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Citation for final published version:

Young, Grace M., Krastins, Digby, Chang, David, Lam, Jeng, Quah, Jing, Stanton, Tony, Russell, Fraser, Greaves, Kim, Kriel, Yuri and Askew, Christopher D. 2021. The association between contrast-enhanced ultrasound and near-infrared spectroscopy-derived measures of calf muscle microvascular responsiveness in older adults. *Heart, Lung and Circulation* 30 (11) , pp. 1726-1733. 10.1016/j.hlc.2021.07.004

Publishers page: <http://dx.doi.org/10.1016/j.hlc.2021.07.004>

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# The Association Between Contrast-Enhanced Ultrasound and Near-Infrared Spectroscopy-Derived Measures of Calf Muscle Microvascular Responsiveness in Older Adults

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Received 2 March 2021; received in revised form 4 June 2021; accepted 6 July 2021; online published-ahead-of-print xxx

## Background and Aim

Contrast-enhanced ultrasound (CEUS) measures of post-occlusion skeletal muscle microvascular responsiveness demonstrate the microvascular dysfunction associated with ageing and age-related disease. However, the accessibility of CEUS is limited by the need for intravenous administration of ultrasound contrast agents and sophisticated imaging analysis. Alternative methods are required for the broader assessment of microvascular dysfunction in research and clinical settings. Therefore, we aimed to evaluate the level of association and agreement between CEUS and near-infrared spectroscopy (NIRS)-derived measures of post-occlusion skeletal muscle microvascular responsiveness in older adults.

## Methods

During supine rest, participants (n=15, 67±11 years) underwent 5 minutes of thigh cuff-occlusion (200 mmHg). Post-occlusion CEUS measures of calf muscle microvascular responsiveness were made, including time to 95% peak acoustic intensity (TTP<sup>95</sup> AI) and the rate of rise (slope AI). Simultaneous measures, including time to 95% peak oxygenated haemoglobin (TTP<sup>95</sup> O<sub>2</sub>Hb) and slope O<sub>2</sub>Hb, were made using continuous-wave NIRS in the same muscle region.

## Results

There were strong correlations between TTP<sup>95</sup> measures derived from CEUS and NIRS ( $r=0.834$ ,  $p<0.001$ ) and the corresponding measures of slope ( $r=0.735$ ,  $p=0.004$ ). The limits of agreement demonstrated by Bland Altman plot analyses for CEUS and NIRS-derived measures of TTP<sup>95</sup> (-9.67–1.98 s) and slope (-1.29–5.23%. s<sup>-1</sup>) were smaller than the minimum differences expected in people with microvascular dysfunction.

## Conclusions

The strong correlations and level of agreement in the present study support the use of NIRS as a non-invasive, portable and cost-effective method for assessing post-occlusion skeletal muscle microvascular responsiveness in older adults.

## Keywords

Gastrocnemius • Post-occlusive reactive hyperaemia • Microvascular reactivity • Capillary blood flow

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## Introduction

Microvascular function is crucial for the maintenance of metabolic homeostasis and cardiovascular health [1]. Contrast-enhanced ultrasound (CEUS) measures of skeletal muscle microvascular responsiveness demonstrate an impairment with age [2,3] and in people with metabolic and cardiovascular diseases (CVD) [4,5]. The routine screening and assessment of microvascular responsiveness has the potential to identify individuals at risk of CVD, as well as being a novel treatment target [6,7].

Real-time CEUS enables the quantitative assessment of skeletal muscle microvascular blood flow, measured as the rate of appearance of a microbubble contrast agent within a region of interest (ROI) [8]. Following a hyperaemic stimulus (e.g. cuff-occlusion), time-intensity curves from the corresponding increase in acoustic intensity (AI) are analysed to obtain parameters reflecting microvascular responsiveness, including time to peak response (TTP), area under the curve (AUC) (a measure of the total response) and the rate (slope) of the response. Post-occlusion microvascular flow responses have excellent test-retest reliability in young and older adults with and without CVD [9,10]. However, CEUS is limited by the need for: intravenous cannulation and administration of a contrast agent requiring medical supervision; sophisticated image analysis procedures; and the costs of ultrasound contrast agents, equipment and analysis software. Therefore, the accessibility and utilisation of skeletal muscle microvascular assessments in research and clinical settings would be improved with an alternative non-invasive and economical method.

