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**Contrast-enhanced ultrasound using bolus injections of contrast agent for
assessment of postprandial microvascular blood volume in human skeletal muscle.**

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30

31 **Abstract**

32 Methods capable of measuring blood flow in a tissue-specific manner are needed. The purpose
33 of this study was to investigate if contrast-enhanced ultrasound (CEUS) using bolus injections
34 of SonoVue® is an useful method for assessing postprandial changes in microvascular
35 perfusion in the vastus lateralis muscle. 10 healthy, young subjects were recruited for this
36 study. 6 subjects participated in washout- and reproducibility protocols to assess washout time
37 of SonoVue® and the reproducibility of the method when measuring microvascular blood
38 volume (MBV). 6 subjects (two of which also participated in the washout- and reproducibility
39 protocols) participated in exercise- and nutrition protocols, to assess the ability of the method
40 to detect changes in MBV in response to these interventions. Intraday variation (Coefficients of
41 variation (CV)) for MBV indices, as assessed by peak signal intensity (PI) or mean plateau signal
42 intensity (mPI), were high (PI: $19 \pm 4.2\%$; mPI: $23 \pm 3.3\%$). The exercise protocol induced
43 significant increases of MBV indices (PI: +113%, $P < 0.0001$; mPI: +218%, $P < 0.0001$) acutely after
44 exercise cessation. There were no changes in MBV indices in response to feeding during the
45 nutrition protocol (PI: $P = 0.51$; mPI: $P = 0.51$). We conclude that CEUS using bolus injections of
46 SonoVue® is not capable of detecting changes in MBV of vastus lateralis in response to feeding.
47 This is probably due to the low reproducibility of the method. However, the method is capable
48 of measuring changes in MBV in response to exercise. This method could therefore be used
49 when investigating exercise-induced changes in microvascular perfusion.

50 **Keywords:** CEUS; Microcirculation; capillary recruitment; harmonic imaging; microbubbles;
51 blood flow.

52

53

54 **Introduction**

55 Skeletal muscle microvascular perfusion is closely coupled to muscle metabolism. Optimal
56 microvascular function is of major importance, due to the capillaries being the main route for
57 delivering and exchanging nutrients, gasses, hormones etc. (Poole et al., 2013). However,
58 investigations of the microcirculation has for many years been problematic in human subjects,
59 due to a lack of adequate methods for accurately measures. Modern advantages in ultrasound
60 sonography have made it possible to use contrast-enhanced ultrasound (CEUS) providing
61 measures of microvascular blood volume (MBV) as estimates of microcirculation for
62 investigating e.g. tissue perfusion (Wei et al., 1998). With the use of a continuous infusion of
63 contrast agent for the CEUS recordings, studies have reported increases in skeletal muscle
64 MBV following food intake (Keske et al., 2009; Mitchell et al., 2013; Vincent et al., 2006) and
65 exercise (Sjøberg et al., 2011; Vincent et al., 2006). Applying a continuous infusion requires a
66 time lapse of 5-10 min prior to assessment and an infusion pump that can handle the
67 phosphorlipid stabilized hexafluoride microbubbles (Mitchell et al., 2013; Sjøberg et al., 2011).
68 The infusion must ensure that the concentration of the contrast agent reaches steady state
69 before measurements can be performed. The demands on time and equipment raised by this
70 method may be challenging in some experimental settings and it would therefore be beneficial
71 if CEUS could be performed using a single-bolus injection of contrast-agent.

72 To date, two studies has, to these author's knowledge, reported the reproducibility of CEUS
73 using bolus injections of contrast agent for investigating skeletal muscle microvascular
74 perfusion (Mulder et al., 2008; Tobin et al., 2010). Both of these studies used the contrast
75 agent SonoVue® (Mulder et al., 2008; Tobin et al., 2010).

