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# Quantification of muscle triglyceride synthesis rate requires an adjustment for total triglyceride content

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**Abstract** Intramyocellular triglyceride (imTG) in skeletal muscle plays a significant role in metabolic health, and an infusion of [<sup>13</sup>C<sub>16</sub>]palmitate can be used to quantitate the in vivo fractional synthesis rate (FSR) and absolute synthesis rate (ASR) of imTGs. However, the extramyocellular TG (emTG) pool, unless precisely excised, contaminates the imTG pool, diluting the imTG-bound tracer enrichment and leading to underestimation of FSR. Because of the difficulty of excising the emTGs precisely, it would be advantageous to be able to calculate the imTG synthesis rate without dissecting the emTGs from each sample. Here, we tested the hypothesis that the ASR of total TGs (tTGs), a combination of imTGs and emTGs, calculated as “FSR × tTG pool,” reasonably represents the imTG synthesis. Muscle lipid parameters were measured in nine healthy women at 90 and 170 min after the start of [<sup>13</sup>C<sub>16</sub>]palmitate infusion. While the measurements of tTG content, enrichment, and FSR did not correlate ( $P > 0.05$ ), those of the tTG ASR were significantly correlated ( $r = 0.947$ ,  $P < 0.05$ ). These results demonstrate that when imTGs and emTGs are pooled, the resulting underestimation of imTG FSR is balanced by the overestimation of the imTG content. **■** We conclude that imTG metabolism is reflected by the measurement of the tTG ASR.—Asghar, R., M. Chondronikola, E. L. Dillon, W. J. Durham, C. Porter, Z. Wu, M. Camacho-Hughes, C. R. Andersen, H. Spratt, E. Volpi, M. Sheffield-Moore, L. Sidossis, R. R. Wolfe, N. Abate, and D. R. Tuvdendorj. **Quantification of muscle triglyceride**

**synthesis rate requires an adjustment for total triglyceride content.** *J. Lipid Res.* 2018. 59: 2018–2024.

**Supplementary key words** in vivo muscle triglyceride synthesis • stable isotope tracers • skeletal muscle lipid metabolism

Skeletal muscle plays an important role in systemic lipid and glucose metabolism (1–3). Increased accumulation of intramyocellular triglycerides (imTGs) or ectopic fat deposition has been reported in obesity, insulin resistance (IR), T2D, and aging (1–5). However, imTGs are neutral lipids and not likely to directly cause IR. The lack of a direct effect of imTGs on insulin sensitivity is, in part, supported by the fact that insulin-sensitive high-performance athletes have high levels of imTGs, a phenomenon named “athletes paradox” (6). Thus, understanding the mechanisms of accumulation of imTGs should assist in understanding the role of imTGs in physiological and pathological conditions, and in establishing novel targets for interventions to prevent the development of IR and related disease states.

The extent of accumulation of imTGs depends on the balance between the rates of its synthesis and breakdown, which can be measured using the stable isotope tracer approach. The incorporation of a tracer (e.g., [<sup>13</sup>C<sub>16</sub>]palmitate) into imTGs can be assessed either by using magnetic resonance spectroscopy (MRS) or by a direct measurement of the tracer enrichment of imTGs in lipid extracts of

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Abbreviations: acyl-CNT, acylcarnitine; ASR, absolute synthesis rate; CNT, carnitine; DEXA, dual energy X-ray absorptiometry; emTG, extramyocellular triglyceride; FSR, fractional synthesis rate; imTG, intramyocellular triglyceride; IR, insulin resistance; MPE, mole percent excess; MRS, magnetic resonance spectroscopy; palmitoyl-CNT, palmitoylcarnitine; TG, triglyceride; tTG, total triglyceride; TTR, tracer-to-tracee ratio; UTMB, University of Texas Medical Branch.

