Arterio-venous balance studies of skeletal muscle fatty acid metabolism: what can we believe?

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Guo Z, Jensen MD. Arterio-venous balance studies of skeletal muscle fatty acid metabolism: what can we believe? Am J Physiol Endocrinol Metab 305: E925–E930, 2013. First published August 13, 2013; doi:10.1152/ajpendo.00346.2013.—The arterio-venous balance (A-V balance/difference) technique has been used by a number of groups, including ours, to study skeletal muscle fatty acid metabolism. Several lines of evidence indicate that, like glycogen, intramyocellular triglycerides (imcTG) are an energy source for local use. As such, the report that increased release of free fatty acids (FFA) via lipolysis from skeletal muscle, but not from adipose tissue, is responsible for the increased systemic lipolysis during IL-6 infusion in healthy humans is somewhat unexpected (26). It appears that given the complex anatomy of human limbs, as to be discussed in this review, it is virtually impossible to determine whether any fatty acids being released into the venous circulation of an arm or leg derive from the lipolysis of intermuscular fat residing between muscle groups, intramuscular fat residing within muscle groups (between epimysium and perimysium, or bundles), or the intramyocellular triglyceride droplets (imcTG). In many cases, it may even be difficult to be confident that there is no contribution of FFA from subcutaneous adipose tissue. This question is fundamentally important as one attempts to interpret the results of skeletal muscle fatty acid metabolism studies using the A-V balance technique. In this Perspectives article, we examine the reported results of fatty acid kinetics obtained using the techniques to evaluate the degree of and how to minimize contamination when attempting to sample skeletal muscle-specific fatty acids.

arterio-venous difference; arterio-venous balance; leg; forearm; skeletal muscle; adipose; fatty acids

Terminology and Expressions

FOR THE STUDY OF MUSCLE FATTY ACID METABOLISM, the leg arterio-venous (A-V) balance technique is commonly employed (17, 19, 20, 22, 26). It employs flexible catheters to collect blood samples from the femoral artery and femoral vein for the purpose of measuring the concentration of fatty acids. This information allows one to determine the net uptake or net release of fatty acids (mmol/l) based on the A-V concentration difference. If the difference is positive, it is a net uptake. If it is negative, it is a net release. Often, plasma or blood flow rate is determined simultaneously to calculate the rates of net uptake and net release of fatty acids across the leg (mmol/min). These parameters only provide one-way information, either release or uptake, but not both. To measure both directions, free fatty acid (FFA) tracers are required. By combining plasma flow, fatty acid concentrations, and the isotopic enrichment (with stable isotope) or specific activity (with radioactive isotope), the unidirectional uptake as well as the unidirectional release of fatty acids can be quantified (13, 25). The term release and uptake with or without a preceding adjective “net” conveys quite different kinetic information. Thus, it is necessary to emphasize that “net release” simply means a positive A-V concentration difference or that the unidirectional release is greater than the unidirectional uptake. In contrast, release or uptake alone refers to the unidirectional processes. In this Perspectives article, the values of unidirectional release or uptake implicitly or explicitly mean that they are derived from tracer studies. Net release either is calculated from the A-V concentration difference without the use of tracers or equals the unidirectional release subtracted the unidirectional uptake.

For A-V balance experiments, the positioning of the arterial catheter does not cause variability in the result because systemic arterial blood is homogeneous in terms of metabolite concentration. In contrast, venous blood varies in metabolite concentration and composition as a function of anatomic location. Femoral vein receives blood from its tributaries along its way to the saphenous femoral junction (SFJ) and beyond. Therefore, femoral venous catheter tip positioned at different levels will give different results of fatty acid release. The issues of femoral venous catheter placement are focused in the discussions below.

