The influence of adipose tissue on spatially resolved near-infrared spectroscopy derived skeletal muscle oxygenation: the extent of the problem

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The influence of adipose tissue on spatially resolved near-infrared spectroscopy derived skeletal muscle oxygenation: the extent of the problem


1 Department of Cardiology, Máxima Medical Centre, PO Box 7777, 5500 MB Veldhoven, Netherlands
2 Department of Applied Physics, Eindhoven University of Technology, PO Box 513, 5600 MB Eindhoven, Netherlands
3 Department of Biomedical Engineering, Eindhoven University of Technology, PO Box 513, 5600 MB Eindhoven, Netherlands
4 Department of Medical Physics, Máxima Medical Centre, PO Box 7777, 5500 MB Veldhoven, Netherlands

E-mail: victor.niemeijer@gmail.com

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Abstract

Objective. Near-infrared spectroscopy (NIRS) measurements of tissue oxygen saturation (StO₂) are useful for the assessment of skeletal muscle perfusion and function during exercise, however, they are influenced by overlying skin and adipose tissue. This study explored the extent and nature of the influence of adipose tissue thickness (ATT) on StO₂. Approach. NIR spatially resolved spectroscopy (SRS) derived oxygenation was measured on vastus lateralis in 56 patients with chronic heart failure (CHF) and 20 healthy control (HC) subjects during rest and moderate intensity exercise with simultaneous assessment of oxygen uptake kinetics (τVO₂onset). In vitro measurements were performed on a flow cell with a blood mixture with full oxygen saturation (100%), which was gradually decreased to 0% by adding sodium metabisulfite. Experiments were repeated with 2 mm increments of porcine fat layer between the NIRS device and flow cell up to 14 mm.

Main results. Lower ATT, higher τVO₂onset, and
CHF were independently associated with lower in vivo StO₂ in multiple regression analysis, whereas age and gender showed no independent relationship. With greater ATT, in vitro StO₂ was reduced from 100% to 74% for fully oxygenated blood and increased from 0% to 68% for deoxygenated blood. **Significance.** This study shows that ATT independently confounds NIR-SRS derived StO₂ by overestimating actual skeletal muscle oxygenation and by decreasing its sensitivity for deoxygenation. Because physiological properties (e.g. presence of disease and slowing of $\tau_{O_2,\text{ons}}$) also influence NIR-SRS, a correction based on optical properties is needed to interpret calculated values as absolute StO₂.

**Keywords:** chronic heart failure, blood, exercise, aging, gender, oxygen kinetics

(Some figures may appear in colour only in the online journal)

**Novelty and significance**

This study shows that adipose tissue thickness (ATT) has a great influence on near-infrared spatially resolved spectroscopy (NIR-SRS) skeletal muscle oxygenation measurements. This is attributable to a decreased measurement sensitivity and the optical properties of adipose tissue itself. Although NIR-SRS is less susceptible to ATT than more conventional NIR-methods (based on modified Beer–Lambert law), measurements should be corrected for ATT by numerical modeling of optical properties of multilayered tissue instead of normalizing for physiological properties (e.g. oxygen consumption and saturation).

**1. Introduction**

Near-infrared spectroscopy (NIRS) is a technique to non-invasively monitor oxygenation in human tissue. Its utility lies in the evaluation of the ratio between perfusion and oxygen consumption in metabolically active tissues. More specifically, it can be used in the assessment of the contribution of O₂ delivery and utilization to limitations of oxidative metabolism in skeletal muscle during exercise (Ferrari *et al* 2011), for instance in patients with chronic heart failure (CHF) (Sperandio *et al* 2009, Bowen *et al* 2012, Niemeijer *et al* 2016).

Contemporary studies mostly use a continuous wave (CW) methodology employing modified Beer–Lambert law (MBL), or the spatially resolved spectroscopy (SRS) approach to attain concentration changes of deoxygenated hemoglobin [HHb] or measures of absolute tissue oxygen saturation (StO₂), respectively. Both $\Delta[\text{HHb}]$ and SRS-derived StO₂ have been used as a proxy for fractional oxygen extraction in skeletal muscle, where StO₂ might be preferable because it can be expressed in absolute values and is essentially less sensitive to skin blood flow changes (Kime *et al* 2013, Messere and Roatta 2013).