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muscle biopsy samples (7–10). The use of MRS is the less invasive approach, but this procedure has not been well-established (10). Measurement of tracer incorporation into the imTGs of muscle samples requires obtaining muscle biopsy samples, a somewhat invasive procedure, but one widely used. The main hesitation for using this method in metabolic studies is the difficulty in isolating the true imTGs (11, 12). In addition to the imTG pool, there is also a pool of extramyocellular TGs (emTGs) of intra-muscular adipocytes, the metabolism and function of which are not well-known (13, 14). The intra-muscular adipocytes or emTG pool is not uniformly distributed in muscles (12, 15–17). When muscle lipids are extracted, unless the emTG pool is precisely excised, the lipid extract contains a mixture of emTGs and imTGs or total TGs (tTGs). Excision of emTGs is difficult and tedious, making kinetic studies involving multiple muscle biopsy samples impractical. On the other hand, if the contribution of emTGs to tTGs is ignored, the emTG-derived FAs will dilute the tracer enrichment incorporated into imTGs, thereby causing underestimation of the fractional synthesis rate (FSR) of imTGs. Therefore, if biopsy samples are obtained at the same time, but from muscle (e.g., musculus vastus lateralis) areas that differ in their emTG pool size, the tracer enrichments of imTGs should vary between the biopsy samples and so should the calculated FSRs (11, 12). This variability should be proportional to the contribution of emTGs in the tTG pool sizes; therefore, if we account for the emTG content, the variability should be corrected. The calculation of the imTG absolute synthesis rate (ASR) requires the use of values of both the FSR and the content of imTGs. However, the muscle lipid extract contains a mixture of both emTGs and imTGs (i.e., tTGs). Thus, if the muscle tTG content is high due to the contribution of the emTG pool, the ASR of the imTGs will be overestimated due to the contribution of emTGs to the measured muscle tTG content. However, the FSR of imTGs should be correspondingly underestimated due to dilution of the imTG tracer enrichment by the FAs of the emTG pool. The single variable affecting the measurement of both the FSR and the ASR is that the same emTG pool is contributing to the tTG pool; thus, the underestimation of the FSR and the overestimation of the ASR should cancel out. We propose to use the tTG pool to calculate the ASR to correct for the emTG-contributed tracer dilution, and thus the calculated ASR should represent the synthesis rate of imTGs. In other words, while this approach does not completely negate the potential effect of emTG contamination, it should correct for the emTG content-driven variability in the imTG FSR of muscle biopsy samples obtained under the same conditions. Therefore, in the current study, we aim to test our hypothesis that the ASR of tTGs is a measure of the imTG synthesis rate that is corrected for the emTG-related underestimated FSR. The underlying assumption is that, when analyzing a muscle biopsy sample containing both imTGs and emTGs, the underestimation of the imTG FSR due to the low tracer enrichment of emTGs is offset by the overestimation of the amount of imTGs due to the contribution of the emTGs to the measured tTG content. To test our hypothesis, we have conducted a

$[^{13}\text{C}_{16}]$ palmitate infusion study, and quantified the tTG FSR and ASR at two different time points (i.e., 90 and 170 min after the start of the tracer infusion). Our rationale is that the tTG ASR should be the same in each sample, but the amount of emTGs will vary. Therefore, a high correlation between the tTG ASR of two samples will reflect that the contribution of varying amounts of emTGs have canceled each other out.

## METHODS

Healthy nonobese women were enrolled in this study. The exclusion criteria were evidence of acute illness, T2D, taking medications that affect lipid metabolism, pregnancy or lactation, a history of substance abuse, or the inability to provide informed consent. All study procedures were approved by the Institutional Review Board of the University of Texas Medical Branch (UTMB), Galveston, TX. All participants provided written informed consent. All study procedures were conducted at the UTMB Clinical Research Center.

Upon enrollment, subjects underwent medical examination and a dual energy X-ray absorptiometry (DEXA) scan to measure whole-body fat mass and fat-free mass. Fasting blood samples were obtained and the plasma levels of VLDL cholesterol, TGs, total cholesterol, HDL cholesterol, and LDL cholesterol were measured using a Vitros 5600 analyzer (Ortho Clinical Diagnostic, Rochester, NY) at the UTMB Clinical Pathology Laboratory. Serum insulin concentrations were determined using an Immulite 2000 insulin system (Siemens Medical Solutions USA, Inc., Norwood, MA), and the glucose concentration using a YSI glucose/lactate analyzer (2300 STAT; Yellow Spring, OH).