Four Pools of Triglycerides in the Leg

Before discussing the implications of venous catheter placement, we provide a brief introduction to the sources of triglycerides in the leg. Most investigators interested in skeletal muscle lipid metabolism focus on the intramyocellular triglyceride (imcTG) pool because of its association with insulin resistance (23). It is a small triglyceride-fatty acid pool (9). We believe that existing data indicate that the imcTG pool is used almost exclusively to provide fatty acids to muscle mitochondria and does not provide for fuel export for the following reasons. In nonexercising humans, imcTG turnover is slow, and therefore, it releases limited amount of fatty acids. These fatty acids are oxidized mostly locally, as skeletal muscle derives ~80% of its energy from fatty acids in the postabsorptive state (4). Also, imcTGs appear to provide the precursor fatty acids for long-chain acylcarnitine synthesis (15), the obligate precursors for mitochondrial long-chain fatty acid oxidation. The imcTG-derived fatty acids may also undergo intracellular reesterification as skeletal muscle takes up glucose and glycerol from the plasma to synthesize imcTG (8). Studies support this assessment. In incubated rat muscle, there is no loss of palmitate labels from the prelabeled imcTG pool (6). In human skeletal muscle, intramyocellular glycerol mobilized from imcTG hydrolysis can be exported, but fatty acids are retained for intracellular metabolism (7). Nonetheless, a more objective estimation of the extent of imcTG-fatty acid released into the venous drainage is to perhaps use a range; i.e., either...
none or all of the FFA efflux from “muscle” is from imcTG as measured using microdialysis technique (below).

In contrast, adipose tissue behaves in the opposite manner. In the postabsorptive state, it releases fatty acids for uptake by other tissues. The other three, larger pools of triglycerides in the leg are thus largely, if not exclusively, responsible for leg FFA release. These pools include the subcutaneous fat, the intermuscular fat, and the intramuscular fat (3). Subcutaneous fat is the predominant site of fatty acid storage in leg, especially in women. Intermuscular fat is typically smaller by comparison but can be quite large in the obese or elderly (Fig. 1). The intramuscular fat is the smallest extramyocellular triglyceride pool. It is also known as extramyocellular lipids detected at 1.5 ppm by NMR proton spectroscopy (21). imcTG is detected at 1.3 ppm simultaneously and termed as intramyocellular lipids. However, the accuracy of these terms is debatable (27). The subcutaneous fat, residing outside the deep fascia, is drained by separate veins (the great saphenous vein and its tributaries) and thus can be in theory excluded from the gross skeletal muscle compartment by careful catheter placement (see below). By comparison, the intermuscular fat resides inside the deep fascia and is mingled with the skeletal muscle groups. As such, it is drained by the same veins as the skeletal muscle. Therefore, the inter- and intramuscular fat are almost unavoidable potential sources of adipose venous drainage “contamination” with respect to fatty acid release via lipolysis. Figure 1 depicts the anatomic relationship among the muscle groups and the fat pools in the thigh.

The Femoral Vein and its Tributaries

There are several major veins at or near the usual femoral venous catheterization site in the groin. These veins drain subcutaneous adipose tissue and the skin of the thigh and the lower anterior abdominal wall. Figure 2 depicts the anatomy of the femoral vein and its tributaries in the catheterization areas. To collect blood that is not draining subcutaneous adipose tissue, the desired site for femoral venous catheter tip placement is distal to the SFJ to avoid the blood coming from the great saphenous vein (GSV). The GSV drains much of the superficial circulation of the leg (i.e., the subcutaneous fat and skin). It starts at the dorsum of foot, ascends to pass the fossa ovalis, and ends at the SFJ ~3 cm below the inguinal ligament (10). Near the point of SFJ, the GSV receives five major tributaries: superficial iliac circumflex, superficial epigastric, superficial external pudendal, posteriomedial vein, and anteriolateral vein. The first three drain the upper thigh and the lower anterior abdominal wall. The other two drain the upper and mid thigh. These vessels carry blood draining subcutaneous adipose tissue and the skin, blood that should be avoided if the goal is to focus on muscle metabolism. Having placed the catheter so as to exclude GSV blood, the next issue is the distance from the SFJ (clearance). The fluid dynamics or turbulence at the point of SFJ within the femoral vein where GSV blood meets femoral venous blood create a localized, nonhomogeneous pool of blood. To avoid this blood, the tip of the catheter must be placed sufficiently downstream of the SFJ to avoid this mixed pool of blood. The third issue is the existence of several perforating veins between femoral vein and the GSV along its way from the lower leg to the SFJ. The blood flow in these perforators is normally unidirectional to but not from the femoral vein because of venous valves that prevent blood backflow in the absence of varicosities. Thus, it is possible that the femoral venous blood at any level may contain blood that is a mixture of subcutaneous adipose tissue and muscle drainage. Together with the issue of inter- and intramuscular adipose lipolysis-derived fatty acids, the question becomes whether it is possible to obtain blood that exclusively drains leg muscle. To examine this question, we review the results of leg muscle fatty acid release relative to the femoral venous catheter positioning.

