



2015 and December 2016. Before the commencement of the study, lower-limb maximal dynamic strength (leg-press 1RM), *vastus lateralis* cross-sectional area (mCSA), and dietary intake (3-d 24-h dietary recall) were assessed. Afterward, participants were randomly allocated to one of the two experimental groups (free leucine supplementation [LEU,  $n = 12$ ] or placebo [PLA,  $n = 13$ ]). Dependent variables were assessed before and after a 12-wk lower-limb resistance training and supplementation intervention.

**Participants.** Participants were considered eligible according to the following criteria: 1) healthy young men age between 18 and 40 yr; 2) absence of chronic diseases and/or musculoskeletal conditions precluding physical exercise; 3) no previous or current use of any androgenic anabolic steroids; 4) previous strength training experience ( $\geq 12$  months); 5) leg-press 1RM  $\geq 3.5$  and  $\leq 5.0$  times their body mass (to ensure a homogenous sample of resistance-trained participants); 6) not using creatine, beta-alanine, protein or amino acid supplements before the study; 7) not on a restrictive diet; and 8) regularly consuming at least  $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$  of protein (assessed by means of a 3-d 24-h food recall). All participants were informed of the risks and benefits of the study before signing the consent form. This study was approved by the Ethical Review Board from the State University of Campinas (35521314.0.0000.5404), and it was registered at <https://clinicaltrials.gov> as NCT02744443.

**Diet and supplements.** A 24-h diet recall was conducted on three separate days (two weekdays and one weekend day) before and after the intervention period by an experienced researcher. We assessed total and relative protein intake ( $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ ), per meal protein and total and per meal leucine intake. A trained nutritionist conducted all procedures, and data were analyzed by the same trained professional using a specific software (Nutritionist Pro® v.7.3; Axxya Systems, Woodinville, WA, USA). Participants were repeatedly encouraged to maintain their habitual diet throughout the experimental period.

Supplementation with leucine/placebo was continuous for the duration of the study. All participants were instructed to take  $10 \text{ g}\cdot\text{d}^{-1}$  of encapsulated free leucine (Ajinomoto®, Tokyo, Japan) or placebo (alanine [Ajinomoto®]) divided into two

5-g doses, one in the morning ( $\sim 8:00$  AM) and the other immediately after training ( $\sim 6:00$  PM). Participants were instructed to maintain a supplementation log (recording date and time of day of each dose taken) to monitor adherence to the supplementation protocol. Supplement packages were coded so that neither the investigators nor the participants were aware of the contents until completion of the analysis.

**Lower-limb 1RM.** Lower-limb 1RM was performed on a  $45^\circ$  incline leg-press machine (Movement Technology®; Bruden, São Paulo, Brazil) and followed previously described recommendations (24). Briefly, 1RM testing consisted of a 5-min general warm-up on a treadmill at  $9 \text{ km}\cdot\text{h}^{-1}$ . This was followed by a specific warm-up on the  $45^\circ$  leg press, which consisted of one set of eight repetitions at 50% of the estimated 1RM, followed by one set of three repetitions at 70% of the estimated 1RM, with a 3-min rest between sets. Participants then had up to five attempts to achieve their 1RM. A 3-min rest period was allowed between attempts. Verbal encouragement was given throughout the tests. Importantly, two familiarization sessions were performed before testing. The 1RM tests were performed on two separate occasions; if values varied by more than 5% between tests 1 and 2, an additional test was performed after a 72-h interval.

**Muscle CSA.** A portable B-mode ultrasound (US) device (SonoAce R3; Samsung-Medison, Gangwon-do, South Korea) using a 7.5-MHz linear-array probe was applied to obtain axial plane images of the *vastus lateralis*. An experienced researcher identified and marked the point corresponding to 50% of the distance between the greater trochanter and lateral epicondyle of the femur. The skin was transversally marked every 2 cm from the reference point toward the medial and lateral aspects of the thigh to orient the placement of the US probe. Sequential US images were acquired by aligning the edge of the probe with each 2-cm mark in the skin. Conductive gel was used during the test to avoid pressure on the muscle tissue.

