobin concentration and oxygenation.

This pilot study in collaboration with Robert Liem, MD is among the first to investigate muscle-specific oxygenation changes during exercise in SCA children. Age-matched control from the previous chapter was used for reference comparison.

II. Methods

Subjects

Seven children (age 13-15; four female) with sickle cell anemia being were recruited from around Chicago, IL for this study. The subjects are under the continued care of a pediatric hematologist. All subjects were black Americans. Average venous hemoglobin concentration was 8.4±1.0 g/dL (mean±SD); reference value for healthy African-American children (11-15 years-old) is 12.6-13 g/dL[74]. Adipose tissue thickness was on average 5.7±2.4 mm. Six of the seven children were taking hydroxyurea regularly as treatment to increase their amount of fetal hemoglobin. Subjects were recruited with an IRB protocol approved by University of Illinois, Chicago where the study was conducted.

Muscle oxygenation data from seven healthy children (age 13-15; four female) from the study in the previous chapter was picked as matched controls. They were

selected to match age, sex, and adipose tissue thickness. All control children were white Americans from around Irvine, CA. Hemoglobin concentrations were not available.

Anthropometric and Body Composition

Standard calibrated scales and stadiometers were used to determine body mass and height. Skin and adipose tissue thickness (ATT) above vastus lateralis was obtained by ultrasonography while subjects were seated with quadriceps relaxed, resting parallel to the ground. ATT was measured from the surface of the skin to the top of the muscle via software-provided digital calipers

Cardio-Pulmonary Exercise Testing (CPET) for SCA Subjects

Ramp-style cycle ergometer exercise test was conducted similar to that in chapter 3 with this notable exception - while healthy subjects from chapter 3 had progressive increases in work rate during exercise test (e.g. power increased by increments of 1 watt) this group's ramp work rate increased 10 watts every 1 minute.

Measurement of Peak Oxygen Uptake for SCA Subjects

After calibration by standard oxygen concentrations, Gas exchange was measured breath by breath using TrueOne 2400 metabolic cart (Parvo Medics, Sandy UT). As with in chapter 3, Peak VO_2 was calculated as the maximum of 20-second rolling average over the last 2 minutes of exercise.

Hemoglobin + Myoglobin Measurements for SCA Subjects

TRS-21 measurements of the *vastus lateralis* was collected every 3 seconds as described in the previous chapter over the course of the exercise test. Tissue water content was estimated as suggested in chapter 2 from the measured adipose tissue thickness with this equation:

$$y = 119.3e^{-0.349x} + 13$$

where x is the adipose tissue thickness in mm y is the estimated water percentage. This water estimate was input to TRS-21 to calculate [oxy Hb+Mb] and [deoxy Hb+Mb]. [Total Hb+Mb] was derived from [oxy-Hb+Mb]+[deoxy-Hb+Mb]. Tissue saturation (StO2) was derived from [oxy-Hb+Mb]/[total Hb+Mb].

Percent change from baseline [Hb+Mb] were compared between SCA and control in two ways:

1) Thirty second averages around 20, 40, 60, 80, 100% peak work
2) Thirty second averages at ~21 VO_2/ kg (average peak work rate for SCA group).

Statistical Analysis

A mixed model was performed with the repeated measurement of stage specified as a discrete values of 0%, 20%, 40%, 60%, 80%, and 100% peak work rate In addition, sex and group (SCA vs control) were included as fixed effects. The full model evaluated main effects of sex, group, and stage and two way interaction of group and stage with VO_2/kg as a covariate. Within-subject correlation was accounted for in the model. Least Square Means were generated for each of the main and interaction effects. Differences in least squared means were evaluated

where a significant effect was found. Significance of the difference between LS Means was judged based on an adjusted p-values. (SAS Software, SAS Institute Inc, Cary, NC)

Group differences for single point outcomes were analyzed using two-tailed student's t-test. Significance was set at $p<0.05$

III. Results

SCA subjects reached lower peak work and lower VO2 peaks compared to their matched controls. Results are summarized in table 4.1.

