Rennes, January 2nd, 2023

Review of the thesis entitled "Insight into the links between sex- and age-related mechanical and biochemical alteration of erythrocytes and their ATP profile in ApoE/LDLR double-deficient mice model" by Mr. Fatih Celal Alçiçek, M.D. under supervision of Prof. Katarzyna M. Marzec

In my opinion, the thesis manuscript by Mr. Fatih Celal Alçiçek meets all the conditions allowing the author to defend orally his Ph.D. thesis. The Ph.D. candidate showed clearly in written form that he has mastered the research topic of his doctorate during his Ph.D. training. He is the author of three articles published in important scientific journals and one patent.

The goal of the Ph.D. thesis was to characterize sex- and age-related alterations of red blood cells (RBCs) related to hypercholesterolemia and atherosclerosis. To this end, Mr. Alçiçek analyzed chosen biochemical, mechanical, and functional properties of red blood cells in females and males of ApoE/LDLR$^{-/-}$ mice, and in their age-matched controls. He did it by applying classical techniques of analysis and vibrational spectroscopic techniques, such as Raman spectroscopy (RS) and Fourier transform infrared spectroscopy (FTIR).

Below, I will overview the content of the thesis, summarize the most important achievements, and finally will focus on eventual shortcomings by asking several questions, which should be addressed during the thesis defense.

The manuscript consists of a very long Abstract (over 6 pages). In my opinion, an abstract should be no more than 1 page long to bring together the most important points of the research. Such a long Abstract makes it difficult to recognize which scientific achievements the candidate considers the most important. The Abstract presented here should be rather named an abridgment of the thesis.

In the "Theoretical Part" which follows the candidate overviews the subject of his studies. The Introduction contains the objectives of the thesis and the main information on atherosclerosis, red blood cell involvement in the disease, the experimental model, which is the double mutant mice strain ApoE/LDLR-/- as well as on conventional and novel methods of red blood cell analysis, with a particular focus on spectroscopical approaches.

In the "Experimental Part" the candidate describes the results obtained while working on a Ph.D. in the laboratory. Each following chapter of this part of the thesis is devoted to the description of results published in three scientific articles co-authored by the candidate. I regret that the papers were not included directly in the thesis, or at least added at the end as an

annex. The description of obtained results, in each chapter, finishes with a short discussion and conclusions allowing to point the most important scientific achievements of the candidate.

In short, in my opinion, four essential groups of results and discoveries of the thesis are as follows.

1. The demonstration that the changes for extracellular ATP levels, MCV, and PS exposure are strongly linked to the basal intracellular ATP levels of red blood cells. Some of these changes were sex-dependent in both control and ApoE/LDLR$^{-/-}$ mice. The higher levels of total lipids and lower levels of unsaturated lipids on RBCs membranes were mainly related to hypercholesterolemia in ApoE/LDLR$^{-/-}$ mice. The author mention, however, that further analyzes are necessary to determine other factors affecting the lipid profile of the RBC membrane.
2. RBCs isolated from ApoE/LDLR$^{-/-}$ mice showed important changes in the secondary structure of hemoglobin and higher levels of 2,3-DPG as compared to the control mice. The alterations in the secondary structure of hemoglobin in ApoE/LDLR$^{-/-}$ mice strongly correlated to the 2,3-DPG levels in RBCs. The author suggests that the 2,3-DPG levels in RBCs increased in the result of a compensatory mechanism in response to the secondary structure alterations.
3. The ATP release was studied by applying iloprost (ILO), 3V cocktail, hypoxia, and low pH. VDAC and PANX1 channel expressions in RBCs membranes were analyzed and inhibitors of these proteins were used before inducers to prevent ATP release from RBCs. Therapeutic concentrations of ILO did not cause a robust ATP release in any mice groups. The expression of VDAC1 was observed at similar levels. The ATP release occurred following the 3V cocktail use indicating the activity of adenylyl cyclase and the intact components of the upstream signal-transduction pathway. It shows that the increase in cAMP stimulates ATP release from RBCs in all studied groups.
4. Hypoxia-induced ATP release from RBCs in ApoE/LDLR$^{-/-}$ mice was higher as compared to age-matched controls. Western blot results suggest higher levels of PANX1 proteins in the RBC cell membrane in ApoE/LDLR$^{-/-}$ mice, which is consistent with the known higher hypoxia-induced ATP release already published. The low pH-induced ATP release from RBCs showed very similar patterns with the hypoxia-induced ATP release from RBCs in all animal groups.

