

Diagnostic Ultrasound Detection of Changes in Femoral Muscle Mass Recovery after Tibial Plateau Levelling Osteotomy in Dogs

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Abstract

Objective The goal of this study was to develop a clinically feasible ultrasound (US) protocol that can detect changes in thigh muscle mass in dogs after stifle surgery. The primary aim of this study was to compare previously described US measurement locations of the canine thigh for detecting changes in muscle mass in dogs recovering from tibial plateau levelling osteotomy (TPLO).

Study Design This was a prospective, exploratory pilot study. Adult dogs ($n = 7$) undergoing pet-owner elected TPLO were enrolled. Twelve different US measurements were performed in triplicate by a single experienced observer. Measurements were performed at 0, 2, 4 and 8 weeks after surgery at a proximal and distal location along the femur. Data from all available time points and locations were analysed for the main effect of time within modalities.

Results A total of 1,008 US measurements were performed. Measurements of the transverse sectional area of the rectus femoris muscle detected significant ($p \leq 0.05$) muscle loss between weeks 0 and 2 at the lateral and medial aspects of the distal location (19% and 15% respectively). Measurements of the thigh muscle thickness were significantly ($p < 0.01$) increased between 2nd- and 8th- week time points at the lateral aspect of the proximal location (26%).


Conclusion The proximal femoral location, measured from the lateral aspect, appears to be the most suitable US measurement for detecting increases in femoral muscle mass in dogs recovering from TPLO. The provided pilot data suggest that further research evaluating this outcome measure is indicated.

Keywords

- ▶ thigh circumference
- ▶ ultrasound
- ▶ muscle recovery
- ▶ tibial plateau levelling osteotomy
- ▶ dogs

Introduction

Cranial cruciate ligament disease is one of the most common reasons for hind limb lameness in the dog, and surgery is generally recommended for medium and large breed dogs.^{1,2}

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Manual thigh circumference (TC) has been used as an objective outcome measure for detecting changes in muscle mass.^{3–6} However, the accuracy of this measurement in dogs has recently been questioned.^{7,8} Muscle mass measurement with advanced imaging computed tomography (CT), magnetic resonance imaging (MRI) and dual-energy X-ray absorptiometry (DEXA) has been advocated as a more objective and

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reliable outcome measure.^{5,9,10} However, the repeated ionizing radiation exposure, high cost, limited access to equipment and requirement for pharmacological immobilization with CT and MRI limit the use of these techniques in dogs. In humans, DEXA-measured lean body mass and muscle thickness measured by diagnostic US were reported to be significantly related.¹¹ However, access to DEXA technology is limited and its estimations are generally restricted to the whole body or an entire limb because it is difficult to determine lean tissue mass of small regions.⁵ Due to these limitations, diagnostic ultrasound (US) evaluation for the measurement of muscle mass as well as its association with limb function and strength has been extensively investigated in humans.^{11–15} In systematic reviews, real-time brightness mode (B-mode) US in humans was found to provide valid measurements of skeletal muscle thickness compared with advanced imaging.^{11,16} Unlike CT or MRI, US is portable, relatively inexpensive, non-invasive and without radiation exposure. Two studies have compared the accuracy of US muscle measurements to advanced imaging modalities in dogs at a single time point.^{17,18} Sakaeda and Shimizu showed a good correlation between US measurements performed at five different locations along the femur and MRI in clinically healthy Beagles.¹⁷ In another study, US muscle measurements of appendicular forelimb muscles correlated well with CT measurements when fiducial markers were used to mark the measurement sites.¹⁸ While these studies show the feasibility and accuracy of single time point US measurements in dogs, no study has evaluated US for its ability to detect serial (i.e. at different time points) changes over time in the clinical setting (e.g. evaluation of muscle mass during recovery from stifle surgery). However, a comparison of serial changes during convalescence is relevant for any clinical study evaluating the effectiveness of treatment over time. In the clinical setting, timely completion of outcome measures is important for an outcome measure to become feasible. Therefore, the goal of this study was to develop a clinically feasible US protocol that can detect changes in thigh muscle mass in dogs after stifle surgery, as a potential alternative to manual TC. The primary aim of the study was to compare previously described US measurement locations for their ability to detect a change in muscle mass in dogs recovering from stifle surgery.