### Preparation of $[^{13}\text{C}_{16}]$ palmitate bound to albumin

The  $[^{13}\text{C}_{16}]$ potassium palmitate [microbiological/pyrogen tested (#CLM-3943-MPT-PK)] was purchased from Cambridge Isotopes Laboratories, Inc. (Andover, MA). Bottles of human 5% albumin were obtained through the UTMB Pharmacy. The mixing of  $[^{13}\text{C}_{16}]$ palmitate in albumin was performed by Investigational Drug Services at the UTMB Pharmacy in a USP 79-compliant clean room using aseptic technique in a biological safety cabinet. Samples of palmitate-albumin solution from each bottle were tested for sterility and pyrogenicity by the Clinical Microbiology Laboratory. Only upon receipt of negative test results were the palmitate-albumin solutions used for studies. All test results were added in the study records.

### The $[^{13}\text{C}_{16}]$ palmitate infusion study

All subjects participated in a  $[^{13}\text{C}_{16}]$ palmitate infusion study as depicted in Fig. 1. This protocol (Fig. 1) was designed to allow the quantification of the FSR and ASR of tTGs using the precursor-product approach. After an overnight fast (~10 h), baseline blood and skeletal muscle samples were obtained. Thereafter, a

	<b><math>\text{U}^{13}\text{C}_{16}</math>-palmitate infusion (IR: 0.06 <math>\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}</math>)</b>			
<b>Time (min)</b>	<b>0</b>	<b>60</b>	<b>120</b>	<b>170</b>
<b>Blood draw</b>	↑	↑	↑	↑
<b>Muscle Biopsy</b>	↑		↑	↑

Fig. 1. The design of the  $[^{13}\text{C}_{16}]$ palmitate infusion study.

continuous infusion of [ $^{13}\text{C}_{16}$ ]palmitate bound to albumin (infusion rate: 0.06  $\mu\text{mol}/\text{kg}/\text{min}$ ; Cambridge Isotope Laboratories, Inc.) was administered for  $\sim 170$  min. Arterialized-venous blood samples were collected from a heated contralateral hand at time points as depicted in Fig. 1. EDTA tubes were used to obtain plasma samples. Skeletal muscle samples were obtained again 90 and 170 min after the start of the [ $^{13}\text{C}_{16}$ ]palmitate infusion.

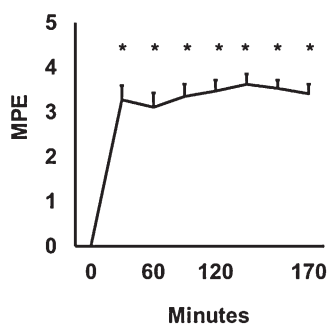
## Muscle biopsies

Muscle biopsy samples were obtained from the lateral portion of the musculus vastus lateralis,  $\sim 10$ – $15$  cm above the knee, using a 5 mm Bergström needle (Stille, Stockholm, Sweden). The procedure was done under aseptic conditions and local anesthesia (1% lidocaine). Any visible fat pieces were removed immediately. A portion of the biopsy sample was immediately frozen in liquid nitrogen and a second portion was quickly rinsed in ice-cold saline, blotted, and then frozen in liquid nitrogen (8, 9). The samples were further stored at  $-80^\circ\text{C}$  for future lipid analyses.

## Sample processing

**Analyses of plasma lipid samples.** Plasma lipids were extracted using a heptane-propanol extraction buffer, and FFAs were separated using TLC plates (Partisil LK5D, Silica Gel 150 Å; Schleicher and Schuell, Maidstone, UK) as previously described (8, 9). After the samples were methyl-esterified, the tracer-to-tracee ratio (TTR) of [ $^{13}\text{C}_{16}$ ]palmitate in plasma FFAs was measured using a gas chromatograph-mass spectrometer (Agilent, Santa Clara, CA) by monitoring the mass-to-charge ratios of 270 and 286 for methyl palmitate. These data allowed us to determine the changes in plasma enrichment of [ $^{13}\text{C}_{16}$ ]palmitate (Fig. 2).

**Muscle lipid analyses.** The lipids were extracted from 30–50 mg of muscle powder overnight at  $4^\circ\text{C}$  in a 1:2 (v/v) methanol:chloroform solution containing 0.05 mg/ml butylated hydroxytoluene. The samples were subjected to a TLC plate to separate muscle FAs and tTGs, as previously described (8, 9). The samples were then methyl-esterified, and the isotopic enrichments ( $^{13}\text{C}/^{12}\text{C}$ ) of TG-palmitates were determined using GC-combustion-isotope ratio MS (Finnigan System; Thermo Fisher Scientific, Waltham, MA). The measured isotopic enrichment of  $^{13}\text{C}$  was multiplied by 17/16 to convert to the enrichment of the [ $^{13}\text{C}_{16}$ ]palmitate because only 16 of the 17 carbons in the palmitate methyl ester molecule are labeled. The background enrichments (i.e., baseline samples) were subtracted from the enrichments of the 90 and 170 min time points to obtain true post-tracer enrichments. The profile of muscle FAs and tTGs was determined using GC flame ionization



