Assessment of the dissertation thesis of Msc. Natividad Alquezar Artieda "Cancer metabolism and its role in the sensitivity of leukemic cells to L-asparaginase" presented within the doctoral study program of Molecular and Cellular Biology, Genetics and Virology.

The dissertation thesis of Msc. Natividad Alqueezar Artieda is focused on metabolic rewiring that is connected with the resistance to asparaginase treatment in childhood leukemia patients as these adaptations have an important impact on the outcome of the treatment. One of the studies presented within this thesis is also characterisation of metabolic changes in ALL patients with JAK2 deregulation. The topic is therefore very important and has significant clinical implications not to mention that it illustrates interesting molecular mechanisms that leukemic cell utilize to escape the treatment..

Formal quality of the thesis

The work is formally well written, the mistakes and typos are very rare. I have a question regarding not listing the manuscripts as integral part of the thesis and rather supplying them as attachments. What was the reason to do so?

Language

The thesis is written in English and I have to say that it is written in good, readable English with broad vocabulary.

Assessment of individual parts of dissertation thesis

1) Introduction

The introduction part summarizes the current knowledge on the main metabolic pathways discussed in the thesis, glycolysis, TCA cycle/oxidative phosphorylation and fatty acid oxidation. Furthermore it describes the mode of action of asparaginase on leukemic cells and metabolic changes that such treatment elicits. It further illustrates the role of cellular signalling pathways that are critical for AA sensing such as the mTOR pathway. I believe that the introduction is succinct, yet comprehensive and mentions all important points that have to be addressed. The only thing to point out is the resolution of some of the Figures as they seem a bit blurred (Figure 5 and 6) but still they are sufficient to present valid information.

2) Aims

The aims of the thesis are clearly defined as four sub aims that do focus on the role of the tumour microenvironment and metabolic rewiring in conferring the resistance to ASNase treatment. Additional sub aims then set to investigate the role of PTEN and PI3kinase signalling pathway and deregulation of JAK2 on leukemic metabolism and the responsiveness to the action of ASNase. They are therefore quite complex but all connected around one topic.

3) Results and Discussion

The work is a sum of four papers, three already published and one in revision. The three publications that have gone through the peer-review process have been already externally assessed and validated so I do not need to comment on these. The last one which is being considered for publication in Hemasphere shows solid data and valid interpretations, at least in my opinion.

The results clearly show that BM cells can donate Asn to the leukemic cells and this mechanism can help to overcome their inherent inability to synthesize Asn. Furthermore, it is evident that the absence of PTEN tumour suppressor leads to activation of AKT and Notch pathways to help them rewire their metabolism

and survive. Of note, cells that have higher glycolytic flux, lower OCR and high MMP seems to be the ones to possess inherent resistance to the ASNase treatment. Of particular interest is then the finding that two different fusion JAK2 proteins have a very different impact on cellular metabolism thus prompting to base the patient stratification not only on genetic testing but also a metabolic one. In summary, the findings of the presented thesis are solid, important and show that exploiting metabolic vulnerabilities that enable them survive the ASNase treatment could be a novel and effective approach.

Questions, notes and comments:

1) The Ph.D. candidate has been able to show that mitochondrial respiration is diminished and glycolytic flux is enhanced in the ASNase resistant group of leukemic cells. My question is whether there is any hint as to what mechanisms is behind this phenomenon. Could that be due to a decrease and diminished assembly of mitochondrial respiratory complexes? Also is the mitochondrial structure affected in these cases, e.g. are mitochondria fragmented and how do the cristae look like?
2) The tool to determine the inner mitochondrial membrane potential was TMRE. Is that superior to the other probe TMRM that is also widely used to asses MMP?
3) Is there any evidence whether ASNase treatment affects ROS production within mitochondria in the sensitive cells and could this be altered in ASNase-resistant cells? Is it known whether the committed cell death is apoptosis or does it involve some other death modality?
4) The leukemic cells can apparently use alternative pathways to obtain energy such as glycolysis and FAO. What about the pentose pathway, is there any evidence whether this is also deregulated in the resistant cells? Since this pathway could provide also reducing equivalents for antioxidant pathways and biosynthetic pathways, it seems an interesting candidate to look at.
5) There is a clear downregulation of Asn and GLn in cells treated by ASNse due to its enzymatic activity. Might it be possible that metabolism of other AA could be also altered, even though indirectly?
6) The author shows that in the co-culture experiments between BM cells and leukemic ones exposing cells to hypoxia did not affect the response and therefore was not used in further experiments. Is it possible that these cells have already activated the hypoxic response irrespective of the oxygen surplus in that *in vitro* experimental setup? Are there any data regarding the role of HIF1α or HIF2α in the resistance to ASNs?

Overall assessment of the Ph.D. thesis

After reading this Ph.D. thesis, I fully agree with its approval in order to grant the Ph.D. title to the candidate. The thesis is clearly written, brings substantial new findings and shows that the Ph.D. candidate is very well able to read the literature, put it into context, carry out the experiments, analyse them and interpret them as well as publish in the peer reviewed international journals. I wish the Ph.D. candidate a successful defence and many more scientific successes in the future.

Prague 02.09.2023 Mgr. Jaroslav Truksa Ph.D.