Materials and Methods

Sample Population and Selection

This prospective study was approved by Colorado State University's Clinical Research Review Board. All study procedures were performed according to standard institutional practices. Privately owned, healthy, medium-large breed, adult dogs undergoing pet-owner elected tibial plateau levelling osteotomy (TPLO) for naturally occurring cranial cruciate ligament disease (CCLD) were offered to be enrolled in the study. Informed consent was obtained from all owners. Criteria for inclusion in the study included clinically unilateral, chronic CCLD, lack of any other neurological or orthopaedic injuries or disease, body weight between 18 and 35 kg and body condition score between 4 and 7 (Nestlé PURINA body condition score scale).¹⁹ Dogs that

were previously (> 1 year prior to enrolment) treated with TPLO for CCLD of the contralateral limb were eligible for inclusion. Dogs underwent a routine TPLO as described elsewhere.²⁰ Surgery was followed by the standard rehabilitation protocol used at the institution (► **Supplementary Appendix B**; available in online version only).

Data Collection

The following evaluations were performed at 0, 2, 4 and 8 weeks: manual TC, US measurements and objective gait analysis. Computed tomography, considered the reference standard, was performed at 0 and 2 weeks. These time points were chosen to confirm the assumption that muscle atrophy would occur during the first 2 weeks of recovery. Manual TC, US and CT measurements were performed at two levels along the femur (► **Fig. 1**) based on their distance from the base (i.e. proximal tip) of the patella.¹⁷ The base of the patella was chosen since the observers felt that this location allows for more accurate palpation compared with the greater trochanter. During the initial visit, the distance from the base of the patella to the greater trochanter was measured manually by a single observer (IF) using a ruler. The two levels along the femur were determined based on locations described in a previous study.¹⁷ The distance measured proximally from the patella was determined as follows: (1) proximal—multiplying the patellar to



Fig. 1 Illustration of the proximal and distal locations (indicated by horizontal lines) where manual thigh circumference, ultrasonographic and computed tomography measurements were performed.

greater trochanter distance in cm by 2/3; (2) distal—dividing the distance from patella to greater trochanter by 2. All measurements were performed at both locations, with the stifle joint at a 135° angle as determined by use of a universal goniometer as previously described.²¹ Measurements for each technique were performed by a single observer blinded to the measurements performed by other techniques or observers. All dogs were sedated for CT and US imaging using dexmedetomidine 2.5 µg/kg and butorphanol 0.2 mg/kg given both intravenously. Following the measurements, the sedation was reversed using atipamezole 25 µg/kg intramuscularly.

Manual Thigh Circumference Data

Manual TC measurements (in mm) at each location were acquired in triplicate by a single observer (IF) using a Gulick II measuring tape (Country Technology, Inc., Gays Mills, Wisconsin, United States) as previously described.^{6,8} The measurements were performed prior to sedation and hair clipping, while the dog was laying in lateral recumbency with the stifle joint at a 135° angle.

Ultrasonographic Muscle Thickness Data

All scans were acquired with dogs sedated (as described earlier) and positioned in dorsal recumbency in a trough with the stifle joint at a 135° angle. The hair was clipped prior to each US exam to allow sufficient probe contact. B-mode US was performed using a 12 MHz linear array electronic transducer and US machine (General Electric NextGen LOGIQ e, Jiangsu, China). Transverse plane images were acquired. The following twelve US measurements of femoral muscle mass were performed based on previous publications and pilot work performed by the authors.^{17,18,22} The transverse sectional area (TSA) of the rectus femoris (RF) muscle measured (in cm²) from the lateral and medial aspects, the RF muscle thickness (the distance from the RF muscle [bisecting this muscle] to the femoral bone) measured from the lateral and medial aspects, and the thigh muscle thickness as the linear distance (both measured to the tenth of an mm) from the superficial aspect of the superficial muscle (avoiding the skin fold and subcutaneous tissue in the measurement) to the bone from both lateral and medial aspects (► Fig. 2).^{16,18,22} The least amount of pressure necessary, in combination with generous amounts of transducer gel for adequate imaging, was applied. Images were obtained with the transducer oriented perpendicular to the long axis of the femur. To minimize the impact of inter-observer variability in this pilot study, all US measurements were performed by a single, board-certified radiologist (LL). Images were obtained in triplicate, resulting in a total of 36 measurements per time point and dog. After image acquisition, measurements (to the tenth of 1 mm) were performed using commercially available software (Philips IntelliSpace Radiology V.4.4.551.4; Philips Healthcare Informatics, Inc. Foster City, California, United States).

