Author's response to reviews

Title: Circadian gene expression and impact of iduronate-2-sulfatase treatment in human fibroblasts from Hunter syndrome

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Version: 2 Date: 15 July 2013

Author's response to reviews: see over
Dear Editor,

Attached you will find a REVISED version of the manuscript MS: 8517379010098279 submitted for publication as a full research article entitled “Circadian gene expression and impact of iduronate-2-sulfatase treatment in human fibroblasts from Hunter syndrome” and that after referee’s suggestion we have now titled “Circadian transcriptome analysis in human fibroblasts from Hunter syndrome and impact of iduronate-2-sulfatase treatment”

All authors have read and approved the final version of the manuscript, which has not been previously published, nor is being considered for publication elsewhere in whole or in part in any language, except as an abstract.

The analysis conducted in this work started from Next generation Sequencing data previously obtained by the Padova research unit as a wider study of whole transcriptome analysis, whose results will soon be published in a separate paper.

Part of the whole transcriptome data, specifically concerning the circadian transcripts, was object of deeper bioinformatic analysis and taken as starting point for further experiments on cell cultures, as described in the present paper.

In the first submission to "Genome Biology" the authorship included only authors who had contributed to these targeted bioinformatic analyses and subsequent experiments, while did not erroneously include other three authors who actively participated in experimental plan design and whole transcriptome data production/analysis. In case of acceptance of our work by "Genome Biology" editorial team, we would have requested the inclusion in the authorship of these authors. Being the redirection process to "BMC Medical Genomics" automatic, we did not have until now the chance to make such changes. Therefore, together with the full acceptance of the revisions suggested by the reviewers and a point-by-point reply to all questions raised, I kindly request you, with the consent of all authors, to make the changes in the authorship, as indicated in the new version of the manuscript.

Looking forward of hearing from you, I remain sincerely yours,

Gianluigi Mazzoccoli MD
RESPONSE TO REVIEWERS

Reviewer's report

Title: Circadian gene expression and impact of iduronate-2-sulfatase treatment in human fibroblasts from Hunter syndrome

Version: 1 Date: 1 July 2013

Reviewer: Gigliola Grassi Zucconi

Reviewer's report:

The study is aimed at verifying an involvement of clock genes in mucopolysaccharidosis Type II (Hunter syndrome), a lysosomal storage disease caused by deficiency of the enzyme iduronate-2-sulfatase (I2S). The study, pursued on fibroblasts of patients affected by Hunter syndrome, shows altered levels of clock gene expression during the disease. In addition, the findings indicate that treatment with idursulfase (the recombinant form of the enzyme) results in transient changes in the expression of these genes. The addressed questions are clearly presented, the methods are adequate and properly described, and the conclusions seem supported by the data (but see point 3 of “discretionary revisions”).

The article therefore reports data of interest also in relation to knowledge on a disease that is at present incurable.

The following issues should, however, be taken into account to improve the manuscript:

Minor Essential Revisions

1. for the ARNTL and NR1D, also the alternative titles BMAL and REV-ERB should be reported

CORRECTED

2. BACKGROUND line 9, “lysosomal” should be corrected

CORRECTED

3. FOCUS line 4, “syndrome” should be corrected

CORRECTED

Discretionary Revisions

1. The Discussion should be shortened and should be more stringent, also with a more rigorous selection of quoted references (the deletion of non essential references would make the text easier to read)

WE AGREE WITH THE REFEREE, THE DISCUSSION SECTION IS RATHER LONG, BUT THE COMPLEXITY OF THE ISSUE REQUIRES EXTENSIVE DESCRIPTION, AS EVIDENCED BY THE OTHER REFEREES
2. Table 1 should be reorganized, presenting only the expression of the genes discussed in the text. The other data should be presented as supplemental material.

WE HAVE TRIED TO ADDRESS THIS SUGGESTION. WE HAVE SELECTED ALL THE CORE CLOCK GENES AND ONLY THE CLOCK CONTROLLED GENES DIFFERENTIALLY EXPRESSED WITH A P VALUE <0.001 (TRESHOD VALUE CONSIDERED TO SELECT GENES FOR THE HYPERGRAPH ANALYSIS), AND WE HAVE TRIED TO ADD A SUPPLEMENTAL TABLE WITH GENES DIFFERENTIALLY EXPRESSED AND P VALUE >0.001, BUT THIS LEAD TO 2 TABLES OF THE SAME COMPLEXITY, SO WE PREFERRED TO MAINTAIN THE ORIGINAL TABLE SUBMITTED

3. At the end of the Discussion, the limitations of the study should be adequately emphasized, and in particular the limited number of patients investigated here, who anyhow suffer from a disease that is clinically heterogeneous in terms of onset, severity and progression.