	SCA	**Control**	**t-test *p* value**
Age (years)	13.6±0.9	13.7±0.7	0.70
Height (cm)	162±4	158±10	0.40
Weight (kg)	51.2±8.8	47.3±8.0	0.45
Adipose Tissue Thickness (mm)	5.7±2.4	6.2±1.6	0.72
Peak Work Rate (watts)	87±14	193±49	**<0.0001***
Peak VO_2/kg (ml/min/kg)	21.6±3	50.8±9.4	**<0.0001***

Table 4.1 Anthropometric and peak work data from SCA vs. age matched controls. All data are mean ± standard deviation.

Percent Change in [oxy Hb+Mb], [deoxy Hb+Mb], [total Hb+Mb], and Tissue oxygenation between the SCA and Control are shown in figure 4.1.

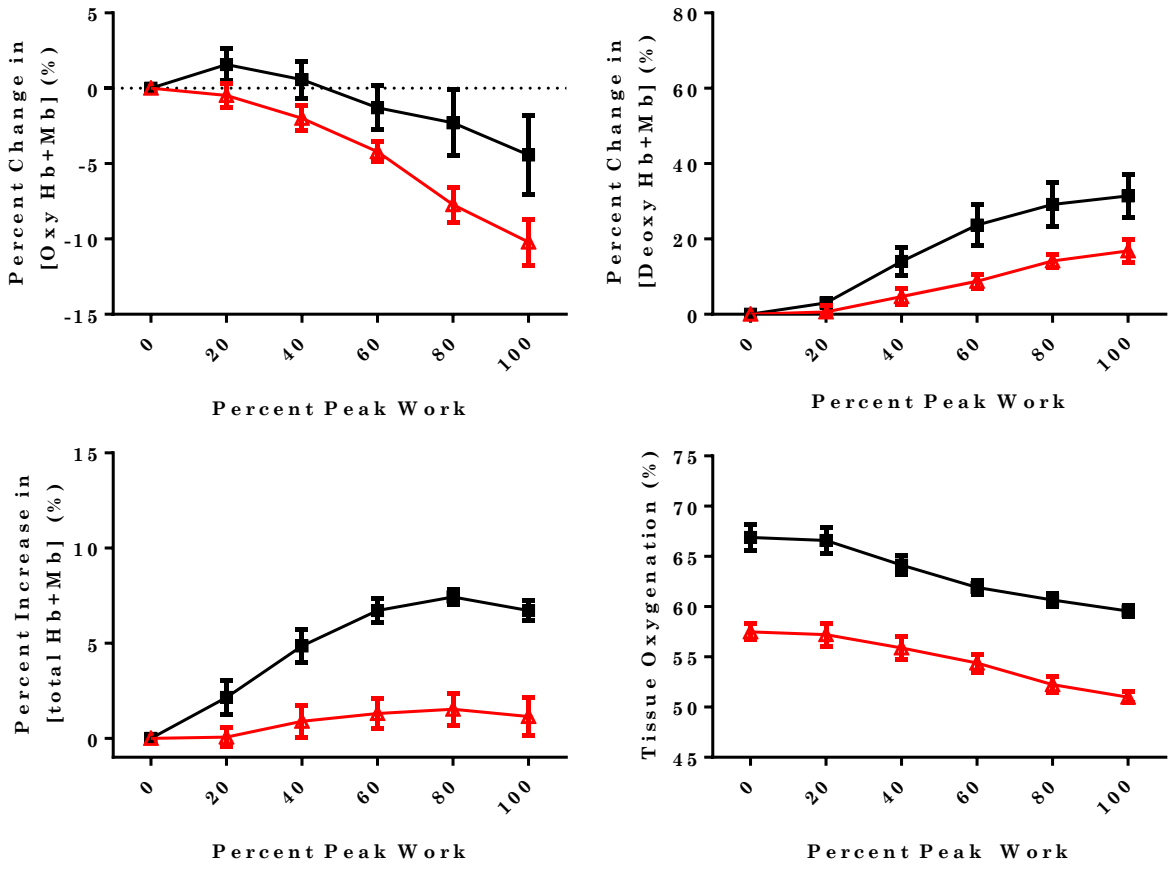

Figure 4.1 Percent change in [oxy Hb+Mb], [deoxy Hb+Mb], [total Hb+Mb], and Tissue Oxygenation during incremental ramp test. Black lines are control, Red lines are children with sickle cell anemia. Mixed model showed significant group*percent work interaction in [deoxyHb+Mb] and [total Hb+Mb] $F(5,60)=4.76$, $p<0.001$; $F(5,60) = 12.07$, $p <0.0001$. [oxy Hb+Mb] and Tissue oxygenation interactions were not significant. All points were mean±s.e.