Although technology has advanced over the years to produce time-domain (TD) and frequency-domain (FD) instruments that allow quantification of the actual tissue optical properties to calculate absolute oxygenated and deoxygenated hemoglobin concentrations, the signal-to-noise ratio and temporal resolution are not as high as for the SRS technique (Ferrari *et al* 2011). Recent studies using evolved TD systems show that manufacturers are closing the gap (Ohmae *et al* 2014), however, the lower costs and practical portability of currently commercially available SRS instruments has not yet been paralleled. Thus, at present, NIR-SRS
provides a highly effective tool to monitor muscle microvascular oxygen exchange during exercise in health and disease. However, when considering between-subject comparisons of skeletal muscle oxygenation, one has to take into account the influence of optical properties of the variable layer of overlying skin and adipose tissue. Indeed, the thickness of the adipose tissue layer has been shown to confound NIRS measurements, distinctly increasing resting oxygenation values (Geraskin et al 2009, Cooper et al 2010, Grieger et al 2013), reducing optical density (Homma et al 1996, Lin et al 2000), and decreasing sensitivity for deoxygenation with greater ATT (Niwayama et al 2000, Feng et al 2001, van Beekvelt et al 2001, Koga et al 2011, Ohmae et al 2014, van der Zwaard et al 2016).

Conventional methods to correct for ATT are based on the assumption of constant tissue properties among measured subjects (e.g. hemoglobin content, oxygen consumption, and oxygen saturation) (Niwayama et al 2000, Ryan et al 2012, Bowen et al 2013). However, this is deemed an unsatisfactory approach when dealing with heterogeneous groups of subjects (van Beekvelt et al 2001, Niwayama et al 2012), as for example CHF patients. To illustrate this, evidence has been found for considerable intersubject variability of tissue hemoglobin content (Myers et al 2009, Adami et al 2015), for reduced skeletal muscle oxygen consumption in CHF (Abozguia et al 2008), and for a relation between resting StO2 and oxygen uptake kinetics in CHF (Bowen et al 2012).

While SRS-NIR derived StO2 is confounded by ATT, it is also influenced by age, gender, disease state and oxygen uptake kinetics (Costes et al 1999, Geraskin et al 2009, Cooper et al 2010, Bowen et al 2012, Grieger et al 2013). The latter associations are considered to mirror physiological differences between, for example, younger and older subjects (Takagi et al 2013), and males and females (Cooper et al 2010, Peltonen et al 2013, Takagi et al 2016). Furthermore, they may reflect pathophysiological changes related to certain diseases (e.g. CHF, diabetes, etc) (Bowen et al 2012, Peltonen et al 2012), and their subsequent consequences on exercise capacity (e.g. slowed oxygen uptake kinetics). However, all of these StO2-related parameters may in turn be associated with ATT, which has previously been demonstrated for age and gender (Perissinotto et al 2002). In this way, ATT may mask or mimic interindividual physiological differences, which is considered to lead to false NIRS-based conclusions (van Beekvelt et al 2001).

Because it is unclear to what extent and how ATT independently influences measured tissue oxygen saturation (StO2) in vivo, it is essential to compare its influence to that of other potentially associated parameters and to isolate it in an experimentally controlled setup. Therefore, this study was designed to assess in vivo StO2 and its relation to ATT in a mixed (i.e. with respect to age and gender) population of CHF patients and healthy control (HC) subjects at rest and during moderate intensity exercise. Furthermore, to isolate the influence of ATT, we measured the effect of changes of overlying porcine ATT on in vitro measured StO2 values in a blood mixture under controlled conditions.

We hypothesize that ATT confounds NIR-SRS parameters, and that there is a difference between StO2 measurements in CHF and HC subjects, necessitating a correction method that does not assume constant physiological tissue properties among individuals.

2. Methods

2.1. Subjects

Fifty-six patients with CHF and twenty healthy active subjects matched for age and body mass index (BMI) were included. Subject characteristics are shown in table 1. Inclusion criteria for CHF patients were stable systolic heart failure on optimal medical treatment for at
least 3 months, attributed to either dilated cardiomyopathy or ischemic heart disease due to myocardial infarction, New York Heart Association (NYHA) functional Class II or III, and left ventricular ejection fraction (as assessed by echocardiograph) \( \leq 40\% \). Examination of healthy subjects was performed to confirm absence of clinical and echocardiographic signs of heart failure. CHF patients and healthy subjects were excluded from the study when they presented with recent myocardial infarction (within the preceding 3 months), angina pectoris at rest, pulmonary, neurological or orthopedic disorders limiting the ability to exercise, peripheral vascular disease and/or clinical signs of decompensated heart failure.

This study represents part of two larger protocols involving exercise testing and training in patients with CHF (Niemeijer et al. 2016, Spee et al. 2016), which were approved by the local Research Ethics Committee of Máxima Medical Centre, Veldhoven, The Netherlands. All study participants gave written informed consent before inclusion, and all procedures were conducted according to the Helsinki Declaration of 1964.