**Fig. 2.** The plasma free palmitate enrichment reached plateau 30 min after the start of the tracer infusion and remained significantly higher (\*) than at 0 min throughout the infusion study. The differences in parameters between different time points were analyzed by one-way repeated measure ANOVA with  $P < 0.05$  considered statistically significant.

detection, and the muscle FA and tTG contents were calculated using the internal standard approach, as previously described (8, 9).

About 30–40 mg of muscle sample were used to isolate acylcarnitines (acyl-CNTs) with  $\text{KH}_2\text{PO}_4$  and acetonitrile/methanol (8, 9, 18). The enrichments of [ $^{13}\text{C}_{16}$ ]palmitoylcarnitine (palmitoyl-CNT) and the profile of acyl-CNT species were assessed using a liquid chromatograph-mass spectrometer (6130 Series Quadrupole; Agilent) as previously described (8, 9, 18). The  $\text{d}_3$ -palmitoyl-CNT (Cambridge Isotope Laboratories, Inc.) was added as an internal standard and used to calculate the intramuscular acyl-CNT content.

## Calculations

The FSR of tTGs, expressed in percent per hour, was calculated using the tracer incorporation method (equation 1):

$$\text{FSR} = \frac{\text{Et2-Et1}}{\text{Ep}(\text{t2-t1}) \times (\text{t2-t1})} \quad (\text{Eq. 1})$$

which is based on the precursor-product approach, as described previously (8, 9, 19). Briefly, where (Et2-Et1) is the enrichment increment of TG-bound palmitate from t1 to t2, and Ep(t2-t1) is the precursor enrichment from t1 to t2. The enrichment of  $^{13}\text{C}/^{12}\text{C}$  in tTG-palmitate served as the product. The enrichment of [ $^{13}\text{C}_{16}$ ]palmitoyl-CNT, calculated as the area under the curve during the tracer infusion, served as the precursor. Both enrichments were expressed in mole percent excess (MPE) (20, 21) using the following equation (equation 2):

$$\text{MPE} = \frac{\text{TTR}}{1 + \text{TTR}} \times 100 \quad (\text{Eq. 2})$$

where TTR is the tracer-to-tracee ratio, calculated using the following equation (equation 3):

$$\text{TTR} = (r_{\text{sa}} - r_{\text{bk}}) \times (1 - 0.0111)^n \quad (\text{Eq. 3})$$

where  $r_{\text{sa}}$  and  $r_{\text{bk}}$  are the ratios of  $^{13}\text{C}/^{12}\text{C}$  in the post-tracer infusion (i.e., biopsy 1 and 2) and the baseline (i.e., background) biopsy samples, respectively, and n is the number of labeled carbons in the [ $^{13}\text{C}_{16}$ ]palmitate (20, 21).

The ASR of imTGs, represented as the ASR of tTGs, was calculated by multiplying the tTG FSR by the pool size of tTGs [micromoles per gram of wet tissue (8, 9)].

## Statistical analyses and data presentation

Data are presented as mean  $\pm$  SEM, unless otherwise stated. The differences between the parameters obtained at 90 and 170 min (biopsy 1 and 2, respectively) were assessed using paired *t*-tests. The relationships between the parameters of interest measured at 90 and 170 min were evaluated by linear regression analyses and used to calculate the intra-class correlation coefficient. Statistical analyses were performed using R statistical software (R Core Team, 2017, version 3.3.3);  $P < 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION

Skeletal muscle lipid metabolism plays a significant role in metabolic health. Excessively accumulated lipid metabolites (i.e., acyl-CNTs, diacylglycerol, and ceramides) have been shown to disrupt intramuscular insulin signaling (21–26). On the other hand, excessively accumulated imTGs



have been shown to be protective and associated with insulin sensitivity. imTGs are considered as neutral lipids, a source for energy production, and not affecting intramuscular insulin signaling (6). Therefore, interventions to increase the accumulation of imTGs may be beneficial in preventing the development of metabolic complications; and thus, approaches to quantitate imTG accumulation are also needed.