During the test, all participants had their legs strapped with a Velcro band to avoid any hip rotation. After data collection, images were reconstructed following the procedures described by Reeves et al. (25). Subsequently, mCSA was measured

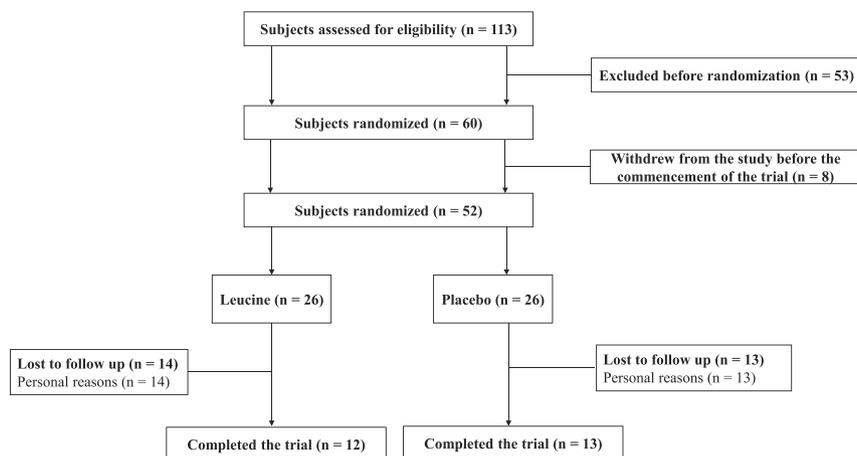


FIGURE 1—Flowchart of subjects.

using ImageJ (NIH, USA). The same researcher, blinded to the intervention, was responsible for all US measures, and the reliability of this procedure has been previously determined and was reported to have a typical error of 0.36 cm<sup>2</sup> and 1.7% coefficient of variation (26). All participants refrained from lower-limb physical training for at least 72 h before US assessment.

**Resistance training protocol.** All participants undertook a supervised, 12-wk, resistance training program comprising two sessions per week. Both incline (45°) leg-press and leg-extension exercises were performed throughout the training program. From weeks 1 to 4, participants were instructed to perform three sets of 12 to 15 repetition maximum (RM) of each exercise, and loads were adjusted accordingly. Training loads increased progressively every 4 wk (weeks 5 to 8: 4 sets of 10–12 RM; and weeks 8 to 12: 5 sets of 8–10 RM). All sessions were performed at the University training center under full supervision of a research team member blinded to the intervention. Subjects maintained their habitual upper-body resistance training routine throughout the intervention on separate nonsupervised days.

**Statistical analysis.** Sample size was calculated using *vastus lateralis* mCSA as the primary study outcome. Analyses were run using G\*Power (3.1.9.2) performing a two-way ANOVA with repeated measures (within-between interaction) considering a medium effect size (ES) ( $f = 0.25$ ) and setting power to 80% ( $\beta = 0.2$ ) with  $\alpha = 0.05$ , which yielded an estimate of  $n = 12$  per group.

Data are presented as means and standard deviation (SD). Data normality was assessed using the Shapiro–Wilk test. Participants' baseline characteristics were tested with independent *t*-tests. A mixed model for repeated measures (group and time as fixed factors and subjects as the random factor) was performed for each dependent variable (i.e., leg-press 1RM and mCSA). Additionally, possible between-group differences in protein consumption in each meal (breakfast, lunch, and dinner) across time were tested using a mixed-model for repeated measures. Whenever a significant *F* value was obtained, a Tukey's *post hoc* test was performed. Potential between-group differences in relative changes (delta) were tested by independent *t*-tests. Fischer's exact test was used to calculate the percentage of participants that could successfully identify group allocation. Effect size and 95% confidence interval (CI) of the difference between groups (Hedges' *g*) was determined for leg-press 1RM and mCSA. An  $ES \leq 0.49$  was considered as "small," between 0.50 and 0.79 as "medium," and  $\geq 0.80$  as "large" (27).

TABLE 1. Baseline characteristics.

	LEU ( $n = 12$ )	PLA ( $n = 13$ )	<i>P</i>
Age, yr	26 ± 4	27 ± 5	0.62
Body mass, kg	76.7 ± 12	79.9 ± 11.5	0.50
Height, cm	177 ± 6	178 ± 8	0.70
BMI, kg·m <sup>-2</sup>	24.4 ± 3.3	25.1 ± 2.9	0.57
Leg-press 1RM, kg	333 ± 69	378 ± 60	0.14
Leg-press 1RM, kg·bw <sup>-1</sup>	4.4 ± 0.6	4.8 ± 0.8	0.19
Protein intake, g·kg <sup>-1</sup> ·d <sup>-1</sup>	1.72 ± 0.43	1.81 ± 0.47	0.62

Values are expressed as mean ± SD.  
bw, body weight.