Fatty Acid Release Affected By Femoral Venous Catheter Placement

We compared the reported results of leg skeletal muscle FFA and glycerol release and uptake obtained using the A-V balance technique. The comparisons are focused on the positioning of the femoral venous catheter tip at various vertical levels in retrograde vs. antegrade direction. To maximize the data homogeneity from different studies and reduce errors of comparisons, we included only postabsorptive data from healthy, lean males at rest. Based on the assumption that imcTG does not release substantial amounts of fatty acids, we assume that if there is no or minimal fatty acid release, the sampled venous blood is not contaminated by adipose fatty acids; i.e., it comes from skeletal muscle drainage. If there is substantial basal fatty acid release, it is highly likely that the sampled blood contains the drainage from adipose tissue. The involvement of adipose fatty acids may also introduce errors to
The absence of basal net fatty acid exchange indicates that the femoral venous catheter tip position needs to be placed distal to SFJ in order to avoid contamination from subcutaneous fat. To our knowledge, this is the greatest clearance employed, the results thus far reported. The femoral arterial catheter was placed 2 cm proximal to the inguinal ligament. Thigh muscle was isolated having avoided FFA contamination via SGV, as Bangsbo et al. (2) did. Therefore, the FFA release rate is considered to represent the combined contribution from inter- and intramuscular fat and imcTG, if any (Fig. 1), approximating what would have been measured as unidirectional FFA release by Bangsbo et al. (2) had they used tracers.

How much of this basal FFA release is from the inter- and intramuscular fat, excluding imcTG? To answer this question, imcTG-fatty acid release must be known. Since this is unknown from either of these studies, it is estimated from another study of human muscle using a microdialysis technique that reported the lipolysis in resting gastrocnemius to be 2 μmol·kg\(^{-1}\)·min\(^{-1}\) (25). The investigators placed the femoral venous catheter 2 cm below SFJ in retrograde direction, thus having avoided FFA contamination via SGV, as Bangsbo et al. (2) did. Therefore, the FFA release rate is considered to represent the combined contribution from inter- and intramuscular fat and imcTG, if any (Fig. 1), approximating what would have been measured as unidirectional FFA release by Bangsbo et al. (2) had they used tracers.

Studies of lipolytic regulation, as they seem to respond only to \(\beta_1\)- and not \(\beta_2\)- adrenergic stimulation (11).

Bangsbo et al. (2) studied a group of males in whom the femoral vein was cannulated in the retrograde direction with the catheter tip positioned 11 cm distal to the inguinal ligament, i.e., 8 cm distal to the SFJ, a clearance considered far enough to avoid blood from SGV. To our knowledge, this is the greatest clearance of femoral venous catheter from the SFJ thus far reported. The femoral arterial catheter was placed 2 cm proximal to the inguinal ligament. Thigh muscle was isolated by restricting blood flow at knee so that only the knee extensor is sampled. In this setting, no net FFA release or uptake (i.e., no net exchange or zero net balance) from the extensor was observed under overnight postabsorptive resting conditions. The absence of basal net fatty acid exchange indicates that release and uptake are equal. Because the catheter placement avoided GSV blood draining the subcutaneous fat, any unidirectional release would reflect the basal fatty acid release from the gross skeletal muscle compartment, i.e., from inter- and intramuscular fat, as well as imcTG, if any. Of note is that the net FFA uptake increased to 44 μmol·kg\(^{-1}\)·min\(^{-1}\) during knee extensor exercise. Unfortunately, FFA tracers were not utilized in this study, and thus the unidirectional release is unknown. However, because of the great clearance employed, the results still provide reasonable reference values for assessing the role of inter- and intramuscular fat contribution to leg FFA release, excluding the contribution from subcutaneous fat. We reasoned that studies reporting similar fatty acid release would suggest no contamination from subcutaneous fat, whereas those reporting greater net release suggest contamination. Because the study did not use tracers, we estimated the leg unidirectional FFA release from another study of participants with similar characteristics under similar conditions. The study observed unidirectional basal FFA release rates averaging 6.5 μmol·kg\(^{-1}\)·min\(^{-1}\) (25). The investigators placed the femoral venous catheter 2 cm below SFJ in retrograde direction, thus having avoided FFA contamination via SGV, as Bangsbo et al. (2) did. Therefore, the FFA release rate is considered to represent the combined contribution from inter- and intramuscular fat and imcTG, if any (Fig. 1), approximating what would have been measured as unidirectional FFA release by Bangsbo et al. (2) had they used tracers.
of the reported values. It provides another reference value for discussions below to assess the degree of contamination in other studies. The implication of this estimate is that a zero or near-zero net fatty acid balance, as reported previously (2, 18, 25), would in fact imply an uptake of fatty acids by skeletal muscle in that quantity. In other words, it can be used to correct for the contamination in studies of fatty acid kinetics. Below, it is used to evaluate whether and to what degree the reported values from other studies are affected by contamination when the femoral catheter is placed at varying distances from the SFJ.