Continuous-wave near-infrared spectroscopy (NIRS) is an affordable, non-invasive and portable method used to assess changes in the relative concentrations of oxygenated haemoglobin (O<sub>2</sub>Hb) and deoxygenated haemoglobin (HHb) in skeletal muscle [11]. Changes in NIRS-derived signals have been used to quantify the microvascular responsiveness to a hyperaemic stimulus, including the TTP and slope of the response [12,13]. Post-occlusion NIRS-derived measures of microvascular responsiveness are well-established [14], reproducible [13,15,16] and have the potential to be adopted into clinical practice. Therefore, NIRS-derived measures of skeletal muscle microvascular responsiveness may provide an accessible, economical and non-invasive alternative to CEUS; however, the relationship between these measures has not been established.

Impaired NIRS-derived post-occlusion microvascular responses have been demonstrated in patients with CVD compared with age-matched controls, characterised by a longer TTP [17,18]. In addition, the rate of responsiveness is slower in older people, with and without CVD risk factors, compared with younger, healthy adults, and is characterised by a lower slope [19,20]. Together these findings indicate that NIRS-derived measures of post-occlusion responsiveness are impaired in populations with, or at risk of, microvascular dysfunction and highlight the potential for NIRS to be used as an alternative method of assessment to CEUS. Therefore,

the primary aim of this study was to determine the level of association and agreement between CEUS and NIRS-derived measures of post-occlusion skeletal muscle microvascular responsiveness in older adults.

## Materials and Methods

This study was conducted in accordance with the Declaration of Helsinki and approved by The Prince Charles Hospital (HREC/18/QPCH/188) and University of the Sunshine Coast (S181201). The study was explained in detail before written informed consent was obtained from each participant. Eligible participants met the following criteria: 40 to 79 years of age; ankle-brachial index (ABI) between  $\geq 0.9$  and  $\leq 1.30$ ; and body mass index (BMI) between  $\geq 18.5$  and  $\leq 30$  kg/m<sup>2</sup>. Participants with a history of anaemia, CVD, diabetes, hypertension (systolic blood pressure [SBP]  $\geq 140$  mmHg and/or diastolic BP [DBP]  $\geq 90$  mmHg) or hypersensitivity to perflutren (an ultrasound contrast agent) were excluded, in addition to current smokers. Nineteen (19) participants who met the inclusion criteria consented to take part in the study. After screening, three participants withdrew from the study due to time constraints and one participant withdrew for an unrelated medical reason. Therefore, 15 (seven female and eight male) participants (age:  $67 \pm 11$  years) completed the study. The same participants also took part in a previously published reliability study [21].

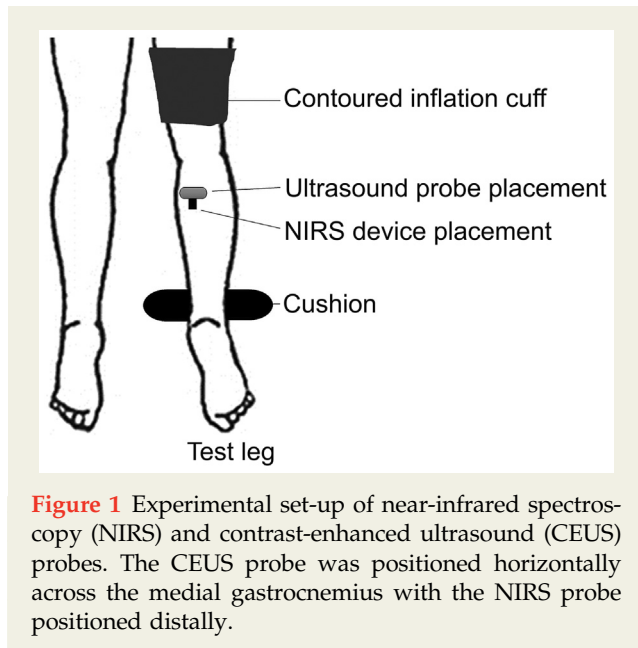
## Overview

Participants refrained from exercise for 24 hours, and caffeine and alcohol for 12 hours before each visit. Testing occurred in a quiet, temperature-controlled laboratory (21–23°C). Initial screening measures including a brief medical history, resting blood pressure, ABI, height, weight and calf-circumference were performed. Participants then attended a familiarisation visit to complete the experimental protocol, and returned to repeat the protocol  $>48$  hours later. Data from the second visit were used for analyses except for one participant whose familiarisation visit data were analysed due to motion artefact during the second visit.

