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Peer Review File

Ataluren Improves Hematopoietic and Pancreatic Disorders in Shwachman-Diamond Syndrome patients: a compassionate program case-series

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have satisfactorily addressed all concerns that this reviewer raised with the original version of the manuscript. The revised manuscript is substantially improved, and in sum stands to provide a critical advance to the field as a proof of concept that read through therapy with ataluren holds the potential to improve hematopoiesis in patients with Shwachman Diamond Syndrome.

Reviewer #3

(Remarks to the Author)
The authors have addressed all concerns and have presented an improved report of the compassionate use of a translational read-through-inducing drug in Swachman-Diamond Syndrome. In particular, the pancreatic responses are more precisely quantified, and the assessment of a hematopathologist is included.

Minor concerns:
1. Marrow cellularly should be quantified by percentage for all participants not just UPN74.

2. The title and first sentence in the new Figure 5 mention digestive enzymes and stool samples which are not in this figure.

Point-by-point response to reviewers

Ataluren Treatment Improves Hematopoietic and Pancreatic Disorders in Patients with Shwachman-Diamond Syndrome

Valentino Bezzerri, Anna Pegoraro, Anca Manuela Hristodor, Genevieve M. Crane, Ilaria Meneghelli, Cecilia Brignole, Christian Boni, Elena Baldisseri, Antonio Vella, Giacomo Menichetti, Roberto Valli, Giovanna D'Amico, Cristina Tecchio, Alice Parisi, Giuseppe Lippi, Angela Mercuri, Simone Cesaro, Seth J Corey, Marco Cipolli

We are grateful to the reviewers for their comments and suggestions. We improved the revised version of the manuscript following your recommendations. The manuscript has been significantly improved and sounds now more convincing in our opinion.

Revisions to the original manuscript have been marked in red throughout the text.

REVIEWER COMMENTS

Reviewer #1 Comments:

Bezzerri and colleagues present results from a provocative compassionate use pilot study of the translational read-through agent Ataluren in 3 patients with Shwachman-Diamond Syndrome (SDS). The rationale for using Ataluren in SDS is strong given that the vast majority of patients with SDS have a heterozygous c.183-184TA>CT variant leading to an in-frame nonsense stop codon change (K62X). Given that the other SBDS allele in patients with SDS is typically a hypomorphic slicing mutation, any improvement in read-through of the nonsense allele would be predicted to improve the ribosome pathophysiology in SDS. This group has previously reported data from ex vivo SDS cell models showing improvement in SBDS protein expression and reduction of ribosomal stress biomarkers in P53 and MTOR pathways. Results of the study presented here show modest but consistent improvement in hematologic/hematopoietic parameters, in vivo demonstration of improved SBDS expression and downstream improvements in activation of ribosomal stress pathways, and significant improvement in pancreatic enzyme levels. Combined, these results demonstrate on-target activity of ataluren for correcting pathophysiology, providing proof of concept for future studies.

R1.1. We agree with this reviewer that this study represents a proof of concept for future studies. According to the reviewer statement, these results demonstrate on-target activity of ataluren for correcting SDS pathophysiology, representing the first attempt of a personalized medicine approach for patients with SDS.

However, the extent of effects seen raises concerns that at least short-term ataluren treatment may not provide marked clinical benefit; whether earlier age or prolongation of ataluren treatment would augment the extent of this therapeutic impact remains unknown.

Major comments:

1. The improvements in hematologic parameters after 1 year of Ataluren therapy are modest, and I would not expect these changes to provide immediate clinical benefit. 2 of 3 patients did show increased ANC, but this ANC improvement left patients in the moderate neutropenia range.

R1.2. At t9 (9 months of treatment) two out of three patients displayed ANC > 1500 cells/mm^3 (see new Figure 3). We clarified this point in results section (lines 110-111) . However, ANC did not improved upon treatment in UPN58. Our hypothesis is that the individual genetic background may influence ataluren activity. However, UPN58 showed a remarkable increase neutrophil maturation (new Fig. 2) and a large improvement in platelet count, indicating that myeloid differentiation was partially improved by ataluren treatment (lines 103-112).

Platelet count improvement was significantly more impressive, though patients went from high moderate thrombocytopenia at baseline (where they would have no substantial bleeding risk) to mild thrombocytopenia. Do the authors expect that longer exposure to ataluren perhaps beginning at an earlier age would lead to more significant improvement in these parameters? Otherwise, the clinical benefit of ataluren would be restricted to preventing MDS onset, a benefit that would require a much longer study to prove.