### 2.2. Exercise protocol

Subjects performed tests on an electromagnetically braked cycle ergometer (Lode Corrival, Lode BV, Groningen, The Netherlands) in an upright seated position. They were instructed to maintain a pedaling frequency of 70 rpm during the exercise phases. Ventilatory and gas exchange measurements (ZAN 680 USB, ZAN Messgeräte, Oberthulba, Germany; calibrated before each test) were recorded breath-by-breath during the entire testing protocols.

The maximal exercise protocol started with a 1 min resting period and 4 min of unloaded pedaling before work rate started to increase with an individualized ramp rate aiming to reach exhaustion within 8–12 min (Fletcher et al. 2013). The test was terminated when the required pedaling frequency could not be maintained due to volitional exhaustion. Peak work rate was the highest registered work rate, and peak pulmonary oxygen uptake (peak VO\(_2\)) and peak respiratory exchange ratio (RER) were the average values of the final 30 s of the maximal exercise test. The gas exchange threshold (GET) was determined by the mean of the independent assessments of two experienced physicians using the V-slope method (Beaver et al. 1986).
Moderate-intensity exercise testing was performed on a different day than the maximal exercise test. It commenced with a 2 min resting period, while the right leg was passively held in a predetermined position. This was followed by a 6 min bout at 80% of the work rate corresponding to the GET, or at 50% of the peak work rate when the GET could not be assessed (Kemps et al 2007).

Subjects were advised to take their medication as usual and to avoid strenuous exercise (48 h), consuming a meal (2 h), and caffeine (4 h) before testing.

2.3. NIRS measurements

NIRS measurements were performed during moderate-intensity exercise with a wireless CW near-infrared spectrophotometer (Portamon, Artinis, Elst, The Netherlands), which employs MBL and SRS with 2 wavelengths of emitting light (760 and 841 nm). The device consists of 3 pairs of light emitting diodes and a detector photo diode, which are configured spatially to provide 3 source-detector distances (30, 35, and 40 mm). By determining the absorption coefficients derived from the slopes of light attenuation at different source-detector distances and wavelengths, an absolute measure of actual tissue oxygen saturation (StO2), the tissue saturation index (TSI), can be calculated. TSI equals the ratio of oxygenated hemoglobin and myoglobin (O2HbMb) and the sum of oxygenated and deoxygenated hemoglobin and myoglobin ([O2HbMb] + [HHbMb] = [totalHbMb]), and is expressed as a percentage. Additionally, after incorporating a differential path length factor of 4 (manufacturer recommendation) in the MBL, changes of deoxygenated hemoglobin ([HHb] in µM) were also reported.

The NIRS device was positioned over the distal vastus lateralis of the right leg, 20 cm proximally from the lateral patellar edge, and fixated with adhesive tape and a Velcro strap. Thereafter, it was occluded from ambient light by dark cloth. Data were sampled at 10 Hz, and stored for off-line analysis.

To obtain an estimate of ATT, the (double) skinfold thickness at the site of NIRS measurement was measured with a skinfold caliper (Harpenden, Baty International, West Sussex, UK) and divided by two (Geraskin et al 2009).

2.4. Blood phantom

Human packed erythrocytes in a saline adenine glucose mannitol (SAGM) solution were heated to 37 °C in a glass beaker and mixed by a magnetic stirrer (Heidolph MR 3001, Heidolph Instruments, Swabach, Germany). Phosphate-buffered saline (PBS) and an intralipid emulsion with a concentration of 20% were added to produce a 300 ml mixture with an intralipid concentration of 1%, and optical properties approximating absorption and reduced scattering coefficients of perfused muscle tissue (Lin et al 2000, Hunter et al 2002, Di Ninni et al 2012). A hematocrit of 6% was chosen, as this was the minimum required to allow blood gas analysis. The blood mixture was circulated (Heidolph Pumpdrive 5001, Heidolph Instrument, Swabach, Germany) via tubing through a custom made flow cell milled from PVC with inner dimensions of 50 × 50 × 85 mm (figure 1). The inside of the flow cell was made black to prevent light reflections. Medical oxygen gas was delivered into the mixture from a cylinder with the use of a pressure reducer and flow meter. The blood mixture reached full oxygen saturation (100% SO2) after 15 min of oxygenation, confirmed by blood gas analysis (ABL90 FLEX, Radiometer Medical, Brønshøj, Denmark) of 1 ml samples taken from a sample point in the tubing distal to the flow cell. NIRS measurements were made with the Portamon device wrapped in one layer of transparent plastic placed on top of the flow cell in direct contact with the mixture. Thereafter, 8 consecutive 60 s measurements were made with fresh porcine fat
layers (LifeTec Group, Eindhoven, The Netherlands) in between the device and flow cell, with a stepwise addition of layers (2 mm) to a total of 14 mm thickness. Care was taken to assure complete contact between layers. The mixture was desaturated in approximately 1 h with the use of 0.1% mass fraction sodium metabisulfite (Na$_2$S$_2$O$_5$), while performing blood gas analysis on 1 ml samples approximately every 5 min. In total, four desaturation courses with new mixtures were measured: 1 without fat and 3 with fat layers of 2, 4, and 6 mm, respectively. On the fully desaturated mixture of the last desaturation course (i.e. 6 mm ATT), another 4 measurements were made with fat layers from 8 to 14 mm in 2 mm steps.