## Experimental Protocol

The leg with the largest calf-circumference was tested with participants lying in a prone position. A diagram of the experimental set-up is shown in Figure 1. A contoured cuff (Hokanson® CC17™, Bellevue, WA, Australia) was secured with an elastic bandage around the proximal thigh. The ultrasound transducer and NIRS optode were secured to the calf, and the test leg supported with cushions to prevent movement. Following 10 minutes of rest, brachial blood pressure (Welch Allyn, Chicago, IL, USA) and resting CEUS and NIRS measures were taken. Participants then underwent a period of thigh cuff-occlusion. The cuff was rapidly inflated to 200 mmHg (VenaPulse® VP-25, ACI Medical, San Marcos, CA, USA) for 5 minutes. Post-occlusion CEUS and NIRS measures were collected simultaneously following





**Figure 1** Experimental set-up of near-infrared spectroscopy (NIRS) and contrast-enhanced ultrasound (CEUS) probes. The CEUS probe was positioned horizontally across the medial gastrocnemius with the NIRS probe positioned distally.

cuff-release, after which blood pressure was measured. Single-lead electrocardiogram and heart rate (Philips Medical Systems, Andover, MA, USA) were monitored continuously throughout testing.

### Contrast-Enhanced Ultrasound

Contrast-enhanced ultrasound measures of the medial gastrocnemius muscle were taken in the transaxial plane for 45 seconds. The ultrasound transducer was secured to the posterior leg with a custom-made foam holder at the level of the largest calf-circumference (Figure 1). Continuous harmonic power-Doppler imaging (Philips Ultrasound Systems, model iE33, Philips Medical Systems, Andover, MA, USA) was performed. A linear-array transducer (Philips, L9-3) was used with a low mechanical index (0.10), 87% gain, 5 cm depth and sampling frequency of 14 Hz. The contrast agent (1.3 mL of DEFINITY®, Bristol-Myers Squibb Medical Imaging, New York, NY, USA), containing lipid shelled octafluoropropane gas-filled microspheres (1.1-3.3  $\mu\text{m}$ ), was activated and mixed to 50 mL with saline, and administered by intravenous infusion (antecubital fossa) at a rate of 200 mL/hr using a syringe pump (Alaris PK, Auckland, New Zealand). The solution was rocked at 40 rpm using a modified mixing platform to prevent sedimentation of the contrast agent (Rowe Scientific Pty Ltd, Sydney, NSW, Australia) and infused for 2 minutes before image acquisition to reach a steady-state concentration. The microbubbles within the field of view were destroyed by a pulse of high-intensity ultrasound (mechanical index = 1.07) immediately prior to image acquisition.

Acoustic intensity (AI) was determined for the analysis of microbubble replenishment (QLAB, Philips Healthcare, Bothell, WA, USA). A rectangular ROI was manually created from a minimum of 1 cm to a maximum of 3.5 cm.

Time-intensity data were exported (Excel 15.0, Microsoft Corporation), with the background intensity set at 0.49 seconds from the end of microbubble destruction to remove the response of larger, faster-filling vessels [22]. The time-intensity curves were fitted to an exponential function [ $y=A(1-\exp^{-\beta t})$ ] where:  $y$  is the AI at time  $t$  (in seconds);  $A$  is the plateau in AI (representative of microvascular blood volume); and  $\beta$  is the rate constant (reflecting the rate of rise in AI) [23]. Microvascular blood flow is derived from the product of  $A$  and  $\beta$  ( $A*\beta = \text{flow}$ ). Curve fitting was performed using Sigmaplot software version 13.0 (Systat Software, San Jose, TX, USA).

