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Peer Review

Review of: "The LIME Framework: A Novel Conceptual NIRS Approach for Quantifying Muscle Metabolic Flexibility via Mitochondrial Oxidative Efficiency"

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1. Regarding the statement “focus on static metrics and do not evaluate the dynamic, adaptive processes occurring at the tissue level”: David Kelley used the leg balance technique to assess skeletal muscle metabolic flexibility. Below are some examples that could be cited to support this point:

Kelley DE, Goodpaster B, Wing RR, Simoneau JA. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. Am J Physiol. 1999;277(6):E1130-E1141.

Kelley DE, Mandarino LJ. Hyperglycemia normalizes insulin-stimulated skeletal muscle glucose oxidation and storage in noninsulin-dependent diabetes mellitus. J Clin Invest. 1990;86(6):1999-2007.

You may wish to clarify how your approach relates to, differs from, or builds upon these earlier dynamic assessments at the tissue level.

2. The statement “is an early marker of insulin resistance, obesity, and type 2 diabetes” needs to be clarified. Do you mean that metabolic flexibility and insulin resistance occur in parallel, or that impaired metabolic flexibility appears before the onset of insulin resistance? Current evidence is more consistent with the notion that metabolic flexibility is a consequence rather than a cause of insulin resistance. Several papers address this; you can see this review paper summarizing the evidence: doi: 10.1111/obr.13131. This temporal sequence must be clearly elaborated, and evidence from original studies should be provided to support whichever interpretation you intend.

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3. It is stated that reduced mitochondrial activity in skeletal muscle limits the switch from fat to carbohydrate oxidation and contributes to insulin resistance. A reference with evidence from original studies must be provided to support this claim. Moreover, there are several studies that do not support this statement. In fact, studies that have compared metabolic flexibility in individuals typically considered as having altered mitochondrial function, or studies that have directly determined mitochondrial function, do not fully support the authors' statement. Here are some examples:

Kelley DE, Mandarino LJ. Hyperglycemia normalizes insulin-stimulated skeletal muscle glucose oxidation and storage in noninsulin-dependent diabetes mellitus. J Clin Invest. 1990;86(6):1999-2007.

Galgani JE, Heilbronn LK, Azuma K, et al. Metabolic flexibility in response to glucose is not impaired in people with type 2 diabetes after controlling for glucose disposal rate. Diabetes. 2008;57(4): 841-845.

Koska J, Ortega E, Bogardus C, Krakoff J, Bunt JC. The effect of insulin on net lipid oxidation predicts worsening of insulin resistance and development of type 2 diabetes mellitus. Am J Physiol Endocrinol Metab. 2007;293(1):E264-E269.

Apostolopoulou M, Strassburger K, Herder C, et al. Metabolic flexibility and oxidative capacity independently associate with insulin sensitivity in individuals with newly diagnosed type 2 diabetes. Diabetologia. 2016;59(10):2203-2207.

van de Weijer T, Sparks LM, Phielix E, et al. Relationships between mitochondrial function and metabolic flexibility in type 2 diabetes mellitus. PLoS ONE. 2013;8(2):e51648.

Given this literature, the statement regarding reduced mitochondrial activity, impaired switching, and insulin resistance should be nuanced and supported by appropriate references, while acknowledging conflicting data.

4. In Figure 1, please specify the meaning of all abbreviations, including BP, $etCO_2$, etc. Abbreviation definitions should be provided in the figure legend.

5. It is mentioned that there is a cutoff for subcutaneous fat thickness (below 1.5–2 cm). Several points require clarification: i) Do you have any data or literature indicating when this cutoff is usually reached according to BMI and sex (two major determinants of body fatness)? ii) What is the relationship between signal quality and subcutaneous fat thickness? In other words, how was this cutoff established? iii) Is it that above this thickness there is no usable signal, and below this cutoff signal quality is always good regardless of the thickness? iv) Or is signal quality a linear (or otherwise graded) function of

subcutaneous thickness? These aspects should be explained, either with empirical data (if available) or with a clear rationale based on previous work.

6. Please specify what objective measures are used to ensure signal quality. For example: a) Are there quantitative criteria (signal-to-noise ratio, stability over time, specific waveform characteristics, etc.)? b) Are there predefined thresholds or quality-control checks that lead to the exclusion or repetition of measurements? A clear statement of these objective criteria is important for reproducibility and for interpreting the robustness of your measurements.

7. The manuscript should provide a power analysis and the primary hypothesis (or hypotheses) to be tested. A clear description of what exactly this study is designed to evaluate is essential to understand the rationale behind the study design and the interpretability of negative or positive findings.

8. It is indicated that, ideally, the following variables should be measured at baseline: blood pressure, heart rate, respiratory rate, SpO_2, end-tidal CO_2 (nasal cannula), and 5-minute heart rate variability (RMSSD). Hydration status (hemoglobin, hematocrit, urine specific gravity) and blood lactate should also be measured. Plasma non-esterified fatty acids (NEFA) would be recorded at baseline as a covariate in between-subject analyses to account for variability in lipid availability; given its role in the causal pathway from substrate selection to k_HHb, NEFA would not be included in the primary within-subject model but would be examined in sensitivity analyses. However, there is no analytical plan describing what you intend to do with these measurements once they are collected. Several issues need clarification: Should some of these variables be similar between groups? If so, what magnitude of difference would be considered “similar”? Would similarity be defined solely in statistical terms (p-values), where sample size and variability heavily influence conclusions? Are there variables that, if they fall below or above certain cutoffs, would lead you to exclude participants or specific measurements from further analysis? For variables used as covariates, please specify which ones and how exactly they will be incorporated into the statistical models (e.g., linear covariates, categorical cutoffs, interaction terms). Because metabolic flexibility is defined as the capacity to adjust fuel oxidation to fuel availability, it seems that FFAs are recommended to be measured with that aim, but it is not clearly indicated how they will be used analytically. Given the importance of metabolic flexibility in your rationale, the treatment of this aspect appears weak. What the paper calls “metabolic flexibility” in the current text captures only one part of the broader concept (the change itself, rather than the adjustment to fuel availability). Furthermore, regarding glucose availability, there is no mention of how it is assessed or incorporated, which is difficult to understand given the nature of the protocol (OGTT). If metabolic flexibility is to be properly evaluated,

both lipid and glucose availability should be considered, and the statistical analysis should elaborate on these aspects, or at least acknowledge this as a weakness of the protocol.