Computed Tomography Thigh Transverse-Sectional Area Data

All CT scans were acquired with dogs sedated (as described earlier) and positioned in dorsal recumbency in a trough

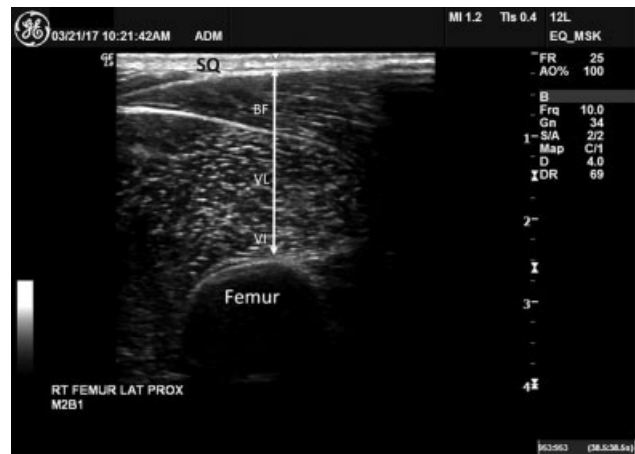


Fig. 2 Ultrasound measurement of muscle thickness at the proximal femur location, taken from the lateral side. The white double head arrow illustrates the borderlines used, avoiding the skin and subcutaneous tissue for this measurement. BF, biceps femoris; SQ, subcutaneous; VI, vastus intermedius, VL, vastus lateralis.

with the stifle joint at a 135° angle. All dogs were scanned with a multislice CT scanner (Philips GEMINI TF Big Bore PET-CT; Philips Healthcare, Koninklijke Philips Electronics N.V., Amsterdam, the Netherlands). Reconstruction algorithms were 1 mm transverse slices in a bone-detail algorithm and 2 mm transverse slices in a standard algorithm. Window width and level were adjusted by the evaluator to allow identification of all landmarks as needed. Multiplanar reformatting was used. Computed tomography measurements were performed using commercially available software (Philips IntelliSpace Portal version 8, Philips Medical Systems, Nederland B.V.) by a single observer (FD). Multiplanar image reconstruction and osseous landmarks (cranial cortex of femur, medial fabella, lesser trochanter, and base of the patella) were used to define locations of each measurement. Each set of CT scans (i.e. 0 and 2 weeks) was evaluated during a single session to minimize variation in measurements between sets. Limb TSA measurements (in mm²) were performed in triplicate. To determine the proximal and distal locations used for US and manual TC measurements, the distance measurements were performed along the sagittal plane axis line from the base of the patella (of the caudal aspect of the patella) using the straight-line tool (ruler).

Objective Gait Analysis

Objective gait analysis was used to evaluate limb function over the study period. A pressure sensitive walkway (HRV Walkway 6 VersaTek System, Tekscan Animal Walkway System; South Boston, Massachusetts, United States; 304.8 cm × 58.4 cm) was used as previously described, at the beginning of each visit, before any physical and orthopaedic examination was conducted, and before any sedation was administered.^{23,24} Ground reaction forces (GRF, including peak vertical force [PVF], vertical impulse [VI] as well as the percentage of body weight distribution (%BWD) were measured.

Statistical Analysis

This was an exploratory study aimed at identifying standardized US measurements that can be used in a clinical setting. The 2- to 8-week time point comparison was defined as the clinically relevant outcome measure based on previous studies suggesting that the 2-week time point represents the time point with the least muscle mass.^{5,6,10} Changes over time across all datasets (CT, US, manual TC and gait analysis) were evaluated using linear effect mixed models and Tukey post-hoc tests where the dog was considered as the random effect and time was the fixed effect. Similarly, a marginal pseudo-R² was calculated to evaluate the strength of the relationships between the various measures using a linear mixed effects models where both the dog and week were entered as random effects. As all measurements were performed in triplicate, the reliability of the measurement was examined using the Intraclass Correlation Coefficient (ICC). Intraclass Correlation Coefficient estimates were calculated based on a mean-rating (k = 3), absolute-agreement, 2-way mixed-effects model which corresponds to Shrout and Fleiss's ICC (2, k) equation. All analyses were conducted using commercially available software (R; R version 3.4.1) with significance set at $p \leq 0.05$.

Results

The reliability was high across all different measures at each location (ICC ≥ 0.99). Therefore, the average of the three replicates was used in all subsequent analyses. Details of the relationship between all measurements is presented in **►Supplementary Appendix A** (available in online version only).

