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

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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).

76 Mulder and colleagues were the first to do CEUS recordings on skeletal muscle using bolus
77 injections of SonoVue® and they derived their protocol based on continuous infusion protocols
78 (Mulder et al., 2008). Following the bolus injection, a high mechanical index (MI) flash was
79 used to destroy the microbubbles within the region of interest (ROI). The reproducibility of the
80 technique was investigated during resting measurements of MBV and microvascular flow
81 velocity (MFV) in the muscles of the forearm. MBV was found to have an acceptable
82 reproducibility, with a coefficient of variation (CV) of 11%, whereas MFV was found to have
83 poor reproducibility (CV=256%). Furthermore, Mulder and colleagues used the technique to
84 demonstrate changes in MBV in response to hyperinsulinemia and exercise (Mulder et al.,
85 2008).

86 Tobin and colleagues investigated the reproducibility of CEUS recordings on abdominal skeletal
87 muscle and subcutaneous adipose tissue using bolus injections of SonoVue® (Tobin et al.,
88 2010). In this study, a high MI flash was not performed. Instead, 4-minutes real-time imaging
89 was recorded from the time of injection. Using this protocol, the researchers used the first
90 phase plateau as an index of MBV. These measurements had a CV of 4%, indicating a good
91 reproducibility in determining MBV in both skeletal muscle and subcutaneous adipose tissue
92 (Tobin et al., 2010). The researchers also investigated changes in MBV in subcutaneous adipose
93 tissue and forearm skeletal muscle in response to an oral glucose load and found an increase
94 of MBV in adipose tissue but not in forearm skeletal muscle (Tobin et al., 2010). This finding is
95 in contrast with earlier findings, reporting increases of 40-70% in muscle MBV following a
96 mixed meal, where it was measured by CEUS using a continuous infusion protocol (Keske et al.,
97 2009; Vincent et al., 2006). Therefore, it still remains unclear if CEUS using bolus injections of
98 SonoVue® is capable of detecting changes in MBV of skeletal muscle in response to ingestion
99 of a mixed meal.

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

106 **Methods**

107 **Participants**

108 A total of 10 young, healthy subjects (3 women and 7 men, 24.3 ± 3.3 years, BMI; 21.6 ± 1.6
109 kg/m^2 , systolic blood pressure; 125 ± 8.8 mmHg, diastolic blood pressure; 69.3 ± 8.7 mmHg,
110 resting heart rate; 57.9 ± 11.0 beats/min [Mean \pm SD]) were recruited through advertisements
111 on social media. Following exclusion criteria were used; BMI >25, smoking, heart disorders,
112 diabetes and daily or frequent intake of medication (oral contraceptives were allowed).

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

116 **Standard preparation**

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

124 **Contrast-enhanced ultrasound protocol**

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

130 The ultrasound gel thickness applied, prevented any pressure of the transducer on the
131 underlying tissue. The contrast agent dry matter (SonoVue[®], Bracco S.p.A, Italy) was dissolved
132 in sterile saline and mixed gently for exactly 30 sec before injection. SonoVue[®] is a suspension
133 of phospholipid-stabilized microbubbles filled with sulphur hexafluoride and is diluted in 4.5 ml
134 0.9% saline solution before injection ($8 \mu\text{l}$ microbubbles ml^{-1}). A bolus of 2.0 ml SonoVue[®] was
135 injected through the antecubital vein followed by an immediate flush of 10 ml 0.9% saline
136 solution. SonoVue[®] contains microbubbles of different sizes, ranging between diameters of 1
137 μm to 10 μm , with a mean of 2.5 μm . The size of the microbubbles is small enough to allow
138 free passage through the capillaries, but large enough to retain in the vascular system (Greis,
139 2004). Therefore the microbubbles will be distributed throughout the entire blood volume, but
140 will not diffuse into the extracellular fluid space (Greis, 2004). After leaving the microbubbles,
141 the gas is exhaled through the lungs, and therefore does not interfere with renal or hepatic
142 excretion pathways (Greis, 2004).

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

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

151 **Study design**

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

157 **Image analysis**

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

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

171 **Protocol A: Determination of washout period and reproducibility**

172 **Subject characteristics**

173 Six healthy, young volunteers (3 men, 3 women, age 24 ± 4.3 years, body mass index 21 ± 4.0
174 kg/m^2 , Systolic blood pressure 129 ± 12 mmHg, diastolic blood pressure 72 ± 18 mmHg, Resting
175 heart rate 63 ± 20 beats/min [mean \pm SD]) took part in this protocol.