We studied nine healthy nonobese women (**Table 1**) to test our hypothesis that the ASR of tTGs is a reliable measure of the imTG synthesis rate that is corrected for the emTG-related underestimated FSR. To do so, we measured several lipid parameters in two muscle biopsy samples obtained 80 min apart during a [ $^{13}\text{C}_{16}$ ]palmitate infusion study (Fig. 1). There were no significant differences in muscle acyl-CNT, FA, and tTG contents, or in the tTG FSR or ASR between biopsies 1 and 2 (**Table 2**), while the enrichment of the precursor (i.e., [ $^{13}\text{C}_{16}$ ]palmitoyl-CNT) was significantly higher in biopsy 2 (biopsy 1 vs. biopsy 2:  $0.707 \pm 0.067$  MPE vs.  $1.141 \pm 0.062$  MPE,  $P < 0.001$ ). The linear regression analyses demonstrated a statistically significant correlation between the measurements of muscle acyl-CNT content (**Fig. 3A**) and the [ $^{13}\text{C}_{16}$ ]palmitoyl-CNT enrichment (**Fig. 3B**). In contrast, although the enrichment of [ $^{13}\text{C}_{16}$ ]palmitate in tTGs was 32% higher in biopsy 2, the difference was not statistically significant (biopsy 1 vs. biopsy 2:  $0.062 \pm 0.021$  MPE vs.  $0.083 \pm 0.019$  MPE,  $P = 0.540$ ). Moreover, intra-individual variability in the enrichment of [ $^{13}\text{C}_{16}$ ]palmitate in tTGs was observed (**Fig. 3C**), which can be explained by noteworthy intra-individual variation in the tTG content between the time points (**Fig. 3D**). These data support the previous reports that the emTG pool is not uniformly distributed in muscles (12, 15–17) and can skew the measurements of imTG parameters.

The above-mentioned variabilities in enrichment of [ $^{13}\text{C}_{16}$ ]palmitate resulted in variability of FSRs between the time points (**Fig. 3E**). This is a commonly observed result (11, 12). Thus, we calculated the ASR of tTGs, which was not different between the time points (**Table 2**). Linear regression analysis demonstrated that the two sets of ASRs of tTGs measured 80 min apart tended to significantly correlate ( $r = 0.639$ ,  $P = 0.064$ ). Sample size analysis indicated that 17 subjects are needed to achieve statistically significant correlation. However, our analysis demonstrated that the data set of subject 5 (**Table 3**) was an outlier (white circle, **Fig. 3F**). When this data set was excluded from the

analysis, a significant correlation was observed between the two sets of ASR values ( $P < 0.001$ , **Fig. 3F**) with the intercept of 0.0761 and with a 95% confidence interval of  $-0.2653$  (lower 95%) and  $0.2538$  (higher 95%), which includes zero point. The slope was 0.990 with a 95% confidence interval of 0.6532 (lower 95%) and 1.3251 (higher 95%), which includes one. The significant association between the measurements with slope and intercept ranges (95th percentile) including one and zero, respectively, demonstrates the lack of over- or underestimation of one measurement over another. The intra-class correlation coefficient was 0.978, which is indicative of an excellent intra-class (i.e., intra-subject) reliability. These data demonstrate that the values for tTG ASR from two samples taken from the same subject, but 80 min apart, were highly correlated (**Fig. 3F**) despite highly variable values for FSR (**Fig. 3E**), suggesting that, when the muscle tTG pool is used to calculate ASR, the emTG portion of the tTG pool corrects the tracer dilution caused by the same emTGs.

For the regression analysis of the tTG ASR, we used the data from eight subjects only, as shown in **Fig. 3F**, and the measurements of tTG ASR were not strongly correlated for subject 5 (**Table 3**). This discrepancy may indicate a problem with the measurement of muscle TG parameters because the measurements of acyl-CNT content and [ $^{13}\text{C}_{16}$ ]palmitoyl-CNT enrichments were significantly correlated between the time points, and these regression analyses included all nine data sets (**Fig. 3A, B**). The discrepancies in the tTG ASR values of subject 5 may be explained by the contamination of the biopsy sample analyses with unlabeled palmitate from the environment, because it is well-established that unlabeled palmitate and stearate are universal contaminants of glassware, solvents, and reagents (27). Therefore, it may be more convenient to use [ $^{13}\text{C}_{18}$ ]oleate tracer to measure muscle TG synthesis. Larger studies need to be conducted to determine whether this is an isolated incident or whether certain factors can cause these types of discrepancies.

