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Dear Prof Komárek,

Re: Review of Doctoral Thesis – MUDr. Michaela Kotrová

Firstly, I would like to thank the 2nd Faculty of Medicine, Charles University, Prague, the specialist board of the Molecular and Cell Biology, Genetics and Virology doctoral program, and the Committee for the Defence of Doctoral Theses for the opportunity to examine this thesis. Professor Trka and the CLIP group have a long-established reputation for the quality of their MRD-based work and it is therefore a pleasure to be asked to review this work.

MUDr. Kotrová should be congratulated on the scope and quality of the work presented in her dissertation and there is no doubt in my mind that this demonstrates the necessary scientific rigor and relevance required for the attainment of the title of PhD. An essential requirement of a PhD is the creation of new knowledge. In building on the previous work and experience of the CLIP group, there are several aspects of the work outlined in this thesis which add significantly to the emerging topic of NGS-based MRD assessment. Currently, the application of NGS technology within the field of haemato-oncology falls into studies looking at either clonality assessment, repertoire analysis or MRD. The work presented in this thesis blurs the boundaries line between MRD assessment and repertoire analysis for prognostication in ALL and as a consequence, represent an original contribution to the knowledge of this discipline.

MUDr. Kotrová's thesis presents an exploration of the clinical utility of NGS technology for MRD analysis firstly for MRD target identification and secondly for MRD quantitation. The approach for MRD target identification is significantly quicker than the conventional methodology and one that as a consequence is believed to be relatively cost effective. It is commendable that an in-house approach to library preparation has been developed rather than relying upon the use of a commercially available kit. The approach described would also appear to have the benefit of being platform independent. In addition, this multi-loci approach is able to identify MRD targets for closely related species and for those patients with sub-clonal populations. A simple approach to MRD quantitation based on the number of reads per clone expressed as a percentage of the total number of reads is presented. This correlates well with qPCR, a technique that is accepted as the current gold standard approach for MRD quantitation. Where discrepancies were observed, these were found to occur at MRD levels below the sensitivities of the two techniques. Analysing what affect these differences in MRD levels between the two technologies would have upon risk-stratification allowed Michaela to demonstrate that NGS provides more a precise risk-group stratification as compared to qPCR.

One of the most striking aspects of this dissertation is the finding that at day 78 the repertoire diversity can be used to predict the risk of relapse where patients with a higher diversity had longer relapse-free survival than those with lower diversity. This diversity was found to be independent of MRD and as a consequence the clinical utility of this NGS-based assessment warrants further investigation. The significance of this finding is such that it was accepted for publication as a letter in the highly prestigious journal Blood.

Michaela also presents a very convincing investigation of the significance that false-positives identified by qPCR play in patients following haematopoietic stem cell transplantation. This extends work previously reported by the CLIP group by Fronkova *et al.,* in 2008. NGS technology was used to demonstrate that low-level qPCR results identified in these patients was a consequence of the non-specific amplification of rearranged Ig/TCR genes. In order to emphasis the point being made, my feeling is that it would have been useful to include the sequences and identities of the Ig/TCR rearrangements that were observed. Having demonstrated a more reliable way of defining low-level MRD in this setting, it would have been interesting to know whether this was also the case for those patients included in the analysis of NGS-MRD and the prediction of relapse. Nine patients without relapse were found to be NGS-negative, qPCR positive at day 33 and it is intriguing to know whether the more precise definition of low-level MRD afforded by NGS-MRD would have been beneficial during frontline therapy.

In addition to the letter published in Blood, Michaela's thesis also includes a series of papers that include her as one of the key authors. Whilst these are not directly related to the topic of this thesis they do demonstrate the breadth of her knowledge and laboratory skills, and importantly, illustrate the contribution she is making to advancing the expertise and reputation of the CLIP laboratory. For completeness, it would have been informative for Michaela to include a short summary of her contribution to each of these published works.

It is also significant to note that Michaela and other scientists from the CLIP group are playing a central role in the standardisation of NGS-MRD and application of this technology within this field through the work they are doing in collaboration with the EuroClonality-NGS consortium. Although this work is yet to be published, it is highly likely that work done through this collaboration will play an important role in exploring the clinical utility of NGS-based MRD, in validating how it should be best utilised in routine laboratory practice and in determining how best it should be implemented in future MRD-based clinical trials.