### Near-Infrared Spectroscopy

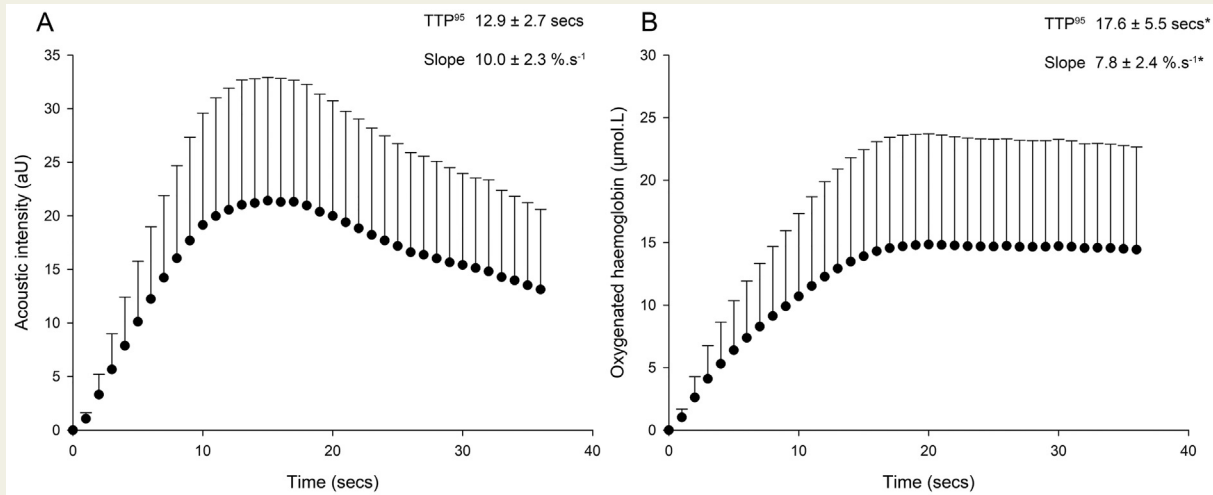
Near-infrared spectroscopy-derived signals for  $\text{O}_2\text{Hb}$ , HHb, total haemoglobin ( $\text{THb}=\text{O}_2\text{Hb}+\text{HHb}$ ) and tissue oxygen saturation ( $\text{StO}_2=\text{O}_2\text{Hb}/(\text{THb})\times 100$ ) were continuously recorded during rest, cuff-occlusion and for 45 seconds following cuff-release with a Portalite device (Artinis Medical Systems BV, Zetten, Netherlands). The NIRS optode was placed over the belly of the medial gastrocnemius muscle distal to the ultrasound probe (Figure 1) and secured using standardised procedures to shield against ambient light contamination. A fixed differential path-length factor of 4.0 was used to correct for photon scattering within the tissue. Data were acquired at 10 Hz via Bluetooth connection (Oxysoft, Artinis Medical Systems BV, Zetten, Netherlands). Data for  $\text{O}_2\text{Hb}$  were exported and used to identify baseline (the average over 60 s prior to each occlusion) minimum (during cuff-occlusion) and maximum (post cuff-release) values from which the magnitude was calculated (maximum  $\text{O}_2\text{Hb}$  – baseline  $\text{O}_2\text{Hb}$ ).

### Microvascular Response Parameters

Both CEUS and NIRS-derived data sets were transformed to 1 Hz for further analysis and comparison. Time to 95% peak ( $\text{TTP}^{95} \text{ AI}$  and  $\text{TTP}^{95} \text{ O}_2\text{Hb}$ ) were calculated as the time from cuff-release to 95% of the maximum response. The top 5% of the hyperaemic response is highly variable and was therefore truncated to improve the reliability of the TTP measure [15]. To allow comparison between the techniques, slope measurements were expressed as a percentage of the total increase from the time of cuff release to the maximum response. The segment corresponding with 25 to 50% of the response was used to calculate the slope ( $\% \cdot \text{s}^{-1}$ ) of AI and  $\text{O}_2\text{Hb}$ . Truncating the initial segment removes the contribution of larger filling vessels and any motion artefact which may occur around cuff-release; therefore, the gradient corresponding with 25 to 50% of the response provides a more reliable measure of slope [15]. Area under the curve (AUC) was calculated as the total accumulative change (AI and  $\text{O}_2\text{Hb}$ ) over 45 seconds following cuff-release.

### Statistical Analysis

Data were evaluated for normality (Shapiro–Wilk). Paired t-tests were used to compare mean post-occlusion measures derived from CEUS and NIRS, including  $\text{TTP}^{95}$  and slope.



**Figure 2** Post-occlusion responses (mean  $\pm$  SD) of **A** Contrast-enhanced ultrasound (CEUS) and **B** Near-infrared spectroscopy (NIRS). TTP<sup>95</sup> time to 95% peak. \*Indicates a significant difference between CEUS and NIRS measure ( $p < 0.05$ ).

Pearson's correlation coefficient was used to assess correlations between measures. The strength of correlations was determined using the following criteria: 0.0–0.19 very weak; 0.2–0.39 weak; 0.4–0.59 moderate; 0.6–0.79 strong; 0.8–1.0 very strong [24]. Bland-Altman plots were used to assess the level of agreement between the CEUS and NIRS-derived measures of TTP<sup>95</sup> and slope [25]. Regression analysis was performed to determine whether the agreement between measures was proportional to the magnitude of the measurement. The coefficient of variation (CV) for NIRS-derived TTP<sup>95</sup> and slope measures was calculated to determine test-retest reliability using the familiarisation and test visit data. Statistical analyses were carried out using SPSS statistical package version 23 (SPSS Inc., Armonk, NY, USA). Data are presented as mean and standard deviation unless otherwise specified.  $P < 0.05$  was considered statistically significant.