R1.3. We thank the reviewer for pointing out this important aspect. Ataluren clinical efficacy was observed improved in younger patients with Duchenne muscular dystrophy. We would expect a more significant improvement in bone marrow cellularity and myeloid differentiation in younger SDS patients as well. We pointed out this observation in the revised text (lines 157-158). An enlargement of the study cohort in a registered international clinical trial will provide responses on this question.

2. The authors note that all 3 subjects underwent BM aspirates/biopsies after 6 months of study treatment, but they only present the primary BM biopsy data and colony assays from the aspirates. Figure 1b does support improved cellularity in UPN26 and UPN58, but the cortical nature of the biopsy at T7 in UPN74 makes comparison to baseline difficult, as there is minimal evaluable marrow space in the T7 biopsy pictures shown for UPN74.

R1.4. We agree with this reviewer that the cortical nature of the biopsy at T7 (6 months of treatment) in UPN74 impaired the comparison to the baseline. Therefore, we collected deeper sections from the bone marrow biopsy in order to exclude a cortical observation. New Figure 1B now clearly shows that UPN74 improved bone marrow cellularity and myeloid maturation upon ataluren treatment. An improved assessment of morphology of BM biopsies was performed by our clinical pathologists and has been reported in results section (lines 91-98).

Did the authors conduct morphologic or flow cytometric data on the BM aspirates in these patients at baseline and at 6 months to demonstrate increasing myeloid content? What happens to percentage of immunophenotypic HSC (CD34+) and myeloid progenitor populations in BM for example?

R1.5. Due to the nature of the compassionate program, we could not collect additional aspirates of BM, but we used a small amount of the aspirates already collected for clinical purposes. In these samples, myelocyte differentiation into neutrophils was assessed before and after therapy by the oncology hematology unit of the University Hospital of Verona. We collected and analyzed these data according to reviewer's suggestion. New Figure 2 shows that neutrophil maturation is remarkably improved both in UPN26 and UPN58 (despite in this patient there was not ANC improvement), whereas UPN74 showed a good myelocyte differentiation into neutrophils already at the baseline, which did not significantly change upon therapy. All these aspects have been discussed in the revised manuscript (lines 103-109).

3. Table 1 shows cytogenetic and array data showing that all 3 treated subjects had 20q deletions that remained stable in size post-ataluren treatment. Were FISH assessments performed to look more sensitively for Chr 7 changes at each timepoint? Was somatic mutation NGS testing performed to look for presence of TP53 mutations, which are the most common mutations in patients with SDS and important biomarkers of MDS risk?

R1.6. Although we agree that aCGH might have a lower sensitivity than FISH analysis in detecting low percentage clones, hypocellular bone marrow specimens limited the possibility of further FISH analyses. According to the reviewer's recommendations, we performed NGS tests to look for presence of *TP53* mutations before and after ataluren treatment. Results are reported in new Supplementary table 2. We did not observe changes in the mutational signature of *TP53* upon ataluren treatment (discussed in lines 81-84).

4. The study does demonstrate reduction in ribosome stress using reduction in Phospho-mTOR as a biomarker. However, they also show using inhibitor studies that MTOR inhibition alone does not improve myelopoiesis in the same manner as is seen with ataluren. To further confirm mechanism of action, this work would benefit from investigation of other correlatives of improved ribosome function, including polysome profiling to prove that impacts of ataluren are due to improvement in ribosome stress.

R1.7. Unfortunately, we could not perform polysome profile analysis because of the scarce human sample amounts obtained in this compassionate program. However, we previously reported that ataluren can improve polysomes, increasing the assembly of 80S ribosomes in LCL derived from SDS patients (Cipolli et al. Br J Haematol. 2023).

5. Improvement in fecal and serum pancreatic enzyme levels with ataluren stood out as the largest improvement from SDS baseline. Can the authors comment on whether these levels

improved enough to allow dose reduction in enzyme supplementation and/or improvement in stools/fat absorption, as measured by other markers?

R1.8. On the basis of our large experience in Cystic Fibrosis and Shwachman-Diamond syndrome, it is challenging to define the minimal level of pancreatic enzymes able to allow a sufficient intestinal fat absorption. Since we know that 10-20% of functional pancreatic gland is enough to generate a sufficient fat absorption, probably it is not necessary to restore the normal levels of pancreatic enzymes to restore pancreatic insufficiency. The decision to stop PERT is generally due to the level of steatorrhea rather than the absolute levels of pancreatic enzymes. We discussed these aspects in the revised manuscript (lines 125-130).

Minor comments:

1. Figure 1a appears to have a labeling misalignment that makes interpretation of the western blot somewhat confusing. Also, did the authors confirm and quantify western blot findings by qPCR?

2. Figure 1c should include the quantification of colony assay outputs and should compare each patient at baseline with respect to HD controls then again at 6 months of ataluren therapy compared to HD controls. The delta compared to healthy control at each time point would be the most direct metric of improved myelopoietic capacity.

