Author's response to reviews

Title: Assessment of the feasibility of exon 45-55 multiexon skipping for Duchenne Muscular Dystrophy

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Author's response to reviews:

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To: The Editor of BMC Medical Genetics

Subject: Manuscript submission

Dear Prof Koutsos,

On behalf of the co-authors, I would like to resubmit our manuscript “Assessment of the feasibility of exon 45-55 multiexon skipping for Duchenne Muscular Dystrophy” by van Vliet et al for publication in BMC Medical Genetics. We apologize for the delay of submitting a response (or rather failing to communicate to you that there would be a delay).

The comments and suggestions of the authors were useful and we have addressed them as much as possible. A point by point outline is given below.

Finally, while we agree with Dr Tuffery-Giraud (one of the reviewers) that only limited data is added to our previous published work on exon skipping, we feel these results are important nonetheless. Numerous researchers are exploring exon 45-55 multi-exon skipping and as one of the leaders in the field, we feel it is important to report that we have explored this as well and did not succeed.

Yours sincerely,

Annemieke Aartsma-Rus

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Response to reviewers and additional changes

1. We have added a conflict of interest statement (no conflict of interest declared)

Dr Bertoni

2. Dr Bertoni states that the results in Figure 2 are difficult to interpret. We agree that they are somewhat complex, but the reason we chose to show them like this, was to underline the complexity of the analysis, the naturally occurring exon 45-55 skipping and the problem of alternative products produced in addition to the aimed product. We would like to point out that there is only a single PCR artifact and that the majority of alternative products result from the skipping of some, but not all of the intended exons. One of the main messages we want to get across is that as a result of the treatment there is dilution of the intended effect (due to all the alternative products produced) and we feel this is reflected in the Figure. Finally, we would like to point out that we agree that a more sensitive method might be able to pick up potential small differences between treated and untreated cells. However, robust and reproducible exon 45-55 exon skipping is needed to restore dystrophin expression at significant levels and therefore we feel that the current PCR method was well suited.

3. Dr Bertoni wonders about the lack of full length dystrophin in Figure 2. As now pointed out in the manuscript, this is due to the length of the expected product. After treatment with the cocktail many cells die (due to toxicity of the transfection reagent) and therefore it is more difficult to pick up the full length, almost 2 kb transcripts. We have been able to confirm the presence of a wild type band for most samples by increasing the extension time and the number of PCR cycles. Note that for some of the samples treated with the linked AON full length products are present. For the Western blot analysis, see point 9 of the other reviewer.

4. Regarding the different chemistries: we chose this chemistry because it works very well in cultured cells. The morpholino chemistry is very hard to transfect (due to their uncharged nature) and while this chemistry is very efficient in inducing exon 23 skipping in the mdx mouse model, it is less efficient targeting human exons (RNA 2007, 13: 1609-1624 and Heemskerk et al submitted)
manuscript). Locked nucleic acids have been shown efficient splice modulators, but they are less sequence specific and bind very efficient to other LNAs (thus using a combination of LNAs is unfeasible). We have now included this in the Results section (end of 1st paragraph).

5. As suggested we have changed the sentence “the most promising” to “a very promising” in the Introduction.

Dr Tuffery-Giraud

6. Regarding the disappearance of the full length bands in the middle section of Figure 2 see point 3 of the other reviewer

7. In contrast to what the reviewer says, many intermediate bands are visible after treatment with the cocktail (ranging from 200-800 nt). It is possible that these look like a smear on a lower resolution figure.

8. The putative exon 45-55 skip RT-PCR bands have been sequenced to confirm their nature to exclude PCR artifacts. This is now included in the Results section (page 8, 2nd sentence) and Legends to Figure 2 (final sentence).

9. As suggested by both reviewers we have performed Western blot analysis for the exon 48-50 deletion patient (the myogenicity of the other line was too low to expect any dystrophin expression). (Methods section added methods for Western blotting, added Figure 3 and Legends to Figure 3. Figure 3 is discussed in the last part of the Results section). As can be seen in the new Figure 3, no dystrophin could be observed before or after treatment of the cells (expected size ~350-365 kD after exon 45-55 skipping).

10. We have made the two suggested changes as they were correctly pointed out mistakes. (changed AON to exon in line 11 of the Results and changed intron 45 and intron 56 into intron 44 and 55 in the background section).