Mulder and colleagues were the first to do CEUS recordings on skeletal muscle using bolus injections of SonoVue® and they derived their protocol based on continuous infusion protocols (Mulder et al., 2008). Following the bolus injection, a high mechanical index (MI) flash was used to destroy the microbubbles within the region of interest (ROI). The reproducibility of the technique was investigated during resting measurements of MBV and microvascular flow velocity (MFV) in the muscles of the forearm. MBV was found to have an acceptable reproducibility, with a coefficient of variation (CV) of 11%, whereas MFV was found to have poor reproducibility (CV=256%). Furthermore, Mulder and colleagues used the technique to demonstrate changes in MBV in response to hyperinsulinemia and exercise (Mulder et al., 2008).

Tobin and colleagues investigated the reproducibility of CEUS recordings on abdominal skeletal muscle and subcutaneous adipose tissue using bolus injections of SonoVue® (Tobin et al., 2010). In this study, a high MI flash was not performed. Instead, 4-minutes real-time imaging was recorded from the time of injection. Using this protocol, the researchers used the first phase plateau as an index of MBV. These measurements had a CV of 4%, indicating a good reproducibility in determining MBV in both skeletal muscle and subcutaneous adipose tissue (Tobin et al., 2010). The researchers also investigated changes in MBV in subcutaneous adipose tissue and forearm skeletal muscle in response to an oral glucose load and found an increase of MBV in adipose tissue but not in forearm skeletal muscle (Tobin et al., 2010). This finding is in contrast with earlier findings, reporting increases of 40-70% in muscle MBV following a mixed meal, where it was measured by CEUS using a continuous infusion protocol (Keske et al., 2009; Vincent et al., 2006). Therefore, it still remains unclear if CEUS using bolus injections of SonoVue® is capable of detecting changes in MBV of skeletal muscle in response to ingestion of a mixed meal.

In the present study we investigated if CEUS using bolus injections of SonoVue® is reliable and a useful method for assessing postprandial changes in microvascular perfusion in the vastus lateralis muscle. As numerous studies have shown large increases in MBV and MFV in response to exercise (Inyard et al., 2007; Krix et al., 2010; Rattigan et al., 2005; Sjøberg et al., 2011; St-Pierre et al., 2012; Vincent et al., 2006), exercise was used as a positive control for the detection of changes in these parameters.

Methods

Participants

A total of 10 young, healthy subjects (3 women and 7 men, 24.3 ± 3.3 years, BMI; 21.6 ± 1.6 kg/m², systolic blood pressure; 125 ± 8.8 mmHg, diastolic blood pressure; 69.3 ± 8.7 mmHg, resting heart rate; 57.9 ± 11.0 beats/min [Mean $\pm$ SD]) were recruited through advertisements on social media. Following exclusion criteria were used; BMI >25, smoking, heart disorders, diabetes and daily or frequent intake of medication (oral contraceptives were allowed).

In all protocols, subjects gave written informed consent. The study was performed according to the declaration of Helsinki II and was approved by the local ethics committee of the Capital Region of Denmark (journal H-4-2014-112).

Standard preparation

All protocols were performed at ~8 am, with subjects arriving to the hospital in the overnight fasted state. Subjects were instructed to refrain from alcohol, caffeine and strenuous activities the day before each trial. Upon arrival to the hospital, the subject was weighed, placed comfortably in the supine position in a bed, and the antecubital vein was catheterized (18 G Venflon, Becton Dickinson, Helsingborg, Sweden). For all protocols, subjects rested in the supine position for 30 minutes before the first CEUS recording was performed. After 20 minutes of rest, blood pressure and heart rate were measured on the contralateral arm.

Contrast-enhanced ultrasound protocol

During the 30 minutes of rest, B-mode imaging was used to find a fixpoint approximately at the mid-portion of the right m. vastus lateralis. Upon determination of an appropriate fixpoint, the precise transducer placement was marked on the skin of the subject and thigh characteristics were drawn on transparent to ensure that the exact same tissue volume was scanned during each recording (inter- and intraday).