TABLE 2. Dietary intake.

	PRE		POST		<i>P</i>
	LEU	PLA	LEU	PLA	
Energy, kcal	2562 ± 455	2438 ± 481	2846 ± 768	2344 ± 552	0.11
Protein, g	129 ± 26	142 ± 33	136 ± 30	143 ± 32	0.60
Protein, g·kg <sup>-1</sup> ·d <sup>-1</sup>	1.72 ± 0.43	1.81 ± 0.47	1.76 ± 0.36	1.81 ± 0.42	0.77
Leucine, g	10 ± 2	11 ± 3	11 ± 2	11 ± 3	0.42
Carbohydrates, g	330 ± 82	279 ± 101	341 ± 123	255 ± 83	0.41
Fat, g	82 ± 18	85 ± 28	106 ± 37	84 ± 27	0.06

*P* value for group–time interaction. Values are expressed as mean ± SD.

SAS 9.3® software (Institute Inc., Cary, NC) was used to perform the statistical analysis. Significance was set at  $P < 0.05$ .

## RESULTS

**Participants.** One hundred and thirteen men answered the study call. Fifty-two were considered eligible according to inclusion and exclusion criteria and were randomized into either LEU ( $n = 26$ ) or PLA ( $n = 26$ ). Twelve (LEU) and 13 (PLA) subjects completed the trial and had their data analyzed (Fig. 1).

Baseline participant characteristics are shown in Table 1. There were no significant differences between groups for any variables ( $P > 0.05$ ). Self-reported supplementation adherence for LEU and PLA was  $97.1\% \pm 5.4\%$  and  $98.7\% \pm 2.0\%$  ( $P = 0.37$ ), respectively. Blinding assessment revealed that nine subjects in the LEU and six subjects in the PLA group correctly guessed their group allocation ( $P > 0.05$ ).

**Dietary intake.** Table 2 describes dietary intake. There were no within- or between-group changes in habitual total protein or leucine (leucine supplementation not included) intake after the 12-wk intervention period ( $P = 0.38$  for group–time interaction). Habitual per meal protein and leucine (leucine supplementation not included) intakes (Table 3) at the main meals (breakfast, lunch, and dinner) were also similar between the two groups across all time points (all  $P > 0.05$ ).

**Lower-limb 1RM.** Leg-press 1RM increased from PRE to POST in both groups (main effect of time;  $P < 0.0001$ ), but no differences were shown between groups (group by time interaction:  $P = 0.31$ ). Moreover, relative changes in leg-press 1RM were also similar between groups (LEU,  $19.0\% \pm 9.4\%$ ;

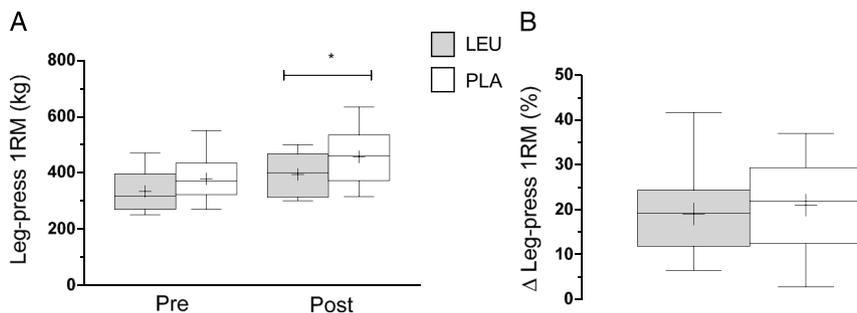
TABLE 3. Per meal protein and leucine intake.

