ADAM MICKIEWICZ UNIVERSITY IN POZNAŃ
Faculty of Biology
Institute of Molecular Biology and Biotechnology

Poznań, March 25, 2025

**Referee report on the doctoral dissertation**

**submitted by MSc Thiliban Manivarma**

**entitled "Regulation of Ferroptosis through 15LOX-1/PEBP1 Complex – Computational Modeling at Molecular Level"**

*prepared under the supervision of dr hab. Karolina Mikulska-Rumińska, prof. NCU and prof. dr hab. Wiesław Nowak*

This doctoral dissertation presents a comprehensive computational investigation of the molecular mechanisms underlying ferroptosis regulation, with a focus on the complex formed by 15-lipoxygenase isoform 1 (15LOX-1) and phosphatidylethanolamine-binding protein 1 (PEBP1). The thesis comprises a short introduction and three research articles systematically exploring different aspects of this important biological process. Two of these articles have already been published in well-recognized peer-reviewed journals – *Free Radical Biology and Medicine* and *Bioinformatics*. The last article is available as a preprint. The candidate is the first author of two of those papers.

The introductory part of the dissertation covers cell death mechanisms, providing context for ferroptosis among other regulated cell death pathways. It then details on ferroptosis, including its discovery, biochemical characteristics, and physiological significance in various diseases. The introduction provides the necessary background to understand the significance of the research and overall motivation for the work. Then, the structural and functional aspects of the key biomolecular systems studied in the thesis, namely 15-LOX-1 and PEBP1 are introduced. This section provides detailed descriptions of the proteins, substrates, and membrane components studied in the investigation of ferroptotic processes. Finally, an introduction to the fundamentals of the computational method is provided. While a theory for molecular docking and MD simulations are presented in a refreshing style with sufficient details, I am missing some overview covering principles of other methods used in the candidate's research, like methods for pathway/tunnel detection, water analyses, or allosteric pathways studies. Despite this minor limitation, the thesis introduction serves as a solid foundation for the research presented in the three articles that constitute the core of the dissertation.

The first article looks into how the 15LOX-1/PEBP1 complex interacts with cellular membranes to facilitate access to the SAPE substrate. Through structural modeling and MD simulations, the candidate showed that membrane association induces conformational

ul. Uniwersytetu Poznańskiego 6,
Collegium Biologicum,
61-614 Poznań
janbre@amu.edu.pl

changes in the complex that expose the catalytic site of 15LOX-1. This enables SAPE to access the catalytic site, where it undergoes peroxidation to form hydroperoxy derivatives that serve as ferroptotic signals. The study also identifies critical residues at the interface between 15LOX-1 and PEBP1, particularly highlighting the significance of the P112 residue in PEBP1, both computationally and experimentally confirming its role in ferroptotic signaling.

The second article describes the development of WatFinder, a computational tool integrated into the ProDy framework for identifying and visualizing protein-water interactions. This tool detects water bridges, clusters, and channels that play crucial roles in protein dynamics and stability. Here, the candidate contributed to the testing of the tool on various biological systems, ensuring its reliability and applicability.

The third study examines how oxygen molecules access the catalytic site of 15LOX-1 under different conditions. Using MD simulations, the author identifies two main oxygen tunnels (Entrance A and Entrance B) and demonstrates that substrate binding and membrane presence significantly influence mechanisms behind oxygen access, i.e., (i) substrate binding induces conformational changes that facilitate oxygen access to the catalytic site, (ii) the membrane helps direct oxygen molecules toward functional tunnels, (iii) two oxygen binding sites exist at the catalytic site that may explain the formation of singly and doubly-oxidized products—in line with lipidomics experiments, (iv) conserved residues across the LOX family suggest evolutionary adaptation, and finally (v) water clusters that may facilitate oxygen transport and product release were identified. Providing very thorough and novel insights into the regulation of 15LOX-1 enzymatic action.

In general, the thesis has a good structure, is well-written and easy to follow. I appreciate the inclusion of graphical abstracts in the summary chapter, as well as the appendices on the unpublished appendix on machine learning for 15LOX-1/PEBP1 hotspot predictions. One downside of the thesis structure is that I could not find in the thesis nor from the provided link the supplementary materials for Article C that was published as a preprint—perhaps these data could be included during the defense.