Sample Population

Seven dogs were enrolled. Mean age (\pm SD) was 7.5 years (± 3.2), mean weight (\pm SD) was 24 kg (± 5.3) and median body condition score was 5 (range: 4–6). Five mixed breed dogs, one Labrador Retriever and one Australian Shepherd Dog participated in the study. A TPLO of the affected limb ($n = 5$ right, $n = 2$ left) was performed without complications. Normal osteotomy healing was documented in all dogs after 8 weeks. No major complications were observed in any of the dogs during the study period. Minor complications observed included seroma formation in 2 dogs, which had resolved by the 2-week time point.

Gait Analysis

All GRF (PVF, VI and %BWD measurements) of the affected hind limb were significantly improved ($p < 0.01$) throughout

Table 1 Ground reaction forces at all time points

Time points	Week 0	Week 2	Week 4	Week 8	p-Value
%BWD	12.1 \pm 3.4 ^a	15.0 \pm 1.4 ^b	15.7 \pm 1.01 ^b	18.7 \pm 1.3 ^c	<0.01
PVF (N)	50.0 \pm 20.3 ^a	61.1 \pm 17.8 ^{a,b}	64.7 \pm 17.8 ^{b,c}	77.1 \pm 17.3 ^c	<0.01
VI (N*sec)	20.7 \pm 17.9 ^a	23.2 \pm 21.62 ^{a, b}	26.3 \pm 19.34 ^{b, c}	28.8 \pm 18.3 ^c	<0.01

Abbreviations: %BWD, percentage of body weight distribution; N, newton; PVF, peak vertical force; SD, standard deviation; VI, vertical impulse. Mean ground reaction forces (\pm SD) of the affected limb at all time points. Within a row, values with different superscript letters (a, b, c) differ significantly ($p < 0.05$; pairwise comparisons) between time points.

Table 2 Relationship between changes in computed tomography, ultrasound and manual thigh circumference

	CT TSA proximal	CT TSA distal
Manual TC proximal	0.77 ^a	0.90 ^a
Manual TC distal	0.55 ^a	0.76 ^a
TMT lateral proximal	0.38 ^a	0.51 ^a
TMT lateral distal	0.36 ^a	0.63 ^a
TMT medial proximal	0.37 ^a	0.57 ^a
TMT medial distal	0.01	0.03
RF TSA lateral proximal	0.87 ^a	0.78 ^a
RF TSA lateral distal	0.44 ^a	0.70 ^a
RF TSA medial proximal	0.51 ^a	0.65 ^a
RF TSA medial distal	0.84 ^a	0.80 ^a
RF MT lateral proximal	0.01	0.04
RF MT lateral distal	0.10	0.19 ^a
RF MT medial proximal	0.04	0.12
RF MT medial distal	0.01	0.00

Abbreviations: CT, computed tomography; MT, muscle thickness; RF, rectus femoris; TC, thigh circumference; TMT, thigh muscle thickness; TSA, transverse-sectional area; US, ultrasound.

Pseudo R² values representing the extent of the relationship between changes in CT, US, and manual TC.

^aIndicates $p \leq 0.05$.

the study period (**►Table 1**). No relationships were found between all GRF and CT, US or manual TC (**►Supplementary Appendix A** [available in online version only]).

Computed Tomography Data

Computed tomography measurements identified a 5% ($p = 0.11$) and 8% ($p = 0.07$) decrease in limb TSA between weeks 0 and 2 at the proximal and distal measurement location respectively. Changes in the CT TSA were strongly related to most of the US measurements as well as to manual TC (**►Table 2** and **►Supplementary Appendix A** [available in online version only]).

Ultrasonographic Muscle Thickness Measurements

A total of 1,008 measurements were performed. Changes in the majority of the US measurements were in moderate to strong relation to CT (**►Table 2**, **►Supplementary Appendix A** [available in online version only]). The US measurements of the TSA of the RF muscle at the distal location, from both lateral and medial, detected significant (19 and 15%