176 **A1: Washout protocol**

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

182 **A2: Reproducibility protocol**

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

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

190 **Protocol B; Microvascular responses to exercise or nutrition**

191 **Subject characteristics**

192 Six healthy, young volunteers (5 men, 1 woman, age 25 ± 4.2 years, body mass index 22 ± 1.0
193 kg/m^2 , Systolic blood pressure 121 ± 9.0 mmHg, diastolic blood pressure 66 ± 7.0 mmHg,

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

196 **Protocol B1 - Nutrition**

197 In this protocol we investigated whether the method was capable of detecting changes in
198 microvascular perfusion in response to feeding. A baseline CEUS recording was performed at
199 time point -15 minutes, as described in section *Standard preparations*. Thereafter, the subject
200 consumed a drink in less than 5 min containing 20 g whey protein hydrolysate (Peptigen IF-
201 3090, Arla Foods Ingredients P/S, Viby J, DK) and 80 g maltodextrin (Fagron Nordic A/S,
202 Copenhagen, DK) at time point 0. The subjects were allowed to sit upright when consuming
203 the drink, but remained in the supine position throughout the rest of the protocol. CEUS
204 recordings were performed again at time points 30 and 60 minutes The experimental protocol
205 is illustrated in fig. 1c.

206 **Strength testing**

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

212 **Protocol B2 - Exercise**

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

218 1 RM. Sets were interspersed with 1 min rest. Immediately after completion of the exercise
219 bout, subjects were placed in the supine position on the hospital bed, and a CEUS recording
220 was performed as soon as possible. All CEUS recordings were initiated within 1 minute after
221 exercise cessation.

222 **Statistics**

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

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

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

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

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

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

243 **Results**

244 **Washout protocol**

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

253 **Reproducibility protocol**

254 To test the reproducibility of the method, we performed CEUS assessments of three occasions
255 with three injections interspersed with 15 minute intervals. We compared two methods for
256 estimating MBV; peak signal intensity and first phase plateau intensity. Mean background
257 signal was 15.7 dB \pm 0.5 dB, mean peak intensity was 17.1 ± 0.8 dB and mean first phase
258 plateau intensity was 16.7 ± 0.7 dB. The coefficient of variation (CV) for measurements
259 including background signal (A + B) were for intraday comparisons (PI CV; $1.8 \pm 0.4\%$, mPI CV;
260 $1.4 \pm 0.2\%$) and interday comparisons (PI CV; $2.9 \pm 0.9\%$, mPI CV; 1.8 ± 0.4). When assessing
261 the signal alone (A) the intraday variation was (PI CV: $19 \pm 4.2\%$; mPI CV: 23 ± 3.3) and interday
262 variation was (PI CV: $27 \pm 9.8\%$; mPI CV: $31 \pm 7.3\%$) (Table 1). Paired t-test did not show
263 significant difference between the CVs of PI and mPI when comparing intraday or interday

264 measurements ($P=0.15$ and $P=0.59$, respectively). Interday variation was not significantly
265 different from intraday variation for any of the measured parameters (PI: $P=0.48$ and mPI:
266 $P=0.38$).

267 **Intervention protocol**

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

270 Average 1 RM in the one legged knee extension exercise was 47.4 ± 14.7 kg, resulting in an
271 average exercise load of 33.1 ± 9.1 kg in 3 sets of 10 knee extension reps at 70% 1RM. Exercise
272 induced acute changes in peak signal intensity (+113%, $P<0.001$), plateau intensity (+218%,
273 $P<0.001$) compared to baseline measurements. All CEUS recordings acutely after exercise
274 cessation exhibited double peaks in signal intensity (as seen in the all-subject mean bolus
275 curve, fig 3).

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

278 **Discussion**

279 The present study demonstrates that CEUS using bolus injections of SonoVue appears to be as
280 reliable as existing techniques for assessing microvascular blood volume in vastus lateralis
281 muscle. This conclusion is based on the finding that the coefficient of variation for our chosen
282 indices of microvascular blood volume (MBV) were comparable to what has been observed in
283 earlier studies using CEUS in other muscles (Mulder et al., 2008; Tobin et al., 2010).