## Results

Participants were normotensive (SBP  $120 \pm 10$  mmHg; DBP  $76 \pm 9$  mmHg) and in sinus rhythm (resting heart rate  $62 \pm 10$  bpm), with a BMI of  $24 \pm 3$  kg/m<sup>2</sup>. The mean post-occlusion responses for CEUS and NIRS are shown in Figure 2. Due to motion artefact, CEUS data of one participant were unsuitable for analysis. Due to a calibration error, NIRS data of one participant were unsuitable for analysis. Therefore, statistical comparisons were made with 13 complete datasets. Both CEUS and NIRS responses increased rapidly, reaching their respective maximum values within 20 seconds of cuff-release. Skeletal muscle microvascular blood flow ( $A^*\beta$ ) derived from CEUS was  $3.22 \pm 1.79$  aU. s<sup>-1</sup> ( $A = 26.43 \pm 14.54$  aU;  $\beta = 0.13 \pm 0.04$  s<sup>-1</sup>). The O<sub>2</sub>Hb magnitude derived from NIRS was  $14.79 \pm 8.3$   $\mu$ mol/L. As presented in Figure 2, time to 95% peak AI (CEUS) was lower than TTP<sup>95</sup> O<sub>2</sub>Hb (NIRS) ( $12.9 \pm 2.7$  s and  $17.6 \pm 5.5$  s, respectively,  $p = 0.001$ ) and slope AI was greater than slope O<sub>2</sub>Hb ( $10.0 \pm 2.3$  %·s<sup>-1</sup> and  $7.8 \pm 2.4$

%·s<sup>-1</sup>, respectively,  $p = 0.002$ ). The test-retest coefficient of variation for NIRS-derived measures was  $8.3 \pm 3.7$  % for TTP<sup>95</sup> and  $9.6 \pm 10.1$  % for slope.

Correlations between the CEUS and NIRS-derived response parameters are shown in Table 1. The rate constant ( $\beta$ ) of the CEUS response, reflecting microvascular blood flow velocity, was positively correlated with the slope of the O<sub>2</sub>Hb response ( $p = 0.04$ ), and inversely correlated with TTP<sup>95</sup> O<sub>2</sub>Hb derived from NIRS ( $p = 0.04$ ). Post-occlusion skeletal muscle microvascular blood flow ( $A^*\beta$ ) strongly correlated with the area under the curve ( $p = 0.02$ ) and magnitude ( $p = 0.02$ ) of the NIRS response.

The association and agreement between the CEUS and NIRS-derived measures of TTP<sup>95</sup> (TTP<sup>95</sup> AI and TTP<sup>95</sup> O<sub>2</sub>Hb) and slope (slope AI and slope O<sub>2</sub>Hb) are shown in Figure 3. There was a very strong correlation between the TTP<sup>95</sup> measures derived from CEUS and NIRS with the corresponding  $r^2 = 0.696$  (Figure 3A). Similarly, slopes of the CEUS and NIRS responses were also strongly correlated with the corresponding  $r^2 = 0.540$  (Figure 3B). Bland-Altman plots show the mean difference (bias) between TTP<sup>95</sup> measures was 3.85 seconds slower (CI: 2.05 s–5.64 s) when derived from NIRS (Figure 3C). Regression analysis indicated that this bias tended to increase with the magnitude of the TTP<sup>95</sup> measure ( $p = 0.04$ ). The mean difference between slope measures was 1.97%·s<sup>-1</sup> slower (CI: 0.97–2.98%·s<sup>-1</sup>) when derived from NIRS (Figure 3D). Figure 3C and D show relatively narrow limits of agreement between CEUS and NIRS-derived measures of TTP<sup>95</sup> ( $-9.67$ – $1.98$  s) and slope ( $-1.29$ – $5.23$ %·s<sup>-1</sup>).