I noted only a few typos and small inconsistencies that do not diminish much the overall high quality of the thesis:

- "high-lighted" should be "highlighted" (p.13)
- "PEBP1 shown in Fig. 5 consist of 187 amino acids length" - should be "consists of"
- "The enzyme converts $Fe^{2+}$ to $Fe^{3+}$, creating a ferric iron-hydroxy complex that abstracts a hydrogen at carbon atom C13 and inserting an oxygen molecule at C15" - should be "abstracts... and inserts"

ul. Uniwersytetu Poznańskiego 6,
Collegium Biologicum,
61-614 Poznań
janbre@amu.edu.pl

- The text alternates between "15-lipoxygenase-1," "15LOX-1," and "15-LOX-1" in different sections, making it harder to follow, similarly, "phosphatidylethanolamine" is sometimes written as "PE" and other times as "phosphoethanolamine."
- Some inconsistent hyphenation, for example, Iron-dependent" vs. "iron dependent", "Molecular-dynamics" vs. "molecular dynamics", "All-atom" vs. "all atom"

**Questions and Comments:**

1. In Paper A, the initial structure of 15LOX-1 was obtained by homology modeling, justified by its 81 % identity to the used template. I wonder if there are any insertions/deletions that would make the task more complex. Also, have you tried to use AlphaFold or related tools for the generation of the model? How does it compare to the homology model?
2. In Paper A, how were the tunnels in 15LOX-1 calculated? Fig 1, points to the Methods section, but I cannot find any details there. I believe it could be informative to see how the tunnel-ensemble geometry changes when the complex interacts with membrane/SAPE, analogously to the evolution of the Theta angles shown in Fig 2.
3. Supplementary materials of Paper B contain five distinct use cases as well as studies on the effects of different parameter adjustments. Some of them seem to fit the information in contribution statements. Could the candidate provide a more exact definition of his contribution to this paper?
4. In Paper C, the candidate comments on the effect of the membrane on the residence of $O_2$ molecules in the active site (Fig 3b) and the directing of $0_2$ molecules: „Contrary, in the presence of a membrane, the $O_2$ molecules due to the particular interactions are more directed toward the entry of the oxygen tunnel leading to the catalytic site (insignificant number of interactions outside the blue/red boxes)." What is the presumed mechanism of this effect?
5. In Paper C, two approaches to delineate the $O_2$ diffusion pathways were employed (molecules bound in the cavity or placed in the solvent). Perhaps I have missed the information or it is in the „inaccessible" supplementary materials, but are the pathways for $O_2$ entry and release equivalent?
6. What is the status of the review process for Paper C, which was submitted to Redox Biology?

ul. Uniwersytetu Poznańskiego 6,
Collegium Biologicum,
61-614 Poznań
janbre@amu.edu.pl

ADAM MICKIEWICZ UNIVERSITY IN POZNAŃ
Faculty of Biology
Institute of Molecular Biology and Biotechnology

In summary, the candidate has demonstrated the quality of his research by being the first author on two provided publications, which constitute a substantial original contribution to the field of protein research, clearly advancing our knowledge of molecular mechanisms responsible for the regulation of ferroptosis. Also, the content of the three articles documents very well that the goals of the thesis were fully reached. Finally, I appreciate the vast amount of computer experiments executed by the candidate, effectively combining different computational techniques, such as docking, MD simulations, perturbation response scanning, electrostatic potential analysis, and conservation analysis.

**As such, I conclude that the thesis presented by MSc Thiliban Manivarma meets all the requirements of a doctoral dissertation as specified in Art. 187 of the Law on Higher Education and Science of July 20, 2018 (as amended) and recommend the thesis for the defense and consequently admission of the candidate to further stages of the procedure towards the award of the doctoral degree.**

.....

**dr hab. Jan Brezovsky, prof UAM.**
Head of the Laboratory of Biomolecular Interactions and Transport

ul. Uniwersytetu Poznańskiego 6,
Collegium Biologicum,
61-614 Poznań
janbre@amu.edu.pl