van Hall et al. (25) specifically compared the retrograde vs. antegrade directions of femoral venous catheter placement, 2 cm distal and 2 cm proximal to the SFJ, respectively. Stable isotope [U-13C]palmitate and D5-glycerol were used to determine unidirectional release and uptake. The basal fatty acid release from the retrograde direction was 6.5 μmol·kg⁻¹·min⁻¹, significantly less than 16 μmol·kg⁻¹·min⁻¹ found in the antegrade direction. Clearly, the antegrade placement results in marked contamination by blood from GSV (subcutaneous fat). By comparison, fatty acid uptake was not very different between the two directions, 8 vs. 11 μmol·kg⁻¹·min⁻¹, indicating that catheter position does not have a major impact on uptake measurements. This study demonstrated clearly that the antegrade cannulation with the catheter placed proximal to the SFJ should be avoided. The study (25) observed a small positive leg FFA balance (uptake) of ~1 μmol·kg⁻¹·min⁻¹, not very different from the zero balance reported by Bangsbo et al. (2). This suggests that a clearance of 2 cm from the SFJ can avoid the blood of GSV almost as effectively as a clearance of 8 cm. Another group employed similar femoral venous catheter placement (distal to SFJ in retrograde direction) and observed essentially a zero net balance (18), again suggesting no contamination and thus a femoral catheter clearance of 2 cm or greater.

The above discussions suggest that 1) the postabsorptive basal FFA release is 5–7 μmol·kg⁻¹·min⁻¹ from the gross leg muscle compartment, likely the result of lipolysis in inter- and intramuscular fat that is unavoidable when the leg A-V balance technique is used; 2) the antegrade direction with the catheter tip placed proximal to SFJ should be avoided since it results in severe contamination; and 3) femoral venous catheter tip clearance should be ≥2 cm. To evaluate these values further in a more quantitative manner, additional reports are discussed below. In another elegant study, van Hall et al. (24) compared varying positions of femoral venous catheter placement for measurements of FFA, glycerol, and glucose concentrations. Placing the catheter tip distal to SFJ consistently provided lower concentrations of fatty acids and glycerol than the proximal position. However, these variations had little effect on lactate and glucose concentrations, as expected. The authors then varied the distance relative to the SFJ. By positioning the catheter ~2 cm below the SFJ (estimated visually from the presented figures), i.e., at the level of two deep veins (the profunda vein and the lateral femoral circumflex vein), the fatty acid and glycerol concentrations were 50% less than at the level of SFJ, which contains blood from GSV. Moving the catheter tip to the level of the inguinal ligament resulted in further increases in fatty acid and glycerol concentrations. A catheter placed at this level essentially sampled the blood, draining virtually the entire leg and a portion of the anterior abdominal wall. This is similar to the setting employed for the measurement of total leg fatty acid release, which is reported to be 14 (range 6–21) μmol·kg⁻¹·min⁻¹ (13). The results demonstrated that fatty acid concentration is a function of the vertical level of the femoral catheter in the femoral vein. The higher it is placed, the greater the concentration (contamination) is. This study confirmed the importance of placing the femoral venous catheter downstream of SFJ. Although we cannot exclude the possibility that catheter clearances of <2 cm may be satisfactory, it seems unwise to risk contamination by the turbulent blood pool at the SFJ. Therefore, for pragmatic reasons, a 2-cm clearance is suggested as the minimum, or greater if technically feasible.