## Discussion

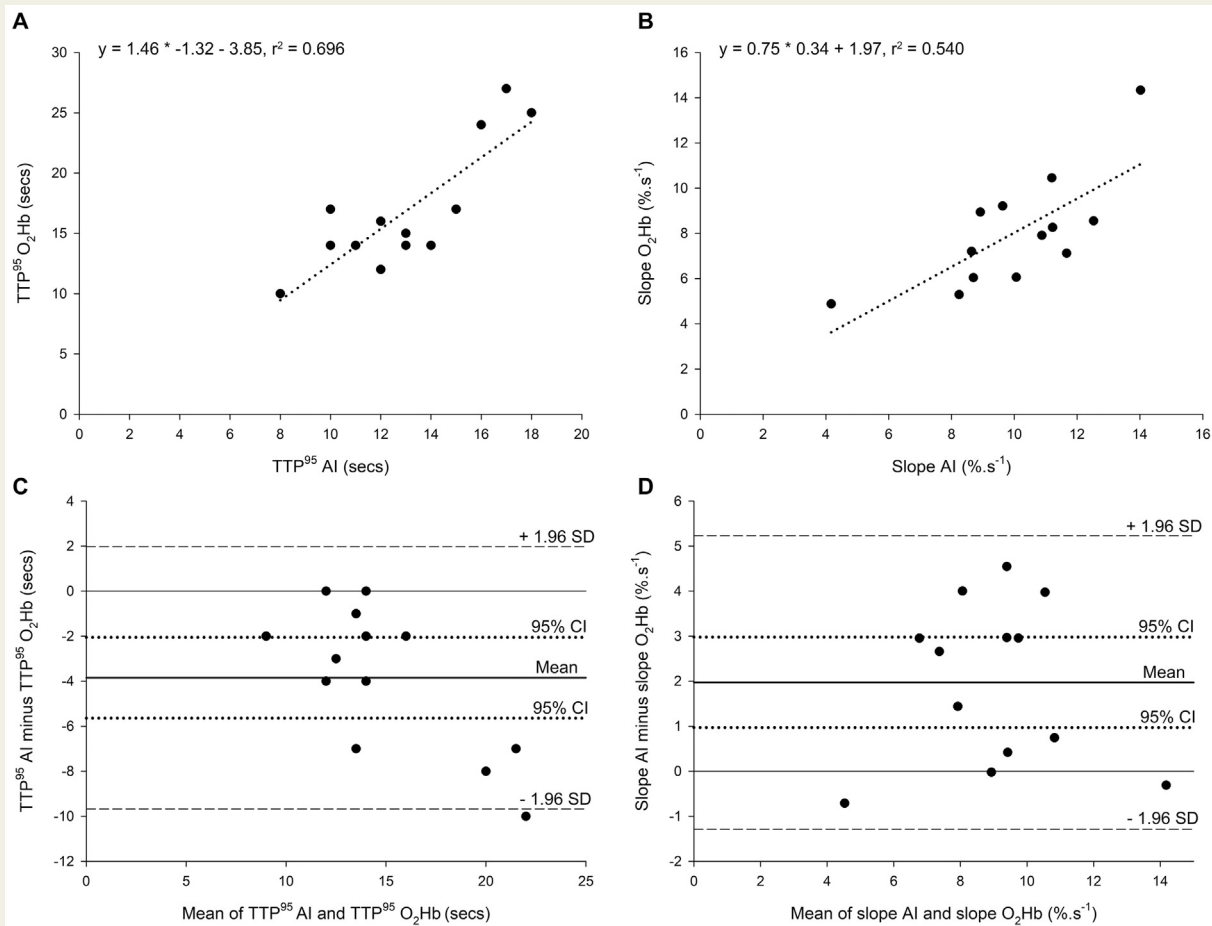
The primary aim of this study was to determine the level of association and agreement between CEUS and NIRS-derived measures of post-occlusion skeletal muscle microvascular

**Table 1** Relationships between post-occlusion microvascular blood flow parameters derived from CEUS and NIRS.

NIRS	CEUS						
	Max (aU)	TTP <sup>95</sup> (s)	Slope (%·s <sup>-1</sup> )	AUC (aU·s)	A (aU)	β (s <sup>-1</sup> )	Flow (aU·s <sup>-1</sup> )
Max (μmol/L)	0.347	-0.123	0.186	0.316	0.313	0.080	0.558 <sup>a</sup>
TTP <sup>95</sup> (s)	0.210	0.834 <sup>a</sup>	-0.629 <sup>a</sup>	0.205	0.238	-0.567 <sup>a</sup>	-0.114
Slope (%·s <sup>-1</sup> )	-0.287	-0.785 <sup>a</sup>	0.735 <sup>a</sup>	-0.270	-0.274	0.575 <sup>a</sup>	-0.063
AUC (μmol·L <sup>-1</sup> ·s)	0.462	-0.117	0.191	0.456	0.438	-0.016	0.622 <sup>a</sup>
Magnitude (μmol·L <sup>-1</sup> )	0.442	-0.333	0.175	0.438	0.414	-0.019	0.602 <sup>a</sup>

Values are correlation coefficients (*r* values).

<sup>a</sup>Indicates *p* < 0.05. CEUS contrast-enhanced ultrasound, NIRS near-infrared spectroscopy, Max maximum value, TTP<sup>95</sup> time to 95% peak, AUC area under curve for 45 secs post cuff-release, Magnitude maximum oxygenated haemoglobin (O<sub>2</sub>Hb) – baseline O<sub>2</sub>Hb.