The final chapter of the "Experimental Part" describes an application part, including the prototyping of the innovative deformation-induced ATP releaser (DIAR), which was successfully completed with the granting of a patent.

The thesis ends with the discussion of limitations of this research and the prospective of potential future works.

The novel results obtained during this Ph.D. will certainly contribute to the extension of understanding of molecular mechanisms of early and late phases of atherosclerosis disease and their reciprocal interactions with RBCs. They should stimulate in future new therapeutic strategies targeting RBCs, hopefully providing novel treatments.

Problems and questions:

1. on page 34 one reads as follows: Nevertheless, to the best of our knowledge, no study was performed on the altered ATP release from RBCs in any atherosclerosis model in the literature.
   No study is performed in the literature, thus, the above sentence is incorrect.
2. on page 35 one reads as follows: Red blood cells (RBCs) are exclusively differentiated to perform their key function – deliver oxygen to each cell within living organism.
   This statement is too strong, because "exclusive" does no leaves an alternative, while in the same sentence the author states the "key function" of RBCs, thus not the only one. This sentence should be reworded.
3. In the reference article describing the mouse model of ApoE/LDLR-/- (Ishibashi et al. PNAS USA 91(4431-35) 1994) the authors state that: all mice used in experiments are hybrids between C57Bl/6J and 129 Sv strains.
   How does the candidate explain the use of C57Bl/6J mice and not hybrids with 129 Sv as the control for his own studies?
4. on page 69 one reads as follows: The RBC membrane skeleton is attached to the lipid bilayer, composed of various type of integral membrane proteins and lipids, mainly cholesterol, phospholipids and sphingolipids.
   There are several inconsistencies in this sentence. It should be rewritten?
5. on page 70 one reads as follows (in bold): The present chapter comprises the second part of the first objective of the present thesis.
   This is very confusing sentence
6. on page 83 one reads as follows: Mature RBCs do not have nuclei and, therefore, are unable to synthesize lipids.
   Why the lack of nuclei blocks lipids synthesis? Please develop this subject.
7. on page 84 one reads as follows: Female sex hormones display a protective role on RBC membrane integrity associated with a decrease in lipid peroxidation. Conversely, male sex hormone testosterone could promote loss of RBC membrane integrity and increased susceptibility to hemolysis.
   Please, comment more on the role of sex hormones in RBCs membrane specificity.
8. on page 85 you mentioned that "mice lack CETP…"
   Please comment more about how this may influence the importance of the mouse model in studying atherosclerosis in humans.
9. on page 85 one reads as follows: As noted above, the total esterified lipids level in RBC membranes is likely to negatively correlation with the plasma HDL…
   It should read "negatively correlate" and not "negatively correlation".
10. on page 99, fig. 8.1 shows a Western blot following SDS/PAGE of PANX1 protein apparently migrating in the gel as monomer and dimer forms.
    What kind of chemical bonds are involved in the dimerization of this protein since it is a dimer in the presence of SDS?

11. on page 125, fig. 9.2 600μm and 30μm are not shown on the same scale. Please, explain and correct it.

In conclusion, the thesis by Mr. Fatih Celal Alçiçek is of substantial scientific merit, the author can defend orally his Ph.D. thesis, and I recommend distinguishing this work.

Jacek Kubiak