2.5. Data analysis

Gas exchange parameters for the constant work rate exercise test were averaged into 10 s sampling intervals after removal of outliers (values >3 SDs from the local mean were omitted) (Kemps et al 2007). Mono-exponential modeling of $\dot{V}_\text{O}_2$ data was performed to characterize the rise of $\dot{V}_\text{O}_2$ (fundamental phase or phase II) during onset by using the non-linear least squares method (Python 2.7, Python Software Foundation, Beaverton, OR, USA) with the following formula (Kemps et al 2007):

$$Y(t) = Y_{\text{baseline}} + A \cdot (1 - e^{-\frac{t-T_d}{\tau}})$$

(1)

where $Y$ equals $\dot{V}_\text{O}_2$, $Y_{\text{baseline}}$ depicts the average value during the last 30 s prior to the exercise transition, $A$ indicates the amplitude during exercise onset for $\dot{V}_\text{O}_2$, $T_d$ is the time delay and $\tau$ is the time constant of the mono-exponential function (in seconds). The first 20 s of the $\dot{V}_\text{O}_2$ response after onset were omitted from kinetics analysis, since it is assumed that the rapid increase of $\dot{V}_\text{O}_2$ during this period (cardiodynamic phase or phase I) represents increased pulmonary blood flow rather than microvascular gas exchange (Benson et al 2013).

NIRS data were filtered using a central moving average filter with a window of 11 data points and resampled into 1 s intervals. Absolute values of TSI during moderate-intensity exercise testing were calculated as the average of the last minute of the resting phase ($\text{TSI}_{\text{baseline}}$).
and the average of the last minute of the exercise phase (TSl_{end-exercise}), as described previously (Niemeijer et al 2017). The difference between TSl_{baseline} and TSl_{end-exercise} was defined as the overall TSI response to the moderate-intensity constant work rate exercise (ΔTSI_{exercise}). Similarly, [HHb] change from baseline to end-exercise was reported as Δ[HHb]_{exercise}. Note that both negative values for ΔTSI_{exercise} and positive values for [HHb]_{exercise} indicate tissue deoxygenation.

TSI flow cell measurements were time aligned with blood gas sampling data and were averaged over the entire 60 s per step of ATT for fully oxygen saturated and desaturated measurements.

2.6. Statistical analysis

SPSS 22.0.0 statistical software (SPSS Inc, Chicago, IL, USA) was used for statistical data analysis. Results are presented as mean value ± standard deviation. Normality was assessed by Shapiro Wilk tests and by skewness and kurtosis of the distribution. Unpaired Student’s t test in case of a normal distribution, or the Mann-Whitney U test when appropriate, were used for between-group comparisons. From literature, variables were selected as possible predictors of StO2 (i.e. TSI) parameters, which were ATT, gender, CHF, age, and oxygen uptake kinetics at onset of moderate-intensity exercise (τVO_{onset2}) (Costes et al 1999, van Beekvelt et al 2001, Geraskin et al 2009, Cooper et al 2010, Bowen et al 2012, Grieger et al 2013). Relations of variables with TSI parameters were evaluated by univariate linear regression analysis and expressed as Pearson’s correlation coefficients. All variables were also used in a multivariate linear regression model (backward stepwise regression, removal criterion P < 0.05). If the multivariate predictions of the TSI parameters were improved by normalized data (logarithmic transformation), these were used instead. The goodness of fit was evaluated using the $R^2$ value. The level of statistical significance was set at P < 0.05 for all tests.

3. Results

Sixty-five males and 11 females with an age range of 43–83 years were included (table 1). ATT was not normally distributed (median 4.3 mm and interquartile range (IQR) 3.4 mm), however not different between CHF and HC groups.