**Figure 3** Association and level of agreement between contrast-enhanced ultrasound (CEUS) and near-infrared spectroscopy (NIRS) measures: Panel A (scatterplot) shows the correlation and B (Bland-Altman plot) shows the agreement between time to 95% peak acoustic intensity (TTP<sup>95</sup> AI) and time to 95% peak oxygenated haemoglobin (TTP<sup>95</sup> O<sub>2</sub>Hb). Panel C (scatterplot) shows the correlation and Panel D shows the agreement between the slope (25-50%) of the CEUS (acoustic intensity, AI) and NIRS (oxygenated haemoglobin, O<sub>2</sub>Hb) responses.

responsiveness in older adults. Significant associations were demonstrated between response parameters derived from CEUS and NIRS in the gastrocnemius muscle, including

strong correlations between measures of TTP<sup>95</sup> (TTP<sup>95</sup> AI and TTP<sup>95</sup> O<sub>2</sub>Hb) and slope (slope AI and slope O<sub>2</sub>Hb). The strong correlations and relatively narrow limits of agreement

between methods in the present study support the use of NIRS-derived measures of TTP<sup>95</sup> and slope to detect clinical differences in microvascular function.

Both CEUS and NIRS-derived responses increased rapidly, reaching maximal hyperaemia within 20 seconds of cuff-release. The magnitude of the CEUS response in the present study is comparable with findings using the same protocol in a similar group of older adults [9]. The magnitude of the O<sub>2</sub>Hb response in the present study is also similar to previous NIRS findings [15]. Post-occlusion hyperaemia is a homeostatic response to the oxygen deficit caused by arterial cuff occlusion, reflecting vasodilatory capacity [26]. As such, the magnitude of the response is related to the magnitude of the oxygen deficit, which is influenced by cuff-occlusion duration [19,21]. The time to peak (TTP) and slope of the hyperaemic response differentiate healthy people from those with microvascular dysfunction, including peripheral arterial disease (PAD) [4,17], type II diabetes [5] and obesity [18]. Our analyses therefore focussed on these parameters.

We found a strong correlation between the CEUS and NIRS-derived measures of TTP<sup>95</sup> (Figure 3A). Based on the significant linear regression, our findings indicate that an increase in TTP<sup>95</sup> AI of 1.00 second is associated with an increase in TTP<sup>95</sup> O<sub>2</sub>Hb of 3.85 seconds and approximately 70% of the variance in NIRS-derived TTP<sup>95</sup> measures can be explained by the variance in CEUS measures of TTP<sup>95</sup> (Figure 3A). Time to 95% peak AI values in the present study were slightly shorter than CEUS findings calculated using TTP (100%) [9]. However, the TTP<sup>95</sup> O<sub>2</sub>Hb values in the present study were comparable to NIRS studies reporting TTP<sup>95</sup> O<sub>2</sub>Hb in similar cohorts [15,27]. Furthermore, the use of TTP<sup>95</sup> improves the reliability of this measure [15], and may have contributed to the strong correlation between the measures of TTP<sup>95</sup> in the present study. We also found a strong correlation between the CEUS and NIRS-derived measures of slope (Figure 3B). Again, based on linear regression ( $r^2=0.540$ ) our findings indicate that a rise in slope AI of 1.00 %·s<sup>-1</sup> is associated with an increase in slope O<sub>2</sub>Hb of 1.97 %·s<sup>-1</sup> (Figure 3B). Slope AI in the current study is comparable with CEUS data in a similar cohort [28]. Taken together and given that both CEUS and NIRS-derived measures of skeletal muscle microvascular responsiveness are sensitive to changes associated with ageing and age-related disease, these findings indicate that NIRS-derived measures of post-occlusion skeletal muscle microvascular responsiveness may provide suitable non-invasive alternatives to CEUS in older adults.

While we found strong relationships between CEUS and NIRS-derived measures of TTP<sup>95</sup> and slope, there were also differences between these measures. Time to 95% O<sub>2</sub>Hb (NIRS) occurred after TTP<sup>95</sup> AI (CEUS), which aligns with the gradient of slope O<sub>2</sub>Hb being lower than slope AI (Figure 2). Bland Altman plot analysis confirmed that the difference between TTP<sup>95</sup> measures derived from CEUS and NIRS increased with the duration of TTP<sup>95</sup>. However, the limits of agreement for TTP<sup>95</sup> and slope measures in the present study are smaller than the differences previously reported between healthy controls and people with CVD

derived from CEUS [9,28–30] and NIRS [27,31]. Furthermore, the test-retest reliability of the NIRS-derived measures of TTP<sup>95</sup> in the present study (CV=8.37±3.7%) is similar to that previously reported for CEUS [10] and NIRS [15]. Therefore, NIRS-derived measures of post-occlusion skeletal muscle microvascular responsiveness may provide a suitable non-invasive alternative to CEUS in detecting the presence of skeletal muscle microvascular dysfunction.