The ultrasound gel thickness applied, prevented any pressure of the transducer on the underlying tissue. The contrast agent dry matter (SonoVue®, Bracco S.p.A, Italy) was dissolved in sterile saline and mixed gently for exactly 30 sec before injection. SonoVue® is a suspension of phospholipid-stabilized microbubbles filled with sulphur hexafluoride and is diluted in 4.5 ml 0.9% saline solution before injection ($8 \mu\text{l}$ microbubbles ml^{-1}). A bolus of 2.0 ml SonoVue® was injected through the antecubital vein followed by an immediate flush of 10 ml 0.9% saline solution. SonoVue® contains microbubbles of different sizes, ranging between diameters of 1 μm to 10 μm , with a mean of 2.5 μm . The size of the microbubbles is small enough to allow free passage through the capillaries, but large enough to retain in the vascular system (Greis, 2004). Therefore the microbubbles will be distributed throughout the entire blood volume, but will not diffuse into the extracellular fluid space (Greis, 2004). After leaving the microbubbles, the gas is exhaled through the lungs, and therefore does not interfere with renal or hepatic excretion pathways (Greis, 2004).

All ultrasound scannings were done by the same investigator, using a handheld linear array transducer (L9-3MHz) and an iU22 ultrasound scanner (Phillips Medical Systems, Bothell, USA). Contrast first harmonic signals were received at 8 MHz with a mechanical index of 0.06. For all subjects, depth was set at 3 cm (except for one subject in protocol A, where depth was increased to 3.5 cm), allowing measurements of the full depth of the m. vastus lateralis. Gain

was set at 90% for each recording. Focus was optimized and standardized for each subject when finding the fix point. Twenty millisecond images were captured consecutively for 2 minutes following each bolus injection.

Study design

The study was divided in 3. First, we performed the washout protocol to determine the washout time of SonoVue® microbubbles. Thereafter we wanted to investigate intra- and interday reproducibility of the method in the reproducibility protocol. Third, we investigated the ability of the method to detect changes in microvascular perfusion induced by either exercise or nutrition, investigated by separate protocols (A1 and A2, B1 and B2).

Image analysis

Image analysis was performed offline using an ultrasound quantification and analysis software (QLAB, Phillips Medical Systems). Image analysis was performed by a blinded investigator. Region of interest (ROI) for analysis was set to include as much as m. vastus lateralis as possible, excluding larger vessels, connective tissue and artefacts appearing on the image. In a ROI, we measured peak signal intensity (PI [dB]), background signal intensity (BI [dB]) and mean first phase plateau signal intensity (mPI [dB]). These measurements are described in detail below.

PI and mPI were used as indices of MBV. Both PI and mPI were measured including (A+B) and excluding background signal (A). PI was defined as the highest measured signal intensity in response to the bolus injection. mPI was defined as the mean signal intensity during the first phase plateau after the peak of wash-in curve. A plateau in signal intensity was defined as a period of minimum 10 seconds where the signal intensity did not change noticeably. BI was calculated as the mean signal before the onset of the wash-in curve.

Protocol A: Determination of washout period and reproducibility

Subject characteristics

Six healthy, young volunteers (3 men, 3 women, age 24 ± 4.3 years, body mass index 21 ± 4.0 kg/m², Systolic blood pressure 129 ± 12 mmHg, diastolic blood pressure 72 ± 18 mmHg, Resting heart rate 63 ± 20 beats/min [mean $\pm$ SD]) took part in this protocol.

A1: Washout protocol

In this protocol, we performed a single CEUS recording as described in section *Standard preparation* at time zero. Subjects arrived at the hospital at time point -30 min and remained in the supine resting position until time point 0 min To detect the minimum time required before microbubbles were no longer detectable in the scanned area, 60 s ultrasound recordings were captured at 10, 15, 20, 30, 40 minutes post injection (See fig. 1a).

A2: Reproducibility protocol

At this point we had determined the washout period, and therefore we knew the minimum time before the CEUS protocol could be repeated.

To test the intraday reproducibility of the method, three CEUS recordings were performed as described in section *Standard preparation*. Based on the findings from protocol A1 (See *Results*), washout intervals of 15 minutes were used between measurements (see fig. 1b). To test the interday reproducibility of the method, the protocol illustrated at fig. 1b was repeated twice within 3-7 days after the washout protocol.