All exercise tests were performed without any adverse events. Peak oxygen uptake was lower in CHF patients and oxygen uptake kinetics (τVO_{onset2}) were faster in HC subjects (table 2). TSl_{baseline} was lower in CHF patients, however TSl_{end-exercise}, ΔTSI_{exercise} and Δ[HHb]_{exercise} were not different between groups. CHF and τVO_{onset} were not significantly correlated with ATT, whereas gender and age were (table 3). From all selected variables from literature, only τVO_{onset} did not correlate significantly with TSl_{baseline}. Furthermore, ATT showed the strongest correlation with TSl_{baseline} and TSl_{end-exercise}, and ATT was the only variable that was significantly correlated with ΔTSI_{exercise} (table 3 and figure 2). The correlation of ATT with Δ[HHb]_{exercise} was stronger than with ΔTSI_{exercise}. When ATT was 7 mm or larger, the proportion of measurements with an exercise response opposite to deoxygenation (i.e. paradoxically increasing TSI and decreasing HHb) was higher for HHb (77%) than for TSI (23%). Smaller ATT (i.e. <7 mm) showed equal paradoxical response rates for both variables (11%). Multivariate regression analysis showed that ATT was the strongest predictor of TSI parameters, specifically after log transformation due to non-normality (table 4). CHF was an independent predictor of TSl_{baseline}, ΔTSI_{exercise}, and Δ[HHb]_{exercise}, while the log of τVO_{onset} was
an independent predictor of TSI_{end-exercise}, \Delta TSI_{exercise}, and \Delta [HHb]_{exercise} (table 4). Gender and age were no independent predictors of TSI or [HHb] parameters.

**Table 2.** Response to maximal and submaximal exercise tests.

<table>
<thead>
<tr>
<th>Variable</th>
<th>CHF</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal exercise test</td>
<td>n = 56</td>
<td>n = 20</td>
</tr>
<tr>
<td>Peak ( \dot{V}O_2 ) (ml·min(^{-1} )·kg(^{-1} ))</td>
<td>19.6 ± 5.7</td>
<td>32.6 ± 7.6(^{c} )</td>
</tr>
<tr>
<td>Peak work rate (W)</td>
<td>124 ± 48</td>
<td>238 ± 48(^{a} )</td>
</tr>
<tr>
<td>Peak RER (l/l)</td>
<td>1.06 ± 0.11</td>
<td>1.11 ± 0.07(^{b} )</td>
</tr>
<tr>
<td>( \dot{V}O_2 ) @ GET (ml·min(^{-1} )·kg(^{-1} ))</td>
<td>14.4 ± 4.4</td>
<td>21.6 ± 4.8(^{c} )</td>
</tr>
<tr>
<td>Submaximal exercise test</td>
<td>59 ± 23</td>
<td>118 ± 28(^{a} )</td>
</tr>
<tr>
<td>( \tau V_{O2onset} ) (s)</td>
<td>59.5 ± 27.2</td>
<td>40.2 ± 11.5(^{b} )</td>
</tr>
<tr>
<td>TSI_{baseline} (%)</td>
<td>67.5 ± 4.9</td>
<td>70.6 ± 5.6(^{a} )</td>
</tr>
<tr>
<td>TSI_{end-exercise} (%)</td>
<td>62.3 ± 7.3</td>
<td>64.1 ± 7.4</td>
</tr>
<tr>
<td>( \Delta TSI_{exercise} ) (%)</td>
<td>−5.1 ± 4.8</td>
<td>−6.5 ± 5.1</td>
</tr>
<tr>
<td>( \Delta [HHb]_{exercise} ) (µM)</td>
<td>3.3 ± 5.2</td>
<td>4.9 ± 5.8</td>
</tr>
</tbody>
</table>

CHF versus HC.

\(^{a} P < 0.05.\)

\(^{b} P < 0.01.\)

\(^{c} P < 0.001.\)

**Table 3.** Pearson’s correlations with near-infrared spectroscopy parameters.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>ATT</th>
<th>TSI_{baseline}</th>
<th>TSI_{end-exercise}</th>
<th>( \Delta TSI_{exercise} )</th>
<th>( \Delta [HHb]_{exercise} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATT</td>
<td>76</td>
<td>0.574(^{c} )</td>
<td>0.627(^{c} )</td>
<td>0.314(^{b} )</td>
<td>−0.565(^{c} )</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>76</td>
<td>0.749(^{c} )</td>
<td>0.423(^{c} )</td>
<td>0.413(^{c} )</td>
<td>0.176</td>
<td>−0.399(^{c} )</td>
</tr>
<tr>
<td>CHF</td>
<td>76</td>
<td>−0.081</td>
<td>−0.267(^{a} )</td>
<td>−0.110</td>
<td>0.122</td>
<td>−0.133</td>
</tr>
<tr>
<td>Age</td>
<td>76</td>
<td>−0.272(^{a} )</td>
<td>−0.230(^{a} )</td>
<td>−0.191</td>
<td>−0.037</td>
<td>0.174</td>
</tr>
<tr>
<td>( \tau V_{O2onset} )</td>
<td>76</td>
<td>0.075</td>
<td>−0.041</td>
<td>−0.115</td>
<td>−0.128</td>
<td>0.083</td>
</tr>
</tbody>
</table>