284 Furthermore, we were able to detect and demonstrate that exercise significantly increased
285 microvascular blood volume acutely after exercise, whereas we could not detect any change in
286 the immediate postprandial period.

287 **Reproducibility**

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

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

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

331 **Intervention protocols**

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

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

338 The positive control, knee extensor exercise, induced a large increase in MBV, as indicated by
339 increases in peak signal intensity (+113%) and plateau signal intensity (218%) acutely after
340 exercise cessation. Due to a lack of any gold standard method of measuring MBV it is not
341 possible to determine which of our parameters for MBV (peak signal intensity or plateau signal
342 intensity) that gives the most accurate estimate. Using the continuous infusion protocol,
343 Sjøberg and colleagues (Sjøberg et al., 2011) found that MBV increased 310% in response to
344 one legged knee extensor exercise at 25 W for 10 min. Vincent and colleagues (Vincent et al.,
345 2006) found that MBV increased approximately ~200% in the muscles of the forearm in
346 response to high-intensity isometric handgrip exercise. Even though our results cannot be
347 directly compared with the above mentioned results due to differences in exercise protocols
348 and muscles investigated, our results seem to be in agreement with prior studies and CEUS
349 recordings using bolus injection can presumably be used for assessing changes in muscle blood
350 volume in response to acute exercise.

351 Surprisingly, we were not able to detect any changes in MBV in response to feeding. Tobin and
352 colleagues were also not able to detect any changes in MBV in response to a 75 g glucose load
353 (Tobin et al., 2010), which is in contrast with prior CEUS studies (Churchward-Venne et al.,
354 2014; Keske et al., 2009; Mitchell et al., 2015, 2013; Timmerman et al., 2012; Vincent et al.,
355 2006). However, most of the latter studies have assessed microvascular perfusion via CEUS
356 using a continuous infusion of contrast agent. Tobin and colleagues suggested that the lack of
357 effect of their feeding protocol on MBV could be due to the feeding stimulus being
358 inadequate. In the present study however, the feeding stimulus was comparable to that of the
359 studies showing an effect of feeding on MBV. Therefore, it seems unlikely that the lack of an

360 effect of feeding on microvascular perfusion in our study is due to an insufficient feeding
361 stimulus. Instead, the lack of an effect is probably due to the either inadequacy of the method
362 to detect the changes, or no effect of feeding on MBV in our subjects. As insulin has been
363 many times to act as a vasodilator in healthy subjects (Dawson et al., 2002; Sjøberg et al.,
364 2011; Timmerman et al., 2010; Vincent et al., 2002), and given that an effect of feeding on
365 MBV in skeletal muscle has been detected in many studies prior to ours, the lack of an effect
366 observed in the present study is likely due to an inadequate sensitivity of our method. Prior
367 studies have found postprandial increases in MBV of 36-67% (Keske et al., 2009; Mitchell et al.,
368 2015, 2013; Vincent et al., 2006). We found an intraday variation for our MBV indices of 19-
369 23%, which therefore might be inadequate in order to detect an effect in the lower range of
370 what has been observed in earlier studies. As there is no golden standard method for assessing
371 microvascular perfusion, it is difficult to verify the validity of our results. Approaching the limit
372 of detection with the bolus injection approach of micobubbles, it would have been valuable to
373 have other measurements of blood flow by e.g. measuring leg blood flow to investigate of
374 feeding had any effect on total perfusion of the leg. However, prior investigations have
375 observed that changes in total leg blood flow are delayed compared to changes in
376 microvascular blood flow (Mitchell et al., 2015, 2013) and hence the microvascular blood flow
377 and blood volume changes are likely to be due to redistribution of microvascular flow (Clark et
378 al., 2006). Changes in a. femoralis blood flow might therefore not be indicative of changes in
379 microvascular perfusion. Alternatively, it would have been interesting to look into the
380 sensitivity of the method by applying a dose-response investigation of vasodilator substances
381 and measuring changes in MBV, and thereby investigate the ability of the method to detect
382 smaller changes in MBV..