Protocol B; Microvascular responses to exercise or nutrition

Subject characteristics

Six healthy, young volunteers (5 men, 1 woman, age 25 ± 4.2 years, body mass index 22 ± 1.0 kg/m², Systolic blood pressure 121 ± 9.0 mmHg, diastolic blood pressure 66 ± 7.0 mmHg,

Resting heart rate 52.7 ± 11 beats/min [mean $\pm$ SD]) took part in this protocol. Two of the subjects had also participated in protocol A.

Protocol B1 - Nutrition

In this protocol we investigated whether the method was capable of detecting changes in microvascular perfusion in response to feeding. A baseline CEUS recording was performed at time point -15 minutes, as described in section *Standard preparations*. Thereafter, the subject consumed a drink in less than 5 min containing 20 g whey protein hydrolysate (Peptigen IF-3090, Arla Foods Ingredients P/S, Viby J, DK) and 80 g maltodextrin (Fagron Nordic A/S, Copenhagen, DK) at time point 0. The subjects were allowed to sit upright when consuming the drink, but remained in the supine position throughout the rest of the protocol. CEUS recordings were performed again at time points 30 and 60 minutes. The experimental protocol is illustrated in fig. 1c.

Strength testing

Strength testing was performed on the same day as the nutrition protocol, after the last CEUS recording had been performed. Subjects had their 1 RM determined on their right leg in a leg extension machine (Cybex®, UK). After warming up on light loads, subjects would perform 2 repetitions on gradually increasing loads interspersed with sufficient rest periods. When the subject was capable of 1 but not 2 repetitions, the load was noted given the 1 RM.

Protocol B2 - Exercise

This protocol was performed 4-7 days after protocol B1, and is illustrated in fig. 1d. Subjects were placed supine on the hospital bed at time point -45 min. At time point -15 min a baseline CEUS recording was performed as described in section *Standard preparations*. Subjects were then placed in the leg extension equipment. At time point 0 min, the subjects would then perform 3 sets of 10 repetitions of unilateral leg extensions with their right leg at 70% of their

1 RM. Sets were interspersed with 1 min rest. Immediately after completion of the exercise bout, subjects were placed in the supine position on the hospital bed, and a CEUS recording was performed as soon as possible. All CEUS recordings were initiated within 1 minute after exercise cessation.

Statistics

In the wash-out protocol, one-way ANOVA and Holm-Sidak's multiple comparisons test was used to evaluate differences in signal intensities for baseline (signal intensity prior to the onset of the wash-in curve of the bolus), mean signal intensity during the bolus curve (mean of signal intensity after onset of the wash-in curve until termination of the recording), and mean signal intensity during the 60 seconds recordings at 10, 15, 20, 30 and 40 minutes post injection.

The reproducibility of the contrast-enhanced ultrasound technique was assessed by calculation of the standard deviation and the corresponding coefficient of variation (CV). CVs were calculated both for intra- and interday measurements using the formula $CV = SD/mean$.

Intraday CVs were calculated for each subject from the variation of the parameters obtained through the CEUS recordings from the 2 reproducibility protocols. Intraday CVs were obtained for both reproducibility protocol day 1 and 2. The average of these two CVs was used as the intraday CV for the subject, and used for the calculation of the mean CV for all subjects.

Interday CVs were calculated using the CEUS recording from the washout protocol and the CEUS recordings at time point 0 from the 2 reproducibility protocols. Furthermore, we tested the effect of bolus injection number by one-way ANOVA and Holm-Sidak's multiple comparisons test.

In the intervention protocols, one-way ANOVA and Holm-Sidak's multiple comparisons test were used to evaluate changes in the measured parameters from baseline to the measured

time point. $P < 0.05$ was considered statistically significant. All data are reported as mean $\pm$ SEM, except subject characteristics, which are presented as mean $\pm$ SD.