R1.9. Revised Figure 1 has been improved according to the reviewer's observations.

Reviewer#3 Comments:

In their manuscript entitled "Ataluren Treatment Improves Hematopoietic and Pancreatic Disorders in Patients with Swachman-Diamond Syndrome" (SDS), the authors provide a report on the compassionate use of a translational read-through-inducing drug to improve two key features of SDS: neutropenia and pancreatic insufficiency. The authors provide excellent scientific rationale for the use of an agent approved for use in Europe in a disease where ~ 50% of patients carry a mutation that may be responsive to this treatment. They report ataluren is well tolerated in 3 patients with SDS and provide clinical data demonstrating response.

R2.1. We thank this reviewer for appreciating our work.

Notably, the patient that had the "best" exocrine pancreatic response did not have a significant improvement in neutrophil counts and the 2 patients with significant improvements in peripheral neutrophil responses had less impressive exocrine pancreatic changes. Although the sample size is limited, the discussion is well written and this report would benefit from more precise quantification of the bone marrow and pancreas responses in each patient.

Major concerns:

1. The authors nicely show Western blots that demonstrate increased SBDS protein in the

bone marrow or peripheral blood of patients treated with ataluren. These data can be strengthened with densitometry to illustrate the relative amounts of SBDS at each time point.
R2.1. Figures have been improved according to the reviewer's observations. Densitometry analysis is indicated in revised Figure 1a.

2. Similarly, while the increase in bone marrow cellularly appears impressive in Figure 1B, it would be helpful to have a pathologist's assessment of the numerical change in the bone marrow cellularly and the myeloid:erythroid ratio.

R2.2. A new statement from our pathologist has been included in the revised main text, accordingly (lines 91-98). Please note that revised Figure 1b has been now improved with deeper sections of bone marrow biopsy from UPN74, strengthening the clinical outcome.

3. The authors show colony assays performed with healthy donor samples and various SDS samples in Figure 1 and Extended Data Figure 1 but do not quantify the colonies observed on each plate. This is particularly important as the authors allude to differences between erythroid and myeloid colony formation after ataluren treatment.

R2.3. We quantified colonies as recommended by this reviewer (revised Figure 1d-e).

4. The change in peripheral blood neutrophil and platelet counts in Extended Data Figure 2b and 2d could be moved to Figure 1 to provide additional evidence of bone marrow response. Were blood counts at the same time post treatment used for each patient?

R2.4. We moved Extend Data Figure 2b and 2d to new Figure 3, accordingly. Blood counts were performed in each patient exactly at the same time post treatment.

5. The changes in mTOR phosphorylation in the peripheral blood are intriguing but it seems the ultimate conclusion is that these changes are secondary to improved SBDS levels and do not correlate with neutrophil response. It would be helpful to include ataluren treated samples in the experiment in Extended data Figure 6 to show that translational read through is distinct from mTOR inhibition in the same samples. Were the authors able to evaluate mTOR phosphorylation in CD34+ bone marrow cells to determine if the drug affects mTOR activation in more immature progenitors?

R1.5. We included ataluren treated samples in revised Supplementary Figure 6, according to the reviewer's suggestion. Unfortunately, we could not perform Phospho-flow analysis for mTOR in BM aspirates because of the scarce human sample amounts obtained in this compassionate program.

6. The authors do not quantify the change in serum amylase for each patient (Figure 2F) or comment on whether the patient with the most impressive response had any change in use of supplemental pancreatic enzymes.

R1.6. Figure 2f has been uniformed with Figure 2e in order to clarify the change in serum amylase upon ataluren treatment for each patient (see new Fig. 4). Pancreatic enzyme replacement therapy (PERT) was not discontinued during ataluren treatment. However, given the impressive results in terms of improvement of release of pancreatic enzymes, we may predict a reduction/stop of PERT in a long-term clinical trial. This has been pointed out in the revised manuscript (lines 125-130).

Minor concerns:

1. Can the authors comment on whether the mutation composition of each patient impacts response to the drug?

R1.7. All SDS patients enrolled have the same *SBDS* genotype (c.183-184TA>CT/c.258+2T>C). However, the genetic background might influence ataluren activity. The amount of *SBDS* transcript, which may vary among different patients, along with the expression of other genes associated with ribosome biogenesis, may affect ataluren efficacy. This has been pointed out in the revised manuscript (lines 155-158).

2. The T0, T4, T7 distinctions for duration of treatment can be confusing. Please just list time of treatment in months.

R1.8. We agree with the reviewer that time of treatment in months is the correct manner to represent data. We edited figures accordingly.