Our data suggest that the stable isotope tracer approach can provide a valid measure of the imTG synthesis rate represented by the tTG ASR. However, our data do not provide insight into the differences in the physiology of the imTG and emTG pools, except that the rate of incorporation of tracer label into these pools is potentially different. The existence of these two pools is well-established; however, their individual significance in skeletal muscle physiology is still not clear. Studies utilizing MRS demonstrated that different physiological states or interventions can affect the distribution, and perhaps the physiology, of imTG and emTG pools (28–30). Training resulted in a significant increase in imTG content with no change in emTG content, while detraining resulted in a return of imTG content to the baseline with increased emTG content (29). Rosiglitazone therapy decreased the imTG/emTG ratio by increasing the emTG pool size (30). One way to determine the differences in imTG and emTG metabolism would be to conduct metabolic studies utilizing both the stable isotope tracer approach and the MRS measurements of the imTG and emTG pools before and after interventions known to affect the distribution of these pools.

TABLE 1. Characteristics of study subjects

Parameters	Values
Age, years	$47 \pm 18$
DEXA body fat, kg	$28 \pm 5$
DEXA body lean mass, kg	$41 \pm 4$
Body mass index, $\text{kg}/\text{m}^2$	$25.7 \pm 1.2$
Plasma TGs, $\text{mg}/\text{dl}$	$98 \pm 91$
Plasma LDL, $\text{mg}/\text{dl}$	$99 \pm 30$
Plasma cholesterol, $\text{mg}/\text{dl}$	$169 \pm 39$
Plasma HDL, $\text{mg}/\text{dl}$	$51 \pm 8$
Fasting serum glucose, $\text{mg}/\text{dl}$	$78 \pm 7$
Fasting serum insulin, $\text{mU}/\text{dl}$	$5 \pm 3$

Data are presented as mean  $\pm$  SD (n = 9).