	Per Meal Protein Intake				
	PRE		POST		<i>P</i>
	LEU	PLA	LEU	PLA	
Breakfast, g	19 ± 5	27 ± 12	19 ± 7	23 ± 10	0.23
Breakfast, g·kg <sup>-1</sup> ·d <sup>-1</sup>	0.24 ± 0.10	0.34 ± 0.17	0.25 ± 0.10	0.29 ± 0.10	0.18
Lunch, g	52 ± 13	52 ± 12	58 ± 18	61 ± 19	0.58
Lunch, g·kg <sup>-1</sup> ·d <sup>-1</sup>	0.70 ± 0.20	0.66 ± 0.15	0.75 ± 0.24	0.77 ± 0.22	0.50
Dinner, g	38 ± 12	43 ± 16	39 ± 14	36 ± 17	0.38
Dinner, g·kg <sup>-1</sup> ·d <sup>-1</sup>	0.50 ± 0.15	0.54 ± 0.19	0.51 ± 0.18	0.46 ± 0.22	0.43

	Per Meal Leucine Intake				
	PRE		POST		<i>P</i>
	LEU	PLA	LEU	PLA	
Breakfast, g	1 ± 0	2 ± 1	2 ± 1	2 ± 1	0.17
Lunch, g	4 ± 1	4 ± 1	5 ± 2	5 ± 1	0.99
Dinner, g	3 ± 1	3 ± 1	3 ± 1	3 ± 1	0.31

Estimated leucine intake values were rounded to whole numbers for precision purposes.  
*P* value for group–time interaction. Values are expressed as mean ± SD.



**FIGURE 2**—Panel A: Lower-limb 1RM in LEU ( $n = 12$ ) and PLA ( $n = 13$ ) groups before (PRE) and after (POST) the 12-wk resistance-training program. Panel B: Relative changes (%) in lower-limb 1RM for both groups. \* indicates  $P < 0.05$  significantly different from PRE (main effect of time). Values are expressed as mean  $\pm$  SD.

and PLA,  $21.0\% \pm 10.4\%$ ,  $P = 0.62$ ; Fig. 2). Finally, between-group ES was small (ES, 0.2; 95% CI,  $-0.6$  to 1.0).

**Muscle CSA.** Significant PRE to POST changes were detected in mCSA for both groups (main effect of time,  $P < 0.0001$ ). No group–time interaction was found for mCSA ( $P = 0.77$ ). In addition, there was no between-group difference in relative changes to mCSA (LEU,  $8.0\% \pm 5.6\%$ ; PLA,  $8.4\% \pm 5.1\%$ ;  $P = 0.85$ ; Fig. 3). Finally, between-group ES was small (ES = 0.1; 95% CI:  $-0.6$  to 0.9).

## DISCUSSION

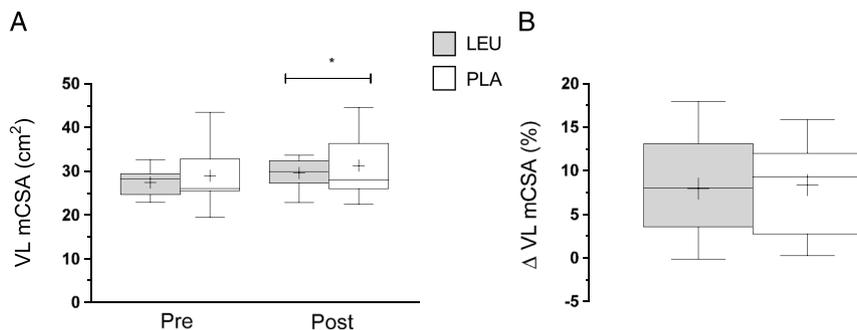
We investigated the effects of twice-a-day high-dose, leucine supplementation on muscle adaptations to resistance training in previously trained individuals. Our findings showed that leucine supplementation was ineffective in augmenting strength and/or hypertrophy after twice weekly resistance training during a 12-wk period.

Leucine has been shown effective at stimulating muscle anabolism via activation of mammalian target of rapamycin complex 1, a central regulator of cellular protein synthesis and mRNA translation (8,28). Indeed, a number of *in vitro* (10,29,30) and *in vivo* (31–33) studies confirm the ability of leucine to stimulate protein synthesis (for a more comprehensive review on the anabolic properties of leucine, please refer to Leenders and van Loon (34)). Collectively, these findings gave rise to a hypothetical application of leucine as a dietary supplement to support muscle anabolism.