Michaela's thesis is well written and is presented in a manner that makes it very readable. The introduction is very thorough and sets the scene very well for the investigation that follows. However, I would perhaps have expected it to be more thoroughly referenced. Then again, this may well reflect my previous experiences of reviewing UK-based PhD theses, which tend to be much longer, rather than highlighting any particular concerns over the scope of Michaela's work and her knowledge. Bearing in mind the CLIP's groups expertise in flow cytometry, a more extensive account of the strengths and weakness of flow cytometry-based MRD assessment could have been of value, particularly as the design of the next set of MRD-based clinical protocols includes an early and rapid assessment of MRD at day 8/15. The search for MRD markers using the conventional Ig/TCR analysis precludes such analysis so an investigation of the application of flow cytometry and NGS-MRD early during treatment would have been of value.

The method section is very comprehensive and gives a full account of the work done. The results for this thesis are also well written and Michaela presents her findings in a clear and concise manner. Where possible, the results and their significance in respect to other published findings are discussed directly. The exception is Figure 26, which presents an analysis of the twenty most abundant V-J combinations found within the buffy coat control performed using the EC-NGS Interrogate software and was done on both the Ion Torrent and Illumina platforms. However, the value of these results is almost lost in Figure 26, which is very difficult to read.

The discussion is concise and highlights the major findings of the project. Of interest is the cost benefit analysis for this approach that is included within the discussion. My feeling is that this would benefit considerably from the inclusion of labour costs. In the assessment given, prices for MRD detection by qPCR are only cheaper if 6-7 time-points are analysed due to the costs of designing and optimising two patient-specific RQ-PCR assays. For the most part, the majority of MRD–based clinical protocols utilise the assessment of MRD at either one or two time-points only and tend not to rely on the analysis at multiple time-points. Michaela also states that after a suitable period of testing in parallel that the CLIP group plans to switch to utilising the NGS approach for MRD target identification. With this in mind, it would be useful to break down the costs further into separate ones for target identification and MRD quantitation.

Data analysis for the project is performed using the Vidjil bioinformatic analysis pipeline (CRIStAL, Université de Lille). As the initial phase of the Vidjil pipeline does not include an alignment of all of the sequences produced relative to germline sequence databases but rather clusters identical sequences before alignment, the Vidjil software presents a fast and reliable bioinformatic pipeline able to perform multiclonal clusterization of V(D)J recombinations from high-throughput sequencing. The speed of this adds to the overall advantage of the NGS-MRD approach utilised in this project. Having had some personal experience of the Vidjil pipeline it would have been interesting to have some data on the fidelity of the classification of junctional regions. Examination of the most recent report by Feret *et al.,* (2016), which outlines a direct comparison of an NGS-based approach with conventional MRD technology in a prospective cohort of patients, the authors report that in comparison to IMGT and IgBlast that Vidjil produces a slightly imprecise positioning of the V(D)J junction (Ferret *et al.,* 2016). Therefore, it would have been of interest if some information regarding the fidelity of the junctional analysis for the patients examined as part of this thesis had been included particularly as Michael's suggests that a switch to NGS target identification is likely to be made soon. However, this and my other comments most likely represent an overly zealous review that stems from this reviewers interest in the subject rather than any particular oversight in the structure and content of Michaela's thesis which is an excellent piece of work.

In summary, the benefits of the methodology for MRD analysis presented in MUDr. Kotrová's thesis are significant and represent important advantages over the current methodology. In addition to the practical advantages of this new approach, the application of NGS-MRD allows more precise risk-group stratification as compared to qPCR and demonstrates a more reliable way of defining low-level MRD following HSCT. The emergence of a potential role for repertoire diversity in MRD prognostication is particularly exciting. As a consequence, these findings are likely to be of interest not just to other MRD-based laboratories but also to clinical centres with an interest in performing MRD analysis but who lack the necessary experience and expertise in Ig/TCR qPCR.

In conclusion, it has been a pleasure to review this thesis. I am delighted to confirm my opinion that it demonstrates the necessary scientific rigor and relevance required and as a consequence, I have no hesitation in recommending that the title of PhD be granted.

Yours sincerely,

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