TABLE 2. The average values of muscle parameters

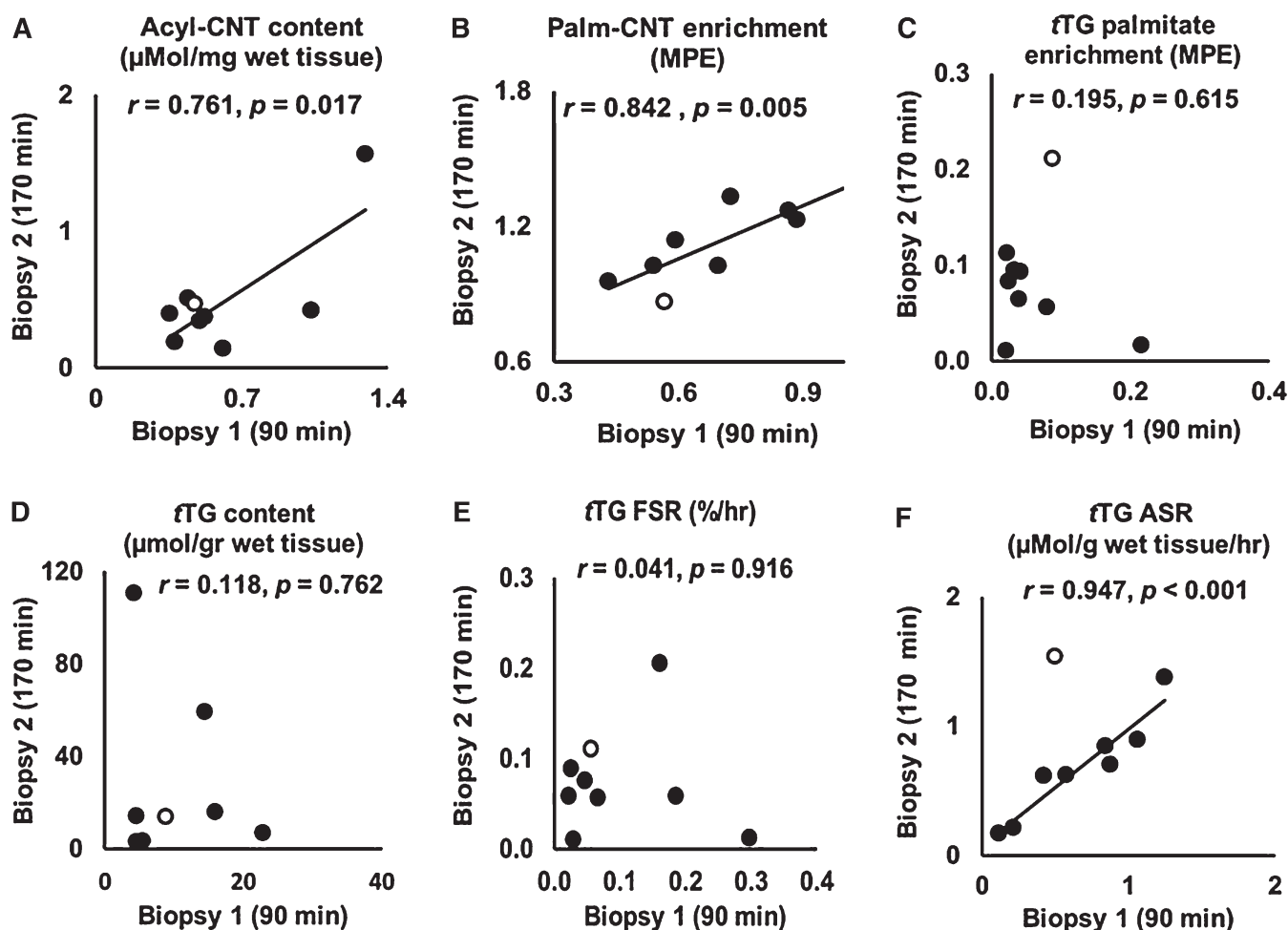
Parameters	Biopsy 1 (90 min)	Biopsy 2 (170 min)	<i>P</i>
Acyl-CNT, $\mu\text{mol}/\text{mg}$ wet tissue	$0.622 \pm 0.107$	$0.491 \pm 0.140$	0.1872
FAs, $\mu\text{mol}/\text{g}$ wet tissue	$0.197 \pm 0.028$	$0.239 \pm 0.035$	0.1055
tTGs, $\mu\text{mol}/\text{g}$ wet tissue	$9.53 \pm 2.22$	$25.63 \pm 12.14$	0.2371
tTG FSR, $\%/h$	$0.099 \pm 0.032$	$0.075 \pm 0.019$	0.5484
tTG ASR, $\mu\text{mol}/\text{g}$ wet tissue/h	$0.649 \pm 0.127$	$0.777 \pm 0.154$	0.3246

Data are presented as mean  $\pm$  SE ( $n = 9$ ). The comparison of values between the time points was assessed by one-way ANOVA with repeated measures (i.e., time);  $P < 0.05$  was considered statistically significant.

The emTGs are considered as intra-muscular adipocytes; thus, if the synthesis rate of TGs in the emTG pool resembles that of adipose tissue, the actual values of the emTG's synthesis rate should be much lower than those for imTGs (31). Thus, a 170 min-long infusion of  $U^{13}C_{16}$ -palmitate should not result in significant incorporation of tracer into the TGs of emTGs. If this is the case, the emTG pool only dilutes the tracer enrichment of the product (i.e., imTGs); and thus, accounting for tTG content should correct for this dilution. Therefore, measuring the imTG ASR using

muscle tTG pool size should be a valid measure of the imTG synthesis rate.

Metabolic studies with an infusion of stable isotope-labeled FAs (e.g.,  $[^{13}C_{16}]$ palmitate) to measure the systemic lipid kinetics are common (8, 9, 20, 32, 33). Our data suggest that obtaining two muscle biopsy samples (90 and 170 min after the start of the infusion) can provide reliable information on skeletal muscle lipid metabolism. The enrichment of plasma TGs prior to the start of the infusion of a labeled FA tracer can be used as a background imTG enrichment.



**Fig. 3.** Linear regression analyses between the parameters measured in biopsy 1 (90 min, x-axis) and biopsy 2 (170 min, y-axis). The muscle contents of acyl-CNT (A) and the enrichment palmitoyl-CNT (Palm-CNT) (B), measured in biopsies 1 and 2 were significantly correlated. No significant correlations were observed in the measurements of palmitate enrichment (C), tTG content (D), and the FSR (E). The measurements of the tTG ASR were significantly correlated (F). One set of data (subject 5, Table 2) was not in accord with the other eight data sets in the analyses of the tTG ASR (F), and was therefore excluded from ASR analyses (denoted as an open circle). The other data from this subject were included in all corresponding regression analyses; however, the data points are denoted as an open circle in all figures representing the regression analyses (A–E).  $P < 0.05$  was considered statistically significant.