In light of the above discussions, it appears that the stimulation of fatty acid release via lipolysis from “skeletal muscle” (femoral venous catheter) but not from anterior abdominal adipose tissue (superficial epigastric vein catheter) during IL-6 infusion in healthy males appears to be inaccurate. Rather, the stimulated systemic lipolysis reflects mainly that of the inter- and/or intramuscular fat, not imcTG (26). In fact, the authors correctly suspected this possibility. In that study, the basal palmitate release was ~1.5 μmol·kg⁻¹·min⁻¹ (based on 7 kg of leg muscle), equivalent to a total fatty acid release of ~6 μmol·kg⁻¹·min⁻¹ (based on one-quarter of fatty acids as palmitic). This is within the estimated range of basal fatty acid release as discussed above, suggesting that the value of 5–7 μmol·kg⁻¹·min⁻¹ is reproducible and thus a reasonable estimate.

**Forearm Arterio-Deep Venous Balance Technique**

The use of forearm deep vein (dV) to sample venous blood draining skeletal muscle is another technique employed in studies of skeletal muscle fatty acid metabolism. Alhborg et al. (1) compared femoral A-V balance vs. forearm arterio-deep venous (A-dV) balance technique in young, lean individuals. The specific catheter positioning is not reported. The basal leg oleic acid release and uptake were found to be 7 and 6 μmol·kg⁻¹·min⁻¹, respectively, and thus a net oleic acid release of 1 or ~3 μmol·kg⁻¹·min⁻¹ of all fatty acids (based on 1/5 of fatty acids as oleic). With the forearm technique, there was a small oleic acid release and a net uptake. In another study comparing the forearm A-dV vs. leg A-V balance techniques, the net fatty acid balance was found to be positive (0.10 mmol/l, net uptake) and negative (~0.10 mmol/l, net release), respectively (17). Multiplied by the blood flow, the rates of net fatty acid balance are 4 and ~4 μmol·kg⁻¹·min⁻¹. These studies seem to suggest lesser probability of contamination by inter- and intramuscular fat in forearm muscle in the areas drained by the cannulated deep vein. Apparently, however, direct evidence is required to confirm this possibility. For instance, another study using the technique reported net fatty acid release at significant amounts from forearm (5). Although the release is claimed to be from the muscle, a possibility of contamination by adipose tissue cannot be ruled out given the relatively high rate of adipose lipolysis in the postabsorptive state. Because the forearm balance technique is utilized less often compared with the leg technique, a more definitive assessment of the technique requires further investigations. On the other hand, it seems clear that an advantage for the forearm technique, even if proven, could be attenuated by questioning
whether forearm muscle serves as a good representation of the whole body skeletal muscle (e.g., smaller mass), although the same question applies to any muscle given the well-known muscle heterogeneity. Another factor for consideration in choosing between the two techniques is the need for supporting imaging procedures for the visualization and accurate placement of the catheter into the deep vein when the forearm technique is used, which is not required for the leg technique. Conceivably, variations in the regulation of forearm adipose lipolysis compared with the systemic level (12) may also have an effect on the accuracy in measuring muscle fatty acid kinetics if the deep venous catheter cannot completely avoid contamination by adipose fatty acids as intended.