Results

Washout protocol

To investigate the washout-period of the SonoVue® contrast-agent, we compared mean baseline signal intensity, mean signal intensity during the bolus curve recorded after the injection, and mean signal intensity at 10, 15, 20, 30 and 40 minutes post injection (Fig 2). Mean signal intensity immediately following bolus injection was significantly higher than mean baseline intensity (Baseline; 15.7 ± 0.2 dB, bolus mean; 17.0 ± 0.3 dB, $P < 0.05$). Mean signal intensities at 10, 15, 20, 30 and 40 minutes post injection were not significantly different from baseline signal intensity. Based on these findings, we decided that a 15 minute washout period was sufficient before injections could be repeated in later protocols.

Reproducibility protocol

To test the reproducibility of the method, we performed CEUS assessments of three occasions with three injections interspersed with 15 minute intervals. We compared two methods for estimating MBV; peak signal intensity and first phase plateau intensity. Mean background signal was $15.7 \text{ dB} \pm 0.5 \text{ dB}$, mean peak intensity was $17.1 \pm 0.8 \text{ dB}$ and mean first phase plateau intensity was $16.7 \pm 0.7 \text{ dB}$. The coefficient of variation (CV) for measurements including background signal (A + B) were for intraday comparisons (PI CV; $1.8 \pm 0.4\%$, mPI CV; $1.4 \pm 0.2\%$) and interday comparisons (PI CV; $2.9 \pm 0.9\%$, mPI CV; $1.8 \pm 0.4\%$). When assessing the signal alone (A) the intraday variation was (PI CV: $19 \pm 4.2\%$; mPI CV: 23 ± 3.3) and interday variation was (PI CV: $27 \pm 9.8\%$; mPI CV: $31 \pm 7.3\%$) (Table 1). Paired t-test did not show significant difference between the CVs of PI and mPI when comparing intraday or interday

measurements ($P=0.15$ and $P=0.59$, respectively). Interday variation was not significantly different from intraday variation for any of the measured parameters (PI: $P=0.48$ and mPI: $P=0.38$).

Intervention protocol

All-subject mean curves for exercise and nutrition intervention are illustrated in fig. 3 and 4 and the results from the intervention protocols are summed up in table 2.

Average 1 RM in the one legged knee extension exercise was 47.4 ± 14.7 kg, resulting in an average exercise load of 33.1 ± 9.1 kg in 3 sets of 10 knee extension reps at 70% 1RM. Exercise induced acute changes in peak signal intensity (+113%, $P<0.001$), plateau intensity (+218%, $P<0.001$) compared to baseline measurements. All CEUS recordings acutely after exercise cessation exhibited double peaks in signal intensity (as seen in the all-subject mean bolus curve, fig 3).

During the nutrition protocol, there was no effect of time on neither peak intensity ($P = 0.51$) nor plateau intensity ($P = 0.51$).

Discussion

The present study demonstrates that CEUS using bolus injections of SonoVue appears to be as reliable as existing techniques for assessing microvascular blood volume in vastus lateralis muscle. This conclusion is based on the finding that the coefficient of variation for our chosen indices of microvascular blood volume (MBV) were comparable to what has been observed in earlier studies using CEUS in other muscles (Mulder et al., 2008; Tobin et al., 2010). Furthermore, we were able to detect and demonstrate that exercise significantly increased microvascular blood volume acutely after exercise, whereas we could not detect any change in the immediate postprandial period.

