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Review of the Doctoral Dissertation of Raghunandan Mahadeva

The doctoral dissertation "*Iron Deficiency Uniquely Reprograms the Amino Acid and Lipid Metabolism of Red Pulp Macrophages to Support Enhanced Iron Recycling Functions*" by Raghunandan Mahadeva was prepared under the supervision of **Dr Katarzyna Mleczko-Sanecka** at the International Institute of Molecular and Cell Biology (IIMCB) in Warsaw, within the Warsaw PhD School in Natural and BioMedical Sciences. The dissertation addresses how splenic red pulp macrophages (RPMs), the principal cell type responsible for erythrophagocytosis and iron recycling, adapt their metabolism to nutritional iron deficiency. This question fits within the research program of the Mleczko-Sanecka lab, whose work over the past several years has substantially reshaped our understanding of red blood cell recycling and macrophage iron handling – a line of research my lab is actively engaged in. This dissertation is a strong continuation of that trajectory and identifies genuinely new and interesting biology, a coordinated, cell-type-specific program of branched-chain amino acid (BCAA) catabolism and fatty acid oxidation that sustains the enhanced erythrophagocytic activity of RPMs under iron restriction.

The dissertation follows the collection-of-articles format, comprising Manuscript 1 (currently a bioRxiv preprint) and Manuscript 2 (published in EMBO Reports), accompanied by an abbreviated written thesis with an introduction, a summary of results, a discussion, a future directions chapter, and a bibliography. There is no independent methods chapter, methods are found only within the appended manuscripts, which is a minor formal limitation but does not hinder the readability. The dissertation is written in clear, fluent English throughout, and I noted no meaningful typographical issues.

I should note, in the interest of transparency, that this format was initially somewhat unfamiliar to me. Having served on more than thirty-five doctoral committees, including as supervisor for seventeen of my own students, I am accustomed to dissertations built around the candidate's own body of work, rather than a compilation of multi-author manuscripts in which the PhD candidate is not always listed first. This is not the convention I am used to evaluating. I understand, however, that the collection-of-articles format is accepted practice at IIMCB, and I do not hold this against the candidate. I would, nonetheless, suggest that a *Table be added, indicating the figure or panel to which the candidate directly contributed (versus that of another collaborator)*, so that the extent of his personal contribution is transparent at a glance.

The Introduction is comprehensive and well organized, moving logically from systemic iron handling to the specialized biology of RPMs and then to the metabolic underpinnings of efferocytosis, before narrowing to the specific rationale for examining amino acid and lipid metabolism. It provides sufficient

background for a non-specialist reader while remaining focused on the questions the thesis goes on to address.

The Results are the strongest part of the dissertation. The candidate first summarizes the laboratory's foundational, collaborative work establishing that iron-deficient (ID) RPMs display enhanced erythrophagocytosis driven by a low hepcidin-high ferroportin (FPN) state acting through SYK signaling, work to which he contributed Seahorse flux analyses, FPN-overexpression experiments, and animal work. I particularly appreciate that the candidate is explicit about which parts of this early work were collaborative and predate his doctoral project (Section 2.1.1), rather than blurring this distinction. He then presents, as his own principal and independently driven contribution (Section 2.1.2), the identification of BCAA catabolism and fatty acid β-oxidation as the metabolic pathways that execute this adaptive program. This section reflects genuinely independent scientific reasoning. The candidate systematically distinguishes RPM metabolism from canonical efferocytic programs described for apoptotic-cell clearance, validates cell-type specificity across Kupffer cells and peritoneal macrophages, and uses two independent BCAT inhibitors and a CPT2 inhibitor, together with in vivo pharmacology and functional readouts, to build a convincing, if still largely pharmacological rather than genetic, case for causality. The candidate's contribution to Manuscript 2, by contrast, is smaller and appropriately described as supporting (formal analysis and investigation) rather than conceptual or leading work. In my view, a single impactful first author manuscript is sufficient for a PhD dissertation.

The Discussion is thoughtful and blends the findings well against the existing efferocytosis literature, noting that the RPM program differs from arginine, tryptophan, or glutamine- centered efferocytic metabolism described elsewhere. The candidate is also acknowledging (appropriately) that SLC7A7/SLC7A8 could mediate BCAA import or export, that the origin of the BCAA substrates has not been directly traced, and that pharmacological inhibition alone cannot exclude off-target effects. The parallel drawn to iron dysregulation in long COVID is an interesting speculation (page 34) but is not well supported and should state " I speculate…".

The Future Directions chapter is a genuine highlight, which I require of my own students. It lays out an ambitious plan detailing isotope tracing of BCAA-derived carbon, conditional Bcat1/Bcat2 deletion, comparison across phagocytic cargoes. It is unusually candid about a technical setback, the inability so far to obtain robust untargeted metabolomics or lipidomics data from RPMs. This kind of honesty about limitations reflects scientific maturity.

I have some questions to which the candidate may respond during the oral defense:

1. Do you have a strategy to determine whether SLC7A7/SLC7A8 primarily import BCAAs into RPMs or export them?

2. Would pre-labeling RBCs before transfusion be one way to trace the origin of the BCAA pool that fuels catabolism during erythrophagocytosis?

3. The FPN D39A calcium-import-dead mutant ruled out direct Ca^{2+} transport by FPN, yet FPN activity is required upstream of Ca^{2+} and SYK. What is your model for how altered iron flux through FPN is sensed and converted into a Ca^{2+} signal?

4. ID RPMs show a modest but significant rise in labile iron and reactive oxygen species alongside a mitochondrial oxidative program driven by BCAA catabolism and fatty acid oxidation, both of which generate additional mitochondrial ROS. Do you view this intersection as a deliberate physiological trade-off, and does it raise a ferroptosis vulnerability for RPMs under sustained iron deficiency?

5. Manuscript 2 shows that Kupffer cells contribute to the clearance of RBC ghosts. Would you predict that the FPN-SYK-BCAA axis you defined in RPMs also operates in Kupffer cells under conditions of pathological hemolysis, even though it is not engaged during nutritional iron deficiency?

I evaluate this PhD dissertation positively. The candidate addresses a physiologically important question within an active and productive research program, applies an impressively broad experimental toolkit, and provides, in the section reflecting his own independent contribution, a convincing case for a new metabolic axis governing red pulp macrophage function during iron deficiency. I do not, however, consider the dissertation to merit distinction (distinguished), given that the central manuscript remains a preprint at the time of submission with four authors contributing equally and the candidate's contribution to the second manuscript is comparatively limited.

I, the undersigned, hereby state that the doctoral dissertation of Raghunandan Mahadeva meets the requirements specified in Article 187 of the Act of July 20, 2018 – Law on Higher Education and Science (c.t., Journal of Laws of 2024, item 1571, as amended). I hereby recommend to the Doctoral Committee of the International Institute of Molecular and Cell Biology in Warsaw to admit Raghunandan Mahadeva to the subsequent stages of the procedure for the conferment of the doctoral degree in the field of natural sciences, in the discipline of biological sciences.

Sincerely,