Table 3 Ultrasound and manual measurements

Measurement parameter	Weeks post-surgery				p-Value
	0	2	4	8	
Manual TC proximal (cm)	31.0 ± 4.6	30.5 ± 4.7	30.6 ± 4.3	31.0 ± 4.8	0.70
Manual TC distal (cm)	26.6 ± 2.7	26.3 ± 2.6	25.5 ± 2.7	25.9 ± 2.4	0.12
TMT lateral proximal (mm)	14.5 ± 5.0 ^{a,b}	12.8 ± 4.4 ^a	12.4 ± 3.9 ^a	16.1 ± 4.9 ^b	<0.01
TMT lateral distal (mm)	15.5 ± 4.8	13.1 ± 4.2	13.9 ± 3.3	15.6 ± 3.7	0.08
TMT medial proximal (mm)	22.0 ± 5.5	20.8 ± 6.5	22.2 ± 5.2	24.2 ± 6.2	0.06
TMT medial distal (mm)	15.6 ± 3.8	15.8 ± 4.0	15.3 ± 4.4	15.8 ± 3.3	0.93
RF TSA lateral proximal (cm ²)	3.8 ± 1.3	3.4 ± 1.2	3.3 ± 1.0	3.5 ± 0.9	0.13
RF TSA lateral distal (cm ²)	3.1 ± 1.3 ^a	2.5 ± 1.1 ^b	2.7 ± 1.0 ^{a,b}	2.8 ± 1.0 ^{a,b}	0.02
RF TSA medial proximal (cm ²)	3.9 ± 1.3	3.6 ± 1.2	3.4 ± 1.0	3.8 ± 1.1	0.12
RF TSA medial distal (cm ²)	3.4 ± 1.3 ^a	2.9 ± 1.1 ^b	2.9 ± 0.9 ^b	3.0 ± 1.1 ^{a,b}	0.05
RF MT lateral proximal (mm)	35.7 ± 5.5	35.5 ± 2.7	36.1 ± 6.0	35.7 ± 4.9	0.99
RF MT lateral distal (mm)	27.5 ± 5.4	26.2 ± 3.6	27.8 ± 5.2	25.1 ± 5.9	0.16
RF MT medial proximal (mm)	32.3 ± 7.1	30.1 ± 4.2	29.7 ± 5.8	32.4 ± 4.0	0.17
RF MT medial distal (mm)	27.1 ± 4.8	24.9 ± 3.0	26.5 ± 5.1	27.0 ± 5.3	0.37

Abbreviations: MT, muscle thickness; RF, rectus femoris; SD, standard deviation; TC, thigh circumference; TMT, thigh muscle thickness; TSA, transverse-sectional area.

Note: Mean ultrasound and manual measurements (±SD) of the affected limb at all time points.

Within a row, values with different superscript letters (a, b) differ significantly ($p \leq 0.05$; pairwise comparisons) between time points.

respectively; both $p \leq 0.05$) muscle loss between weeks 0 and 2 (► **Table 3**). A significant ($p < 0.01$) increase in thigh muscle thickness between the 2- and 8-weeks time points was identified by the US measurements taken at the proximal location from lateral (26%, ► **Table 3**). The remaining measurements showed no significant change for any time point comparison.

Manual Thigh Circumference

Manual TC measurements did not detect significant muscle loss or gain between any time points (► **Table 3**). Manual TC values showed strong relation to CT and weak relation to the various US measurements (► **Table 2**, ► **Supplementary Appendix A** [available in online version only]).

Discussion

The results of this study define a clinically feasible protocol for the utilization of US as an outcome measure for detecting muscle mass changes over time after TPLO. Based on our data, the proximal thigh muscle thickness should be considered as the primary measurement location in future studies when US is used as an outcome measure evaluating muscle mass changes after TPLO surgery during the first 8 weeks of recovery.

It has been previously suggested that dogs demonstrate femoral muscle loss by 2 weeks after stifle surgery and will begin to show muscle recovery by week 8.^{5,10,25} Our findings support this suggestion and US measurements were able to quantify these changes objectively. In humans, US measurements of the RF thickness and TSA were found to significantly relate with DEXA and CT and were also strongly associated

with limb function and strength during recovery.^{11,15,26,27} To the best of the authors' knowledge, no similar studies have been published in dogs. In our study population, measurements of the RF thickness did not reveal any significant changes between time points. The RF TSA did reveal significant muscle loss during the first 2 weeks, yet did not detect muscle gain during the subsequent recovery. It is possible that this reflects the individual response of the RF muscle in dogs. Another possible explanation may be related to the fact that the RF is the only quadriceps muscle that also acts as a hip flexor; it has been reported to undergo a different pattern of muscle atrophy and recovery compared with the vastus medialis and lateralis in dogs after 10 weeks of rigid immobilization of the stifle.^{28,29} However, further research is needed to evaluate these suggestions.