TABLE 3. Individual values for intramuscular enrichments and pool sizes of lipids and the tTG FSR and ASR

Subject ID	Biopsy 1 (90 min)					Biopsy 2 (170 min)				
	Enrichment (MPE)		tTG FSR (%/h)	tTG Pool Size	tTG ASR	Enrichment (MPE)		tTG FSR (%/h)	tTG Pool Size	tTG ASR
	tTG-palm	[ <sup>13</sup> C <sub>16</sub> ]palm-CNT				tTG-palm	[ <sup>13</sup> C <sub>16</sub> ]palm-CNT			
1	0.0394	0.5925	0.0665	15.96	1.0612	0.0641	1.1380	0.0564	15.86	0.8936
2	0.0413	0.8845	0.0467	4.57	0.2135	0.0935	1.2299	0.0760	2.86	0.2174
3	0.0240	1.0665	0.0225	5.09	0.1146	0.0832	1.4264	0.0584	2.95	0.1721
4	0.0873	0.5406	0.1616	5.41	0.8737	0.2110	1.0266	0.2056	3.41	0.7014
5	0.0318	0.5664	0.0562	8.82	0.4954	0.0950	0.8643	0.1100	14.03	1.5423
6	0.0796	0.4300	0.1851	4.56	0.8439	0.0562	0.9555	0.0588	14.36	0.8441
7	0.0203	0.6955	0.0292	14.42	0.4215	0.0106	1.0247	0.0103	59.40	0.6144
8	0.0218	0.8647	0.0252	22.76	0.5734	0.1129	1.2702	0.0889	7.03	0.6245
9	0.2163	0.7268	0.2977	4.18	1.2436	0.0166	1.3320	0.0125	110.74	1.3814

The imTG pool size is expressed in micromoles per gram of wet tissue and ASR is expressed in micromoles per gram of wet tissue per hour. ID, identification; [<sup>13</sup>C<sub>16</sub>]palm-CNT, [<sup>13</sup>C<sub>16</sub>]palmitoyl-CNT; tTG-palm, total triglyceride palmitate.

Additionally, as mentioned above, the measurements of muscle acyl-CNT content (Fig. 3E) and [<sup>13</sup>C<sub>16</sub>]palmitoyl-CNT enrichment (Fig. 3F) correlated significantly between the two time points. These data demonstrate that the emTG pool may not affect the muscle acyl-CNT parameters; however, careful consideration should be given to the timing of sample collection because the incorporation of label into palmitoyl-CNT continued to increase throughout 170 min of tracer infusion (Table 3, Fig. 3A). Moreover, because acyl-CNT metabolism may represent a segment of muscle lipid dynamics, its evaluation may help in the understanding of the kinetics of muscle lipid metabolism.

The potential limitations of this study are: 1) a small group of nonobese women under fasting conditions were studied, so other populations under different conditions (e.g., hyperinsulinemic-euglycemic clamp) must be studied; 2) we did not assess the imTG breakdown rate, which potentially can be calculated using a decay curve and an additional muscle biopsy sample; and 3) this approach does not exactly distinguish the emTG pool within the tTG pool, which would have been an ideal situation. However, the fact that, from all the measurements (Fig. 3C–F) that can be affected by the contribution of emTGs into the tTG pool, only the measurement of the ASR (Fig. 3F) was significantly correlated, suggests that accounting for the tTG pool size can correct the emTG-related variability in outcome measures.

In conclusion, our data demonstrate that the potential dilution of imTG tracer enrichment in the calculation of the imTG synthesis rate (i.e., FSR) can be corrected for varying contributions of emTGs to the observed synthetic rates if the emTG content is taken into account by calculating the tTG ASR. This finding suggests that imTG metabolism can be reliably quantified using the labeled FA infusion (e.g., palmitate or oleate tracers) approach. Future studies to determine the kinetics of imTG metabolism in association with the distribution of the imTG and emTG pools are warranted. These studies should help us better understand the physiological and metabolic significance of these two pools. 

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