Despite consistent evidence supporting the acute effects of leucine on MPS stimulation, chronic studies have failed to show a beneficial effect of leucine supplementation on muscle mass in either healthy (20,21) or in elderly individuals with type-2 diabetes (19). In acute studies, leucine has also been shown to be ineffective as an anabolic supplement when combined with other amino acids or carbohydrate (35). More recently, Aguiar et al. (22) showed similar gains in muscle mass and strength in untrained young participants after either leucine ( $3 \text{ g}\cdot\text{d}^{-1}$ ) or placebo supplementation accompanied by resistance training.

To the best of our knowledge, this is the first study to examine the effects of multiple daily doses of leucine in young resistance trained individuals consuming an amount of protein ( $\geq 1.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ ) that should, theoretically, provide a maximal anabolic stimulus (23). In accordance with previous findings (22), we observed increases in muscle mass and strength resulting from resistance training, with no additional effect of leucine supplementation. These data challenge the notion that leucine may play an additive role in supporting anabolism, at least when dietary protein intake is optimal.

One may speculate that the timing of supplementation may impact the results. Importantly, supplemental leucine was offered in the lower protein-containing meals of the day, thus mitigating timing-related issues. Furthermore, to significantly stimulate MPS, all essential amino acids (EAA) are required (36,37), thus, at least theoretically, limiting the effect of between-meal supplementation. The relationship between the concentration



**FIGURE 3**—Panel A: *Vastus lateralis* cross-sectional area (mCSA) in LEU ( $n = 12$ ) and PLA ( $n = 13$ ) groups before (PRE) and after (POST) the 12-wk resistance-training program. Panel B: Relative changes (%) in *vastus lateralis* cross-sectional area (mCSA) for both groups. \*  $P < 0.05$  significantly different from PRE (main effect of time). Values are expressed as mean  $\pm$  SD.

of circulating EAA (particularly leucine) and MPS assumes a dose-dependent, but saturable fashion (despite persistent amino acid availability) (38,39), suggesting an upper limit of amino acid usage for MPS before excess is diverted toward deamination, urea synthesis, and amino acid oxidation (as described by the muscle full hypothesis) (38–41). In this respect, it seems reasonable to speculate that any possible anabolic effect of supplementary leucine may be overridden by the availability of sufficient protein required to maximally stimulate MPS. In fact, 5 g of leucine may be “easily” obtained by the ingestion of approximately 200 g (~7 oz.) of red lean meat or 50 g of whey protein isolate.

Albeit further research is needed, it was recently suggested that the MPS response to protein and exercise may be potentially influenced by the amount of muscle mass involved in exercise, whereby a greater muscle mass utilized would require a higher amount of exogenous amino acids to optimize the anabolic response (42). In the present study, upper and lower-limb exercises were performed on separate days. Therefore, despite the high per-meal protein intake observed in our study (i.e.  $>0.24 \text{ g}\cdot\text{kg}^{-1}$ ), it is unknown whether our data can be extrapolated to a whole-body (i.e.; lower- and upper-limb exercises in the same training session) resistance training condition.

Despite a null outcome, the present study adds to, and extends, our current knowledge in one of the most targeted consumers of “muscle-building” supplements: recreationally trained individuals consuming high protein diets. An additional consideration should be made in respect to between-subject response variability. Notwithstanding previous demonstration of greater effectiveness of protein supplementation in those with

a lower starting muscle mass and/or strength (43), this does not seem to be the case in our study. The ANCOVA analysis using both baseline and strength values as covariates did not influence data interpretation (data not shown). Moreover, the number of nonresponders (gains below coefficient of variation) was similar between the two groups (LEU:  $n = 2$  and PLA:  $n = 1$ ).

Notably, our results cannot be extrapolated to individuals with a suboptimal protein intake; however, we speculate that in the absence of adequate dietary EAA, and given their inability to be endogenously produced, the EAA necessary to support muscle building could be obtained via muscle protein breakdown. In this theoretical scenario where additional leucine intake is not paralleled by adequate dietary intake of all other EAA, no additive gains in muscle hypertrophy and strength would be expected. Evidence to support this hypothesis is not currently available. More importantly, further studies with clinically compromised patients, for whom leucine supplementation may play a larger role in mitigating muscle mass loss (e.g.; sarcopenic elderly and bed-rested or immobilized individuals), are warranted.

In conclusion, leucine supplementation did not enhance muscle strength and mass accrual in resistance trained, young men consuming an optimal protein intake.

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The authors have no conflict of interest to declare. The results of the present study do not constitute endorsement by ACSM. Finally, all authors herein declare that all the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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