WE HAVE ADDED THIS TO THE END OF THE DISCUSSION SECTION

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**
I declare that I have no competing interests

**Reviewer’s report**

**Title:** Circadian gene expression and impact of iduronate-2-sulfatase treatment in human fibroblasts from Hunter syndrome

**Version:** 1  **Date:** 28 June 2013

**Reviewer:** Ana C Anzulovich

**Reviewer’s report:**
This is a novel and very interesting study that shows an extensive transcriptome analysis performed in fibroblasts from healthy subjects and mucopolysaccharidosis type II patients before and after idursulfase treatment. The authors also evaluated clock genes expression after serum-shock-induced synchronization of normal human fibroblasts and fibroblasts from patients affected by the Hunter syndrome before and after of idursulfase treatment.

In opinion of this reviewer, novelty and methodology are the main strength of this work. The methods are, not only, appropriate and clearly described, but also advanced, including an in vitro model of Hunter syndrome, Next-Generation Sequencing technology, qRT-PCR analysis and the use of bioinformatic tools for a semantic hypergraph-based analysis of circadian gene expression.

Results obtained from that analysis will have a high impact on the understanding of the molecular bases of mucopolysaccharidosis disease, bringing up potential new target genes (clock and clock-controlled genes) for therapeutic strategies. Discussion and conclusions are well balanced and adequately supported by the present data as well as it is by the authors’ and others’ previous work.

Minor Essential Revisions
1-In the Abstract, at the end of the Background paragraph, I suggest to complete the sentence regarding the aim of the work as it follows: ...We aimed to evaluate the expression of CG and downstream CCGs in HS, before and after idursulfase treatment.
CORRECTED

2-In the Background section, on page #3, first line of the 3rd paragraph, I would say ‘circadian expression’, or ‘temporal orchestration’ instead of ‘functioning’ of the clock gene machinery.
CORRECTED

3-All along the Results section of the mns, please, unify groups’ denomination among text, tables title, tables body and figures for example, for the Control healthy group, you might always use C or the whole word ‘Control’ in every text, table and figure where it corresponds. The same for the Hunter syndrome group.
CORRECTED (the acronyms are explained in all the locations)

4-For a better understanding of the results description, it would be helpful if the authors mentioned the group against to which the comparison is made, for example in the subsection “Evaluation by NGS technology of core clock gene expression levels in normal fibroblasts and fibroblasts of patients affected by Hunter syndrome after 24 hours of treatment with idursulfase (T1/C)”, they could write: In Hunter syndrome fibroblasts after 24 hours of treatment with idursulfase ........ genes showed lower expression levels in comparison to controls. In the next paragraph: The genes ARNTL .......... in Hunter syndrome fibroblasts after 24 h treatment, in comparison to healthy human fibroblasts. In the following paragraph: The expression level of CLOCK ........ between normal and idursulfase treated diseased fibroblasts or did not reach the p<0.001 threshold. Another example, in “Evaluation by NGS technology of changes of clock gene expression levels in fibroblasts of patients affected by Hunter syndrome after 24 hours of treatment with idursulfase (T1/H)” In Hunter syndrome fibroblasts after 24 hours of treatment with I2S, ........ showed lower expression levels, in comparison to HS fibroblasts before treatment. The genes CLOCK ........ showed higher expression levels in Hunter syndrome fibroblasts exposed to 24 h treatment with I2S.
And in that way, or similar, to continue describing the following set of results.
CORRECTED AS SUGGESTED BY THE REFEREE

5-In “Evaluation by qRT-PCR after Serum-Shock Induced Synchronization of changes of clock gene expression levels in normal fibroblasts and fibroblasts of patients affected by Hunter syndrome after 24 hours of treatment with idursulfase”
After synchronization by serum shock and qRT-PCR analysis, statistically significant differences were evidenced in the mRNA expression levels of ARNTL2 at 10h (p=0.036), PER1 at 4h (p=0.019), PER2 at 10h (p=0.041) and 16h (p=0.043). Please, indicate in comparison to what group those results are statistically significant (Figure1).
CORRECTED
6-Given that some methods explanations are included in the Results section, my suggestions are: 1) move description of Qualitative hypergraphs building from Results (page #7) to Materials and Methods section, 2) the following paragraphs from the next subsection: “This simple agglomerative weighting function takes the weights of the edges connecting any two nodes in input, and gives a unique value in output. Constitutively, it gives more and more importance to the pairs that are connected by multiple edges” and “[Equation] ...where n is the number of edges connecting any two nodes A and B, and i refers to the ith edge” should be also moved to M&M, as well as 3) the explanation of the weighted topology indices calculation “We recall that the un-weighted formulation of closeness between any two nodes ................., thereby discriminating between reliable and not-reliable interaction paths”.
WE HAVE MOVED SOME ITEMS IN THE METHODS SECTION