\(^{a} P < 0.05.\)

\(^{b} P < 0.01.\)

\(^{c} P < 0.001.\)

\*In vitro experiments showed that measurements on fully saturated blood mixture (100% SO\(_2\)) without an adipose tissue layer produced a TSI value of 99.5% (figure 3). Desaturated blood (1.2% SO\(_2\)) corresponded to a TSI of 0%. Adding adipose tissue up to 14 mm resulted in a regression towards a TSI value of 73.5% for 100% SO\(_2\), and a TSI of 68.0% for 0% SO\(_2\) (figure 3). The deoxygenation course without adipose tissue showed that the average absolute deviation of TSI from actual SO\(_2\) was 5% until SO\(_2\) dropped below 42% from whereon TSI was equal to 0% (figure 4). Deoxygenation courses with progressive ATT showed increasing average error of TSI with respect to SO\(_2\) (21%, 23%, and 29% for ATT of 2, 4, and 6 mm, respectively). Linear regression analysis of TSI as a predictor of SO\(_2\) showed correlation coefficients of (\( R^2 \)) 0.94, 0.99, 0.97 and 0.99, and an intercept with the line of identity at 80.7%, 84.0%, 73.7%, and 74.9% for 0, 2, 4, and 6 mm, respectively.
Figure 2. Relation between adipose tissue thickness (ATT) and (a) resting tissue saturation index (TSIbaseline), (b) TSI at the end of moderate-intensity constant work rate exercise (TSIend-exercise), (c) response of TSI to moderate intensity exercise ($\Delta$TSIexercise), and (d) response of deoxygenated hemoglobin concentration to moderate intensity exercise ($\Delta$[HHb]exercise) for healthy control (HC) subjects and chronic heart failure (CHF) patients.
4. Discussion

4.1. Influence of ATT on StO2

In this study we demonstrate that adipose tissue layer thickness greatly influences NIR-SRS derived StO2 at rest and during exercise. ATT explained 33% ($R^2$) of variability of resting tissue oxygenation in human measurements when ATT ranged from 1 to 17.5 mm. In multivariate regression analysis ATT was the strongest independent predictor of StO2. In addition, the presence of CHF and slower oxygen uptake kinetics were independently related to lower StO2, suggesting (patho-) physiological differences among subjects are reflected in tissue oxygenation. In contrast, relations between age, gender and TSI parameters were mostly explained by inter-individual ATT differences. Ex vivo isolation of the influence of ATT showed that adipose tissue has optical properties that limit the range of measured TSI parameters from as little as 2 mm thickness, and probably even less.
Previous research focusing on the influence of ATT on NIR-SRS parameters in vivo showed a similar increase of resting StO₂ with ATT in a mixed group (i.e. 10 males and 10 females) of young subjects (Geraskin et al 2009, Grieger et al 2013). In addition, Cooper et al (2010) demonstrated a positive association between percentage body fat and StO₂ that was only evident for young male subjects, but not for young females. However, relations between local ATT and StO₂ were not reported, limiting comparability between studies. Furthermore, measurements performed by Costes et al (1999) suggest a relation between aging and resting oxygenation. However gender and age differences with respect to StO₂, other than explained by ATT variability, were not seen in the present study. Gender-related and age-related physiological differences between subjects of for instance muscle mass, capillary density, hemoglobin content, and muscle oxidative capacity may have contributed to the observations of Costes et al (1999) and Cooper et al (2010). Similarly, the present results of a lower TSIbaseline for CHF, and an independent association of CHF with StO₂, may be explained on the basis of pathophysiological consequences of the disease, as for example reduced skin blood flow (Andreassen et al 1998), and compromised vascularity (Richardson et al 2003).