Reproducibility

To assess intra- and interday reproducibility of the CEUS method, we performed repeated measurements during resting conditions on the same day, as well as on different days, respectively. There was no difference in intra- and interday reproducibility when using peak intensity or mean plateau intensity for measuring MBV. The bolus curves obtained in this study was very different between subjects, with some bolus curves exhibiting a good plateau phase, while others had no clear plateau phase. This difference between bolus curves could potentially cause data interpretation to be highly investigator-dependent. In the present study, bolus curves were analyzed by a blinded investigator, which we suggest is crucial for this type of data analysis. As peak intensity represents the highest signal intensity obtained in the bolus curve, this method for estimating MBV is not investigator-dependent. Our findings therefore indicate that peak signal intensity could be used instead of mean plateau intensity when measuring MBV by CEUS using bolus injections of SonoVue®. The coefficient of variation (CV) of our measurements of peak signal intensity and plateau signal intensity were comparable to that observed in prior studies (Mulder et al., 2008; Tobin et al., 2010). Tobin and colleagues reported a CV of 4% while Mulder and colleagues found a CV of 11% when measuring signal intensity including background signal (A+B) (Mulder et al., 2008; Tobin et al., 2010). Compared to these studies, we found a numerically lower CV (1.4%). However, as changes in microvascular blood volume in response to vasodilatory stimuli are assessed by the ratio of A from the intervention and A obtained from baseline recordings, the reproducibility of the parameter A by itself is therefore more relevant than when combined with B , being the background noise of the probe without presence of microbubbles, as the parameter $A+B$. B is very large compared to A , and will contribute minimally to the variation of the total signal ($A+B$). This effectively causes a slightly larger SD to be divided by a far larger mean signal intensity, thereby lowering the CV. Therefore, we suggest that the CV of the actual

measurement should be given only by including the A parameter. We got a CV here on 19% for peak signal intensity and 23% for plateau signal intensity. We cannot though, compare the reproducibility of our method with CEUS using a bolus injection protocol to CEUS using a continuous infusion protocol as there are no available studies reporting the reproducibility of the latter protocol.

To assess interday reproducibility of the method, we compared measurements obtained on three separate days under comparable conditions. When being very thorough with identifying and repeating the scannings at the same area and ROI, we found that CVs for peak signal intensity (27%), plateau signal intensity (31%) were not significantly different for interday measurements compared to intraday measurements. These results indicate that reproducibility of the method is not compromised when comparing recordings obtained at different days. Weber and colleagues (Weber et al., 2006) found that signal intensity obtained through the use of CEUS recordings (with a continuous infusion protocol) in resting subjects correlated with capillary fiber contacts in human skeletal muscle. Capillarization increases over the course of a prolonged training period by 10-50% in the number of capillaries per fiber (Hoier and Hellsten, 2014). Therefore, although CEUS could potentially be used as a method of estimating changes in capillarization after e.g. a training protocol. the CEUS method as performed in this study probably does not have the required interday reproducibility to detect such changes.

Intervention protocols

Having verified the reproducibility of the CEUS measurement using a bolus injection, we performed experiments to investigate if the method was capable of detecting acute changes in microvascular perfusion in response nutrition. Furthermore, we used exercise as a positive control, to investigate if the method was capable of detecting larger changes in MBV. Hence,

we performed CEUS recordings after intake of a protein-carbohydrate drink, and after the execution of one-legged knee extension exercise.

The positive control, knee extensor exercise, induced a large increase in MBV, as indicated by increases in peak signal intensity (+113%) and plateau signal intensity (218%) acutely after exercise cessation. Due to a lack of any gold standard method of measuring MBV it is not possible to determine which of our parameters for MBV (peak signal intensity or plateau signal intensity) that gives the most accurate estimate. Using the continuous infusion protocol, Sjøberg and colleagues (Sjøberg et al., 2011) found that MBV increased 310% in response to one legged knee extensor exercise at 25 W for 10 min. Vincent and colleagues (Vincent et al., 2006) found that MBV increased approximately ~200% in the muscles of the forearm in response to high-intensity isometric handgrip exercise. Even though our results cannot be directly compared with the above mentioned results due to differences in exercise protocols and muscles investigated, our results seem to be in agreement with prior studies and CEUS recordings using bolus injection can presumably be used for assessing changes in muscle blood volume in response to acute exercise.