Mixed model analysis on [oxy Hb+Mb], [deoxy Hb+Mb], [total Hb+Mb], and tissue oxygenation across percent peak work rate had significant group * percent work interaction in [deoxyHb+Mb] and [total Hb+Mb] $F(5,60)=4.76$, $p<0.001$; $F(5,60) = 12.07$, $p <0.0001$ respectively. [oxy Hb+Mb] had no significant group effect or group*stage interactions. Tissue oxygenation trended towards a significant group

effect p=0.05. Using the adipose tissue thickness vs.[total Hb+Mb] results from chapter 2 as reference values, both control and SCA groups had resting [total Hb+Mb] that were ~ 100% predicted for their individual ATT (SCA: 104±17%, Control: 102±17%, p=0.78)

Comparisons of changes in [Hb+Mb] between groups were made at the same normalized oxygen uptake of ~21 ml/kg/min (figure 4.2). This was the peak oxygen uptake measured in the SCA group.

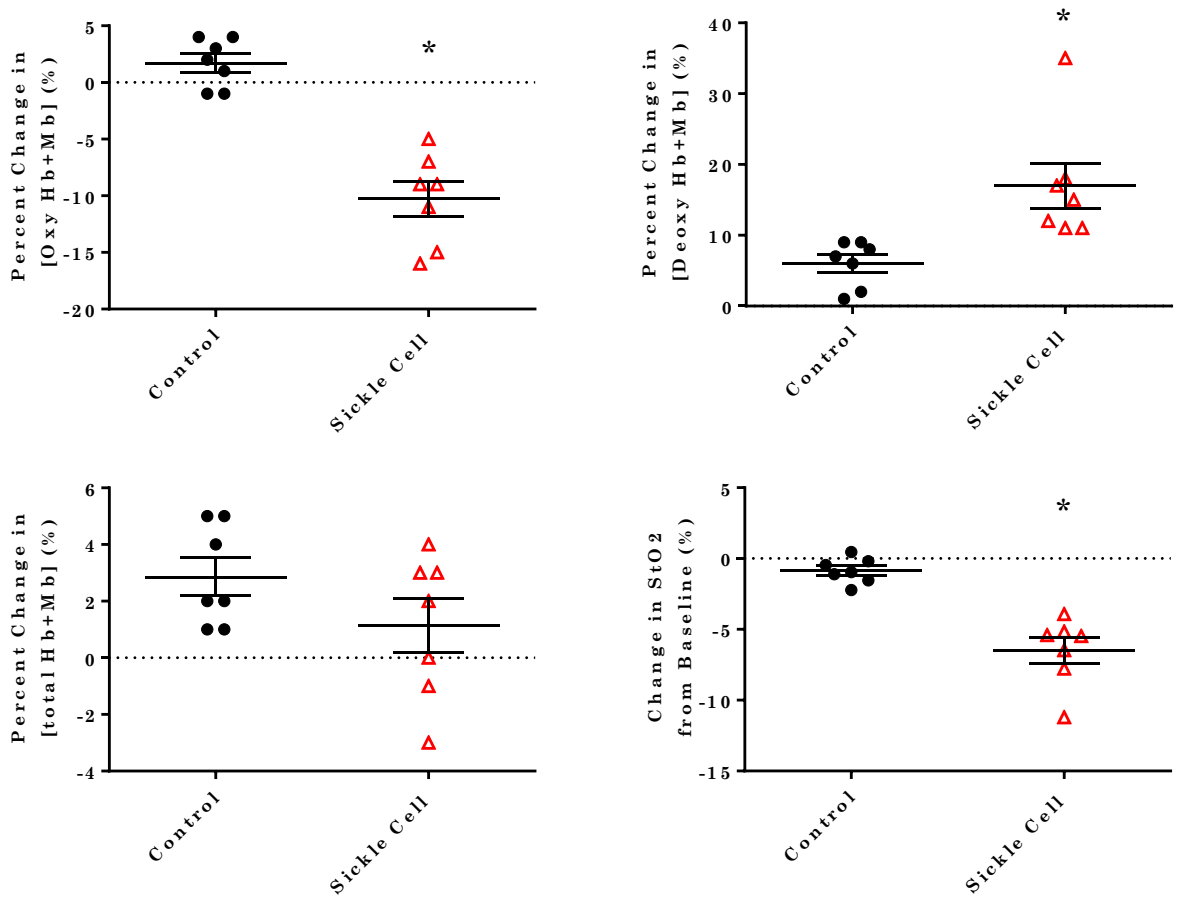

Figure 4.2 Percent change in [oxy Hb+Mb], [deoxy Hb+Mb], [total Hb+Mb], and stO_2 at oxygen uptake of 21 ml/kg/min in children with sickle cell anemia compared to healthy controls. 2-tailed t-test p<0.001 vs. control.

At VO2/kg of 21ml/kg/min, SCA had significantly greater percent decrease in [Oxy Hb+Mb], greater percent increase in [deoxy Hb+Mb], similar increases in [total Hb+Mb] and greater desaturation (greater decrease in tissue oxygenation from baseline), all two-tailed student's t-test, $p < 0.001$.

IV. Discussion

Children with sickle cell anemia (SCA) had markedly different patterns of muscle deoxygenation during incremental ramp test compared to aged-matched controls. When compared across percent peak power, even with a statistical model controlling for VO2/kg differences, children with SCA did not have as large a percent increase in [total Hb+Mb] - reflecting an inability to increase the diffusive capacity of the microvasculature. This blunted percent increase in [total Hb+Mb] wasn't compensated by a higher resting [total Hb+Mb] (i.e. percent increase wasn't lower due to greater starting [total Hb+Mb] in children with SCA). This behavior could be expected the SCA group's anemia. Furthermore, because of their anemia, children with SCA is described to be in a cardiopulmonary hyperdynamic state with higher resting heart rate, higher resting blood pressure, and higher resting cardiac output[72]. Regardless of the mechanism, this demonstrates that children SCA cannot meaningfully increase their muscle [Hb+Mb] to increase diffusional surface area and increase oxygen supply during exercise.

Children with SCA display a markedly lower oxygen saturation at rest (even lower than that of end exercise in healthy controls) and decrease throughout the exercise test. However, a few factors cause the comparison of oxygen tension and