7-In Table 1, translate ‘anche detto’ to ‘also known as’ in the first column.
CORRECTED

8-Please, check figures order, Figure 1 is duplicated and it also appears as figure 2, then all following ones, figures 3-10, have wrong numbering and they don’t correspond to their mention in the text nor to their figure legends.
THE FIGURES WERE UPLOADED CORRECTLY, AND THE SUBMITTED PROVISIONAL PDF WAS CORRECT AND CORRECTLY APPROVED. THE MISTAKEN VERSION IS RELATED TO TECHNICAL PROBLEMS NOT IMPUTABLE TO THE AUTHORS

9-Please include a more detailed description of figures 5, 6 and 9, in order to make their comprehension easier.
CORRECTED

"Figure 5: Heat map of the clock gene expression fold changes between untreated patients and control cases (H/C), patients 24 or 144 hours after treatment and control cases (T1/C and T2/C), treated and untreated patients (T1/H and T2/H). Cyan segments and a density plot quantify the magnitude of intra class and global FCs, respectively."

"Figure 6: Heat map of the clock-controlled gene expression fold changes between untreated patients and control cases (H/C), patients 24 or 144 hours after treatment and control cases (T1/C and T2/C), treated and untreated patients (T1/H and T2/H). Cyan segments and a density plot quantify the magnitude of intra class and global FCs, respectively."

"Figure 9: Hierarchical clustering of the weighed interaction networks. Gene are clustered according to the proximity of their expression profiles, evaluated by an Euclidean distance metrics.

10-In the Discussion section, on page #10, in the 2nd paragraph, the authors write: “... An example is represented by SERPINE1, which showed higher expression levels in basal conditions, increased after 24 hours of idursulfase treatment, and decreased after 144 hours...”. They should clarify in comparison to which group SERPINE1 showed higher expression levels in basal conditions.
CORRECTED in HS fibroblasts when compared to control fibroblasts

11-Although the writing is acceptable and the mns is of a quite easy reading, authors should revise and correct some grammar and spelling mistakes. For example:
1) 'core clock genes' should be changed for 'clock core genes' on page #2, at the beginning as well as at the end of the 3rd paragraph, and on page #3 in the 4th paragraph;
WE DO NOT AGREE WITH THIS SUGGESTION (see Cho et al., Nature 2012)
2) 'We evaluated also the expression levels of...' should be said 'We also evaluated the expression levels of...';
CORRECTED
3) instead of 'After synchronization by serum shock and...' they should write 'After serum shock synchronization and...';
CORRECTED
4) 'dysregulation' should be written 'deregulation';
CORRECTED
5) on page #9, 2nd paragraph, '... in peripheral tissues peak CRY1 mRNA expression is delayed...' could be changed to 'in peripheral tissues, peak of CRY1 mRNA expression is delayed...' or 'in peripheral tissues CRY1 mRNA expression peak is delayed...';
CORRECTED
6) on page #10, in 2nd paragraph '...at this time point the genes CSNK1E, CSNK2A1, CSNK2B, NR1D2, and TIMELESS showed...' should be corrected 'at this time point, CSNK1E, CSNK2A1, CSNK2B, NR1D2, and TIMELESS genes showed...';
CORRECTED
7) on the same page, in the last paragraph: '...to at least a biological process or pathway...' change by '...to at least one biological process or pathway...'.
CORRECTED

Discretionary Revisions
1-Given the significance of this work, I´d suggest modify the title including any reference to the methodology, for example an option could be: Circadian transcriptome analysis in human fibroblasts from Hunter syndrome, before and after treatment with idursulfase.
CORRECTED IN THIS WAY Circadian transcriptome analysis in human fibroblasts from Hunter syndrome and impact of iduronate-2-sulfatase treatment

Level of interest: An article of outstanding merit and interest in its field
Quality of written English: Needs some language corrections before being published
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests

Reviewer's report
Title: Circadian gene expression and impact of iduronate-2-sulfatase treatment
The manuscript by Mazzoccoli et al., entitled “