Surprisingly, we were not able to detect any changes in MBV in response to feeding. Tobin and colleagues were also not able to detect any changes in MBV in response to a 75 g glucose load (Tobin et al., 2010), which is in contrast with prior CEUS studies (Churchward-Venne et al., 2014; Keske et al., 2009; Mitchell et al., 2015, 2013; Timmerman et al., 2012; Vincent et al., 2006). However, most of the latter studies have assessed microvascular perfusion via CEUS using a continuous infusion of contrast agent. Tobin and colleagues suggested that the lack of effect of their feeding protocol on MBV could be due to the feeding stimulus being inadequate. In the present study however, the feeding stimulus was comparable to that of the studies showing an effect of feeding on MBV. Therefore, it seems unlikely that the lack of an

effect of feeding on microvascular perfusion in our study is due to an insufficient feeding stimulus. Instead, the lack of an effect is probably due to the either inadequacy of the method to detect the changes, or no effect of feeding on MBV in our subjects. As insulin has been many times to act as a vasodilator in healthy subjects (Dawson et al., 2002; Sjøberg et al., 2011; Timmerman et al., 2010; Vincent et al., 2002), and given that an effect of feeding on MBV in skeletal muscle has been detected in many studies prior to ours, the lack of an effect observed in the present study is likely due to an inadequate sensitivity of our method. Prior studies have found postprandial increases in MBV of 36-67% (Keske et al., 2009; Mitchell et al., 2015, 2013; Vincent et al., 2006). We found an intraday variation for our MBV indices of 19-23%, which therefore might be inadequate in order to detect an effect in the lower range of what has been observed in earlier studies. As there is no golden standard method for assessing microvascular perfusion, it is difficult to verify the validity of our results. Approaching the limit of detection with the bolus injection approach of micobubbles, it would have been valuable to have other measurements of blood flow by e.g. measuring leg blood flow to investigate if feeding had any effect on total perfusion of the leg. However, prior investigations have observed that changes in total leg blood flow are delayed compared to changes in microvascular blood flow (Mitchell et al., 2015, 2013) and hence the microvascular blood flow and blood volume changes are likely to be due to redistribution of microvascular flow (Clark et al., 2006). Changes in a. femoralis blood flow might therefore not be indicative of changes in microvascular perfusion. Alternatively, it would have been interesting to look into the sensitivity of the method by applying a dose-response investigation of vasodilator substances and measuring changes in MBV, and thereby investigate the ability of the method to detect smaller changes in MBV..

In conclusion, we found that CEUS using bolus injections of SonoVue appears to be as reliable as existing techniques for assessing microvascular blood volume in the vastus lateralis muscle.

However, our results also demonstrate that CEUS using bolus injections of SonoVue® is not capable of detecting changes in skeletal muscle microvascular perfusion in response to feeding, likely due to the method having inadequate sensitivity. Given that a large number of prior studies have detected postprandial changes in MBV using continuous infusion protocols of contrast agent (Keske et al., 2009; Mitchell et al., 2013; Vincent et al., 2006), we therefore recommend to use such a protocol if feeding-induced changes in microvascular perfusion is of interest. Our method was capable of detecting changes in microvascular perfusion in response to exercise, and could therefore potentially be used if exercise induced changes in microvascular perfusion is of interest.

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Conflict of interest

The authors have no conflict of interest.

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477 TABLE 1

Intraday CV (%)			Interday CV (%)	
	<u>Peak intensity</u>	<u>Plateau intensity</u>	<u>Peak intensity</u>	<u>Plateau intensity</u>
<u>A</u>	19 ± 4.2	23 ± 3.3	27 ± 9.8	31 ± 7.3
<u>A + B</u>	1.8 ± 0.4	1.4 ± 0.2	2.9 ± 0.9	1.8 ± 0.4

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481 TABLE 2

	Exercise protocol		Nutrition protocol		
	<u>Baseline</u>	<u>0 min</u>	<u>Baseline</u>	<u>30 min</u>	<u>60 min</u>
Peak intensity (dB)	1.7 ± 0.1	3.6 ± 0,1*	1.3 ± 0.2	1.1 ± 0.1	1.1 ± 0.1
Plateau intensity (dB)	0.9 ± 0.1	2.7 ± 0.1*	0.9 ± 0.1	0.8 ± 0	0.9 ± 0.1

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492 FIGURE 3

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496 FIGURE 4

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