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

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

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399 **Conflict of interest**

400 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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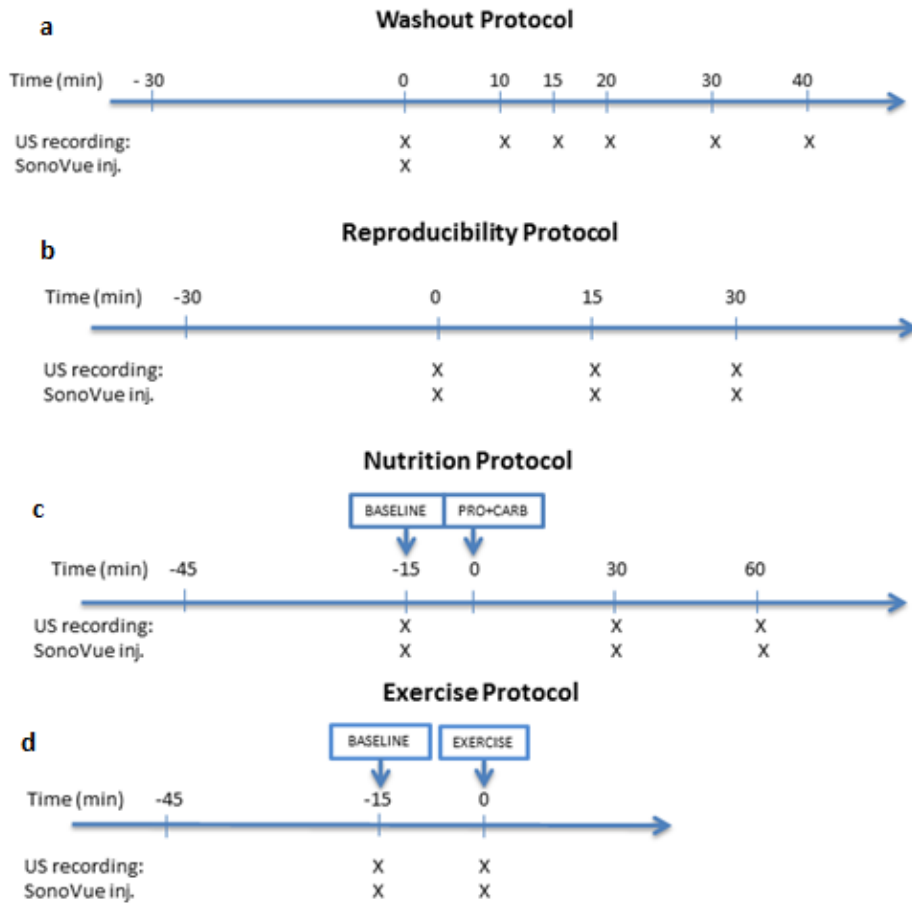
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481 TABLE 2