Instrumented gait analysis is a well-established and extensively used methodology to objectively compare lameness before and after various orthopaedic surgical procedures including the stabilization of CCLD in dogs.^{30,31} In the present study, it was used to verify continued limb improvement over the study period. While objective gait analysis has not directly been correlated with muscle mass increase in dogs, the authors' assumption was that increased limb function would indicate a normal recovery and in turn this should be associated with continued increase in thigh muscle mass.³² We found no relationship between changes in GRF and US; however, this may be related to the small sample size.

Manual TC is a simple, frequently used outcome measure.³⁻⁶ In the study population, this measurement method did not detect significant changes in muscle mass at any time point. Potential limitations associated with manual TC measurements

that may explain this finding include the variability in the location of the measuring tape, the degree of limb extension and tension applied to the tape, positioning, hair regrowth and lack of sedation.^{6–8} The strong relation of manual TC with CT in this study is easily explained by the fact that both measure the perimeter of the thigh (i.e. TSA and TC). Also, both methods include the skin and subcutaneous tissue in their measurements, which may explain the lack of significant changes during the recovery. As previously reported in humans, subtle differences in TC may actually represent significant muscle mass changes measured by US.³³ Our results suggest that US may be more sensitive than manual TC measurements; however, larger clinical trials are needed to confirm this finding.

To evaluate the accuracy of a technique, comparison to a gold standard is ideal. In humans, CT is considered a gold standard.¹¹ Therefore, CT was used in this study to determine the time point comparison most likely to show a significant difference between measurements (i.e. 0–8 or 2–8 weeks). Due to financial limitations, CT was not performed at week 4 and 8, which is a limitation of our study. Computed tomography showed muscle loss between weeks 0 and 2; however, this was not significant. This may be explained by the difficulty to accomplish the same positioning of the limb in serial CT scans or the small sample size.

Based on our subjective experiences, several limitations when using the US for measurement of muscle thickness have to be considered. First, the variable pressure of the transducer on the underlying musculature may alter measurements. An effort was made to minimize this confounding factor by using a single, experienced, board-certified radiologist. Second, the angle of incidence of the transducer with respect to the muscle is difficult to standardize and small differences in the angle may influence the measurements. Third, there is a lack of clear landmarks (such as consistent vessels or bony prominences) in the area examined that would allow for the confirmation of repeatable positioning of the transducer at different time points. Unfortunately, the inability to accomplish consistent positioning of the patient is the main concern with any serial outcome measure of muscle mass over time. Fourth, in some dogs, the difference in echogenicity of the muscles with respect to the adjacent fascia was less well defined, making the margin of the muscle more difficult to identify and accurately measure. Lastly, variability in the shape of the RF muscle (round to ovoid to oblong) was observed between dogs which may make the measurement of the RF muscle thickness, but not TSA, difficult to standardize. Based on the study results, the proximal femoral location, measured from the lateral aspect, appears to be the most suitable for detecting increases in femoral muscle mass. This location also has the potential to be performed without sedation, since the dog can be positioned in lateral recumbency.¹⁷ The measurements of the TSA of the RF at the distal location showed a decrease in muscle mass between weeks 0 and 2; however, it did not detect a significant increase between weeks 2 and 8. Additionally, the measurements of muscle thickness at the lateral proximal location revealed the highest percentage of change. Lastly, the lateral measurements were subjectively easier to

perform than TSA of RF and medial muscle thickness measurements. Overall, based on these results, we recommend prioritizing the proximal location; however, more studies with a larger sample size are needed to confirm this suggestion.

This study was designed to identify one or two measurement locations described by Sakaeda and Shimizu that should be defined as the primary outcome measure in future studies.¹⁷ In this study population, US measurements of muscle thickness of the proximal femur were found to offer the greatest feasibility for clinical trials evaluating muscle mass changes in dogs recovering from TPLO. Our study provides pilot data; however, further work is required to validate the proposed clinical protocol. Future research could focus on improving methods for consistent positioning (such as using custom-made foam beds as previously described), evaluating the variability between observers and less experienced observers and whether sedated and non-sedated measurements differ.^{34,35}

Author Contribution

Ilan Frank, Felix Duerr and Linda Lang contributed to the study conception, the study design, the acquisition of data, data analysis and interpretation, drafting or revising of the manuscript, and approved the submitted manuscript. Brian Zanghi and Rondo Middleton contributed to the study conception, the study design, data analysis and interpretation, drafting or revising of the manuscript, and approved the submitted manuscript.

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Conflict of Interest

Brian Zanghi and Rondo Middleton are employees of Nestlé Purina Research, St. Louis, Missouri.

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