9. The standardization procedure for locating the device should be better described, including: How the site is identified and marked. Whether anatomical landmarks are used. How reproducibility of probe placement is ensured between and within subjects. You should also explain why that specific body region (brachioradialis) was chosen. Is there any possibility of including a larger muscle region (e.g., leg muscles such as the vastus lateralis)? The manuscript emphasizes the relevance of skeletal muscle for insulin-stimulated glucose disposal. While skeletal muscle is indeed a major site of glucose uptake under supraphysiological insulin conditions (e.g., in the euglycemic-hyperinsulinemic clamp), under more physiological conditions—such as after an OGTT or mixed meal—the liver is also a major organ disposing of glucose (see pivotal work by John Gerich). The selection of the brachioradialis does not appear fully consistent with the idea that muscle is being measured because it is quantitatively important for glucose disposal. The brachioradialis is a relatively small muscle. It should therefore be elaborated why a smaller muscle was selected instead of a larger, more metabolically representative muscle group.

10. Regarding Figure 4, there are several methodological and conceptual issues: From a technical standpoint, correlation analysis should not be conducted when the data distribution is not uniform across the range of values (e.g., when there is a cluster with limited spread in one region and sparse data elsewhere). More importantly, the final goal of this analysis should be made explicit. Is the intention to develop a marker that could replace HOMA? This connects to your earlier statement that metabolic flexibility "is an early marker of insulin resistance". If this is true, impaired metabolic flexibility should emerge before insulin resistance, making metabolic flexibility a predictive marker of insulin resistance. In that case, the value of metabolic flexibility would not simply be as an alternative marker of insulin resistance, but as a tool for early detection. Moreover, in that scenario, MF may not necessarily relate to HOMA. This raises the question: why do we need another marker of insulin resistance when we already have established ones for research settings (e.g., clamp-derived indices, HOMA, Matsuda index)? Additionally, HOMA is primarily a marker of hepatic insulin resistance under conditions of low insulin, as originally elaborated by Matthews (Diabetologia 1985). In contrast, your approach appears to be a marker of muscle metabolic flexibility (and possibly muscle insulin resistance) under conditions of higher insulin levels. Muscle and hepatic insulin resistance may not evolve in parallel in the pathophysiological sequence, which likely explains why concordance among different markers of insulin sensitivity (e.g., clamp vs. HOMA, or HOMA vs. Matsuda) is far from 1.00 and usually only moderate. This can certainly be

due in part to analytical variability, but it is also reasonable to propose that such imperfect associations have physiological meaning. Therefore, even if NIRS-derived measures (assuming they are markers of insulin resistance or metabolic flexibility) do not correlate strongly with HOMA, this does not necessarily imply that they are not valid markers of insulin resistance in a setting more closely related to muscle insulin sensitivity. In that case, they should be compared against more appropriate reference methods, such as: The euglycemic-hyperinsulinemic clamp, or Muscle-related indices derived from OGTT, such as the Matsuda-muscle approach (for example, the decrease in glucose after OGTT as elaborated by Abdul-Ghani: doi: 10.2337/dc07-0622).

11. There is no clear analysis plan for validation. In other words, it is not specified what procedure and what outcomes will be used to conclude that the method is valid. You mention a hypothetical plot of HOMA-IR vs. LIMEFlex (%), but it is not elaborated: What specific outcomes would define that LIMEFlex (%) is a valid measure relative to HOMA-IR? Will validity be based on statistical criteria alone (e.g., a given p-value)? If so, the conclusion would depend heavily on variance and sample size. Or will it be based on an effect-size criterion (e.g., a minimum correlation coefficient, or other measure of agreement)? It would be helpful to explicitly state the validation criteria and the rationale behind them.

12. It should also be elaborated why LIMEFlex (%) is calculated in the following way: (LIMECarb−LIMELip)/LIMELip×100. There are many ways of calculating changes (see this paper: doi/10.1080/00031305.1985.10479385), and it would be appreciated if more explanation is provided regarding the selection of this specific formulation. In addition, readers unfamiliar with this approach would benefit from an explanation of the meaning of negative values, which arise when LIME-lip exceeds LIME-cho (or vice versa, depending on your definition). Furthermore, it is not indicated whether LIMELip or LIMECarb have independent utility on their own: Although these components are mentioned, the manuscript does not elaborate on expectations regarding their separate associations with HOMA (or other indices). Clarifying whether LIMELip and LIMECarb are themselves of interest, or whether only the combined LIMEFlex index is intended to be used, would improve conceptual clarity.

13. Finally, the manuscript should provide the strongest available evidence regarding what exactly this technique measures physiologically, and its concordance with reference methods. Are there validation studies comparing NIRS-derived signals (or equivalent metrics) with gold-standard measures of muscle oxygenation, blood flow, substrate use, or insulin sensitivity? What is the strength of those associations, and in what populations and conditions were they observed? A more explicit link between your proposed

measure, its physiological interpretation, and its agreement with accepted reference methods is essential to convince the reader of its validity and relevance.

Declarations

Potential competing interests: No potential competing interests to declare.