	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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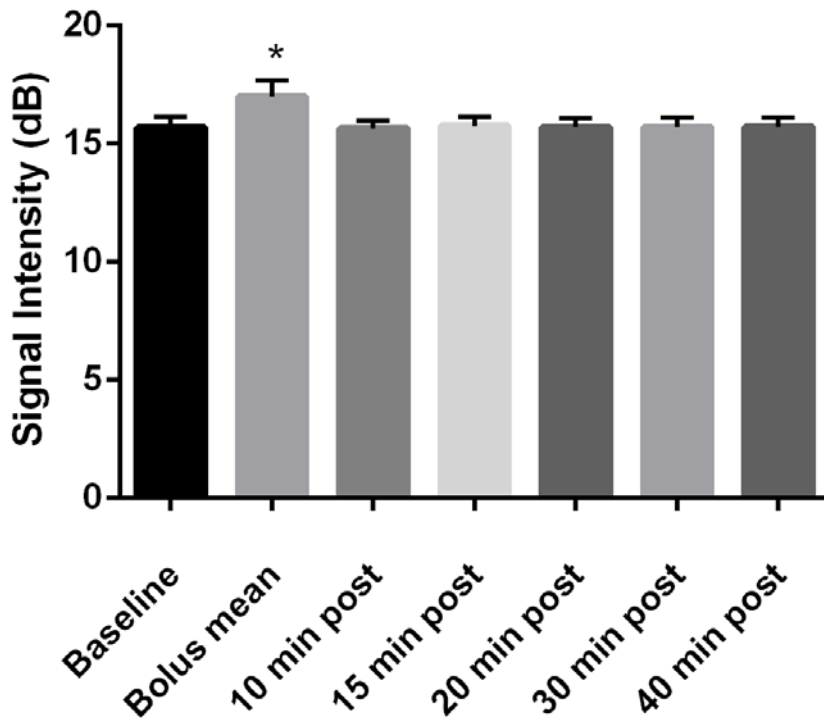
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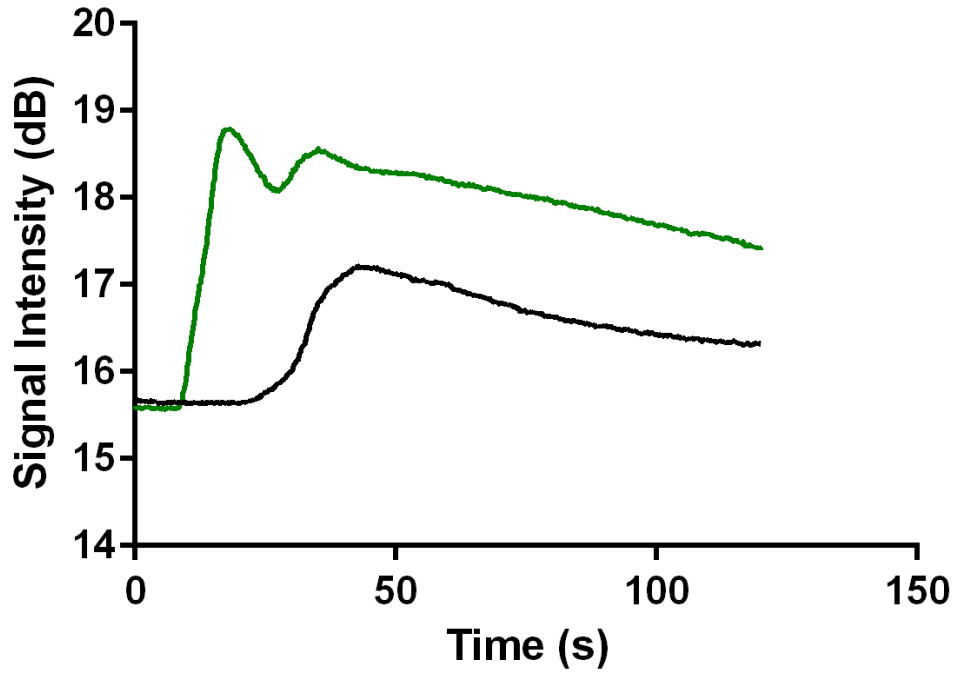
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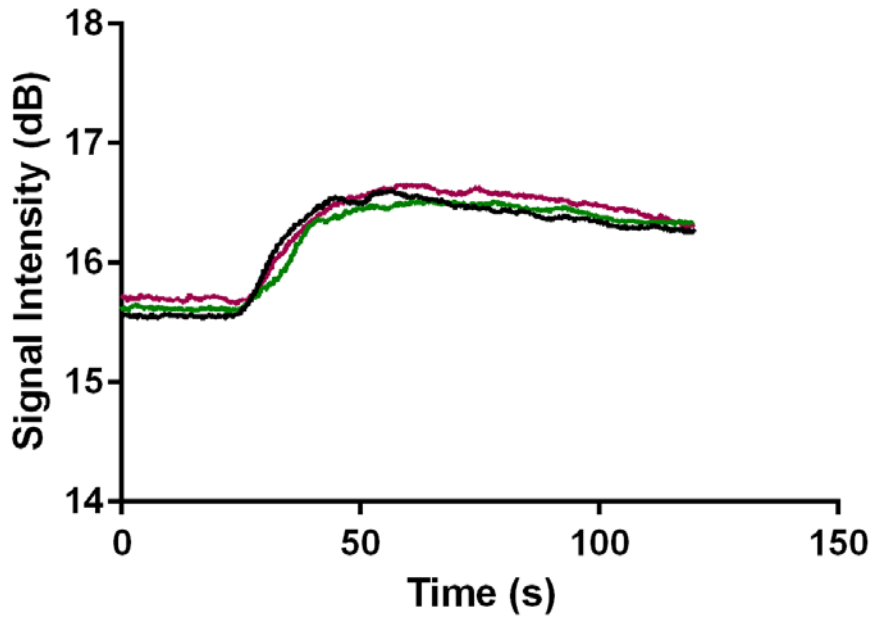


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496 FIGURE 4



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