Several explanations can be given for the relation between in vivo StO₂ and ATT by considering oxygenation of hemoglobin (the major NIR-light absorber in adipose and muscle tissue) and other tissue light absorbers. First, actual in vivo muscle hemoglobin oxygenation is most likely lower than oxygenation of hemoglobin in adipose tissue. Adipose tissue contains highly oxygenated blood (at least higher than muscle tissue) because of a lower oxygen consumption rate and higher blood flow (Niwayama et al 2000, van Beekvelt et al 2001). Second, Goossens et al (2011) demonstrated that subjects with greater ATT have even higher hemoglobin oxygenation in adipose tissue than lean subjects, at least in fasting conditions, amplifying the aforementioned effect. Third, as clarified by the in vitro experiment, exsanguinated adipose tissue itself has NIR-light absorbing qualities that lead to StO₂ outcomes specifically...
in the range of 68–74%. ATT would therefore increase TSI values when actual tissue hemoglobin oxygenation is lower than the specified range. This is more likely to occur during skeletal muscle deoxygenation, for example due to exercise. As such, it may in part explain the stronger relationship between ATT and TSIend-exercise than between ATT and TSIbaseline. Indeed, the in vitro deoxygenation experiment confirmed such a concept by showing that when ATT is at the group’s average value (~5 mm), TSI values lower than 74% tend to be overestimations of SO2. More specifically, with smaller ATT overestimation was less pronounced and occurred at higher SO2. With the device used in this study, the difference between TSI values and actual StO2 increased when actual StO2 became lower than 81–84% for 0–2 mm ATT and lower than 74–75% for 4–6 mm ATT.

Skeletal muscle properties that may oppose the observed relation between measured StO2 and ATT include an oxygenation gradient within the skeletal muscle tissue layer. Deeper muscle regions commonly have a greater proportion of oxidative fibers compared to superficial regions, leading to a greater degree of oxidative energy metabolism and a concomitantly greater sensitivity for vascular control (Grassi and Quaresima 2016). Correspondingly, Koga et al (2015a) found higher StO2 in the deeper regions of the rectus femoris than in the superficial regions, coinciding with slower and less profound deoxygenation profiles. Smaller ATT would then lead to higher StO2 values. However, we did not find such an effect probably due to a greater influence of optical properties of adipose tissue itself on measured StO2.

A finding in the present study that warrants further exploration is the independent relation between TSIend-exercise, ΔTSIexercise, and pulmonary oxygen uptake kinetics. That is, after correction for ATT, speeding of $\tau V\dot{O}_2$ onset was related to a higher StO2 during exercise. Additionally, after subsequent correction for CHF, faster $\tau V\dot{O}_2$ onset was also related to a smaller difference between resting and exercising StO2 (i.e. $\Delta$TSIexercise). Analogous to the lesser degree of deoxygenation in deeper, more oxidative muscle regions found by Koga et al (2015a), a greater proportion of oxidative muscle fibers in subjects with faster oxygen uptake kinetics can also explain the relation between the degree of measured deoxygenation and speed of pulmonary oxygen uptake kinetics in this study (Poole and Jones 2012). However, while there was a relation between TSIend-exercise and oxygen kinetics, the relation between resting StO2 and $\tau V\dot{O}_2$ onset reported by Bowen et al (2012) in their group of CHF patients could not be confirmed. The univariate analysis in the current study did not show such a relationship, nor did the multivariate analysis. The fact that resting oxygenation was lower in their CHF patients (57.2 ± 7.2% versus 67.5 ± 4.9% in our study) could have led to a greater sensitivity of oxygen uptake kinetics to resting oxygenation, slowing oxygen uptake kinetics in a more O2 availability/delivery dependent way (Poole et al 2011).

4.2. Differences between SRS-based and MBL-based parameters

Our results showed less susceptibility to the influence of ATT for SRS-derived StO2 than for MBL-derived [HHb]. Although this study is the first to perform such a comparison, previous authors have concluded that the SRS-methodology is less affected by superficial tissue layers. Messere and Roatta (2013) reported that MBL-based blood volume indices (i.e. total hemoglobin) were more affected by cutaneous warming than those based on SRS (i.e. tissue hemoglobin index), while sensitivity to exercise-induced changes of blood volume was similar between methodologies. In explaining their results, they argued that NIR-SRS more or less excludes the common pathway of light for spatially separated emitter-optode pairs (i.e. in the superficial layers), instead it focuses on analysis of the differential signal between pairs (i.e. in the deeper layers). This may also explain why we observed a large portion of measurements
(77%) with a paradoxically decreasing [HHb] during exercise in case of an ATT of 7 mm and larger, whereas this rate was far lower for TSI (23%). While this observation indicates that TSI is more sensitive to skeletal muscle deoxygenation than [HHb], it simultaneously demonstrates a lower sensitivity of TSI for increases of skin and adipose tissue oxygenation due to skin blood flow changes (Kime et al. 2013). Another noteworthy advantage of NIRS-SRS over MBL-derived [HHb] is that it is not calculated with an assumed constant differential path length factor (DPF), which can potentially vary during exercise.

4.3. Correction for ATT

Based upon the present and previous results it is evident that a correction for ATT is needed if NIR-SRS derived StO2 parameters of individual subjects are to be compared in a meaningful way. In contrast to suggestions by other authors (Yang et al. 2005), the in vitro experiments show that even a minimal ATT of 1 mm is expected to influence StO2 measurements to a great degree. Therefore, when a group of study subjects is heterogeneous with respect to age, gender, disease state or oxidative capacity, a correction method for ATT needs to incorporate the optical properties of the measured tissues. Correction can consist of direct measurement of optical properties (e.g. by means of a laser rangefinder) (Niwayama et al. 2012). However, the correction method that is most widely used is based on Monte Carlo simulations of a two- or multiple layered tissue volume (Lin et al. 2000, Niwayama et al. 2000, Grieger et al. 2013). Such a method employs look-up tables for determination of optical tissue properties at each wavelength that is used, and thereby corrects for the reduced measurement sensitivity due to ATT. However, the optimal composition of look-up tables and their validity are poorly investigated and should be subject of future studies. Furthermore, correction through Monte Carlo simulations still relies on the assumption of a constant reduced scattering coefficient ($\mu'_s$), and hence, on an unchanging tissue composition (e.g. constant hemoglobin content). However, recent work by Koga et al. (2015b) using NIR-TRS revealed $\mu'_s$ varies during exercise and whole body heating. Moreover, tissue composition may be variable between subjects due to differences in hemoglobin content (Myers et al. 2009, Adami et al. 2015). Consequently, studies on the error of calculated StO2 associated with variable $\mu'_s$ are needed as well.

4.4. Limitations

Before definite conclusion can be drawn, several issues should be addressed. First, we did not measure ATT with ultrasound (US), but by means of skinfold caliper measurements, which could reduce reliability. However, Geraskin et al. (2009) showed favorable agreement of US measured ATT with caliper readings. Second, porcine adipose tissue was exsanguinated and inactive. Optical properties of human adipose tissue in vivo may be different from those of exsanguinated inactive porcine adipose tissue. However, this experimental setup was chosen because it allowed isolation of the separate influence of adipose tissue. Third, a higher hematocrit was used in the phantom experiment (i.e. 6%) than what is thought to arise in resting muscle tissue, although no standard exists for in vivo tissue hemoglobin content (Myers et al. 2009). However, since no myoglobin was used in the in vitro experiment, replacing it with hemoglobin may have compensated the absorption that would be otherwise caused by myoglobin. Fourth, a blood mixture with an intralipid concentration of 1% provides optical properties approximating absorption and reduced scattering coefficients of perfused muscle tissue. However, given the poor relation between SO2 and TSI (figure 4) at lower oxygenation (below 40%) without overlying adipose tissue (ATT = 0), scattering of NIR light may not
have met the requirement of sufficient saturation of the photodetector. And finally, young subjects (younger than 40 years of age) were not included, limiting generalizability of the conclusions to older groups. However, the large range of 43–83 years provided sufficient contrast to detect an influence of age.

5. Conclusions

This study shows for the first time the independent and isolated influence of ATT on skeletal muscle oxygenation measured with NIRS. Although NIR-SRS derived StO2 was shown to be less susceptible to the influence of ATT than MBL-based NIRS derivatives (i.e. [HHb]), overlying adipose tissue still confounds approximation of skeletal muscle oxygenation to a great degree. Specifically, the optical properties of (exsanguinated) adipose tissue give rise to an SRS-derived StO2 outcome in the range of 68–74% for the device used in the present study. This outcome influences calculation of actual StO2 proportionally to the thickness of the adipose tissue layer. However, when NIR-SRS measurements were corrected for ATT in a multivariate analysis, the presence of disease and slowing of oxygen uptake kinetics were related to lower TSI values. This indicates that SRS-derived StO2 cannot be corrected for ATT by assuming constant tissue properties among measured subjects (e.g. hemoglobin content, oxygen consumption, and oxygen saturation), since these are likely subject to physiological variability. Therefore, the obvious violation of the assumption of tissue homogeneity needs to be addressed in future studies by assessing the validity of corrections based on Monte Carlo simulations or by incorporating the influence of the actual optical properties of measured tissues into the calculation of tissue oxygenation.

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Author contributions

VMN, JPJ, TD, RFS, EJM, HMCK, and PFFW were all contributors to the conception and design of the study. VMN, JPJ, and RFS conducted all experiments and performed all data collection. VMN, JPJ, TD, and RFS performed all data analysis. VMN prepared the first draft of the manuscript. All authors edited, and agreed, the final version.

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