Slope AI (CEUS) represents the rate of post-occlusion hyperaemia (microvascular blood flow), while TTP also represents the maximal microvascular filling capacity, reflecting capillary density and morphology. The responsiveness of larger upstream vessels also contributes to CEUS measures of TTP [28]; thus slope measures may provide a more relevant measure of microvascular responsiveness, particularly when making between-group comparisons. Slope O<sub>2</sub>Hb reflects the rate of local tissue oxygenation [16,32]. The transportation of O<sub>2</sub>Hb is largely dependent on microvascular blood flow; therefore, associations between the post-occlusion measures derived from CEUS and NIRS were expected (Figure 3A and B). However, the transportation of O<sub>2</sub>Hb is also influenced by oxygen transport kinetics within the peripheral microvasculature, including the capacity to reoxygenate haemoglobin and myoglobin [13]. Therefore, O<sub>2</sub>Hb measures are also dependent on the rate of skeletal muscle metabolism and mitochondrial function. The slower rate of rise in O<sub>2</sub>Hb, compared with the rate of rise in AI, reflects the time taken to resaturate Hb in addition to the influx of O<sub>2</sub>Hb (reactive hyperaemia). These physiological differences may partly explain the delay in TTP<sup>95</sup> O<sub>2</sub>Hb compared with TTP<sup>95</sup> AI (Figure 3C) and the lower rise in slope O<sub>2</sub>Hb compared with slope AI (Figure 3D) observed in the present study.

## Limitations

The present study is limited to assessing CEUS and NIRS-derived post-occlusion measures of skeletal muscle microvascular blood flow responsiveness in older adults. While there is currently no gold standard measure of skeletal muscle microvascular responsiveness, post-occlusion measures of reactive hyperaemia have traditionally used strain-gauge (venous occlusion) plethysmography. Plethysmography assesses whole-limb blood flow, including the contribution of the macrovascular and cutaneous circulations, in addition to the skeletal muscle microvasculature. Plethysmography measures have previously been compared with CEUS measures of calf muscle microvascular blood flow [9,10]. Post-occlusion whole-leg blood flow (plethysmography) correlated with post-occlusion calf muscle microvascular blood flow (CEUS) in older adults with and without peripheral arterial disease ( $r=0.84$ ,  $p<0.01$ ) [9]; however, no relationship was found between the same measures in younger and older adults ( $r=0.39$ ,  $p=0.07$ ) [10]. This is likely due to the strong contribution of macrovascular function to plethysmography derived measures of reactive hyperaemia.



The confidence intervals of the mean difference between CEUS and NIRS-derived measures of TTP<sup>95</sup> (Figure 3C) and slope (Figure 3D) reflect the limited sample size in the present study of older adults [33]. Further studies are needed to investigate whether the variance between CEUS and NIRS-derived measures would differ in clinical populations with known microvascular dysfunction. We aimed to reduce variance in the present study by using a longer (5 mins) cuff-occlusion stimulus, which is associated with more reliable measures than shorter occlusion periods [21] and exercise [9]. We aimed to reduce the potential difference between measurement depths of the two methods by analysing CEUS data between 1.5 cm and 3 cm, which is comparable to the penetration depth of skeletal muscle by near infrared light. While we did not measure adipose tissue thickness, calf limb circumference was not associated with CEUS or NIRS-derived measures and all the participants had a normal BMI.

## Conclusions

Near-infrared spectroscopy derived measures of post-occlusion skeletal muscle microvascular responsiveness were strongly associated with CEUS measures in older adults. Furthermore, the limits of agreement between the CEUS and NIRS-derived measures were smaller than reported differences between people with and without CVD. This level of agreement and the strong correlations in the present study support the use of NIRS as an alternative non-invasive, portable and cost-effective method for assessing post-occlusion skeletal muscle microvascular responsiveness in older adults.

## Funding Sources

This study was supported by the School of Health and Behavioural Sciences at the University of the Sunshine Coast.

## Declarations of Interest

None.

## Acknowledgements

We thank Bailly Causon, Adrien Chanteau, Jana Bromand and Colin Gavagan for assisting with data collection/handling and figures; University of the Third Age and Men's Shed for assisting with participant recruitment; and the participants for their time.

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