Summary and Suggestions

Although the femoral A-V balance technique is relatively straightforward to perform, the anatomy of the femoral vein and the existence of multiple triglyceride pools associated with leg muscle make using the technique a challenge for the investigation of skeletal muscle fatty acid metabolism. It is virtually impossible to completely avoid sampling blood that drains adipose such as the inter- and intramuscular fat, except in those individuals with extremely low body fat, such as the late legendary martial arts icon Bruce Lee or the most medalized Olympian Michael Phelps, both of whom have been said to have nearly zero or very low body fat. Moreover, the contamination may occur from the subcutaneous fat via the perforator veins. Additional contamination of potentially much greater magnitude comes from the massive subcutaneous fat via the great saphenous vein if the catheter tip is placed above the saphenofemoral junction. The use of isotopic tracers will allow measurements of FFA uptake, which is primarily by skeletal muscle in most adults, although leg adipose tissue does take up FFA directly (16). It is realized that although the contaminations are independent of blood flow, i.e., the latter does not change the presence or absence of the former, adding blood flow into the equation (A-V concentration balance \times blood flow) may “magnify” the problem, especially for women, obesity, or the elderly, where the absolute errors are greater proportional to the degree of the contamination. Also, experimental approaches to measure leg blood flow, such as by dye dilution or thermodilution, may prove difficult to validate when applied to the more limited compartments sampled by the retrograde catheter placement. Although the femoral A-V balance technique has been used for many years, there has been no consensus-driven standardization as to how femoral venous catheter placement should be performed to limit the contamination by adipose tissue. Because of this lack of consensus, the procedure has been performed with variations among laboratories. It is not surprising that results also vary, which may result in confusion as to which conclusions can be properly drawn with regard to fatty acid metabolism in skeletal muscle.

From the results discussed above, we suggest some guidelines for future studies. First, the femoral catheter sampling tip should be placed at a minimum of 2 cm distal to the saphenofemoral junction if the goal is to minimize sampling blood of the great saphenous vein draining the subcutaneous adipose tissue. Second, isotopic tracers are needed to distinguish uptake and release. Third, the contribution of FFA from the inter- and intramuscular fat and from the perforator veins is estimated to be in the range of 5–7 \( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \) at rest in the postabsorptive state. These values seem reasonable as they are comparable with the unidirectional leg fatty acid release determined with the aid of fatty acid tracers (19). Therefore, for studies to be performed under the similar metabolic conditions (postabsorptive, resting healthy lean men), this release rate can be used for correction. For instance, an observed zero net balance of fatty acids or a lack of basal fatty acid uptake (22) would be taken to represent an uptake of \(-6 \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\). In case of a negative net balance, e.g., \(-3 \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\) (release), it would imply an uptake of \(3 \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\) instead. In cases of positive net balance, e.g., \(3 \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\), the correct value may actually be \(9 \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\). Among these scenarios, zero or nearly zero balance appears to be a more likely expectation. In addition to the above studies that reported such results (2, 18), the basal systemic fatty acid appearance rate (equal to uptake under steady state) in healthy lean men and women of 5.8 \(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\) (9) also supports this assessment. Fatty acid uptake by leg skeletal muscle could be somewhat higher than this rate. However, the counterbalance to this is the fatty acid uptake by leg adipose tissues (16). Thus, the actual uptake by leg muscle may not deviate greatly from this rate. Therefore, a small positive balance can be expected (18, 25). It is cautioned, however, that because there are no gold standard methods or definitive reference values available at present for the study of leg muscle fatty acid kinetics, some assumptions are made that may lead to speculations. Also, the estimates of basal leg fatty acid release are established on the basis of no release of imdTG-fatty acids to the muscle drainage. Therefore, they are not considered error free, as this assumption is unproven. In addition to using the basal leg fatty acid release as a proxy for correction of the observed balance results, which may conceivably introduce errors, another approach would be to actually quantify this release for the populations being studied as a component of the main studies. For instance, it could be done as a pilot study. In using either approach, the estimated basal leg fatty acid release (5–7 \(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\)) established herein for the healthy lean males can be used as a reference or start point for other populations. The forearm arterio-deep vein balance technique appears to involve less contamination under certain conditions, but not always. Whether this is due to smaller mass of inter- and intramuscular fat or less adipose fatty acids through perforator veins is unknown, which is a very interesting topic to clarify. In choosing from the two techniques, factors needing consideration include the feasibility of performing the more technically demanding forearm deep vein catheterization procedures and the required imaging support. Potentially, the representativity of forearm muscle vs. leg muscle to the whole body skeletal muscle is another factor to consider.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Z.G. contributed to the conception and design of the research; Z.G. and M.D.J. analyzed the data; Z.G. and M.D.J. interpreted the results of the experiments; Z.G. prepared the figures; Z.G. drafted the manuscript; Z.G. and M.D.J. edited and revised the manuscript; Z.G. and M.D.J. approved the final version of the manuscript.
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