muscle pO2 between the SCA and control groups to be challenging. First, Hb-S has a "right-shifted" oxygen dissociation curve, sickle hemoglobin are less likely to be bound to oxygen at the same pO_2 compared to healthy hemoglobin. Second, there is some evidence of greater increase in blood lactate shortly following the onset of exercise in patients with SCA[75]. This will cause a further desaturation of hemoglobin at a given pO_2. Third, few studies in patients with SCA have shown a greater drop in arterial hemoglobin saturation during exercise - potentially reducing the availability of oxygen even further. Last, six of these patients were taking hydroxyurea as a treatment to increase their fetal hemoglobin which are "left-shifted" compared to healthy adult hemoglobin. These opposing factors make the extrapolation of an oxygen tension in microvasculature difficult. Given the compounding factors that influence oxygen dissociation in children with sickle cell anemia, microvascular pO_2 at this lower tissue saturation could be the same, lower, or even greater compared to controls. That said, given the small variability in hemoglobin/myoglobin saturation in our small sample - these differences don't appear to be individual specific and might vary similarly in the population.

The big takeaways from this pilot study are that NIRS provides an intriguing tool to investigate hemoglobin/myoglobin saturation at the muscle during exercise. During our acute exercise challenges, at the superficial VL, there didn't seem to be a big buildup of hemoglobin/RBC in microvascular capillaries. The measurements of [total Hb+Mb] showed a clear indicator of inadequate oxygen supply during exercise in SCA children. From the similar decrease in StO2 during exercise, there doesn't

appear to be obvious problems with oxygen extraction. All subjects stopped due to complaints of leg fatigue and not pain from severe vasoconstriction.

These pilot data on children with sickle cell anemia provide compelling evidence for the usefulness of TR-NIRS as a tool to assess muscle oxygenation. Although additional, more invasive, measurements might have to be taken to better understand the NIRS signal the sickle cell population, this non-invasive tool gives a snapshot a functioning muscle's tissue microvasculature that previously was not available. Specific experiments would have to be designed to test the clinical relevance and potential biomarkers available but would be invaluable in designing evidence-based exercise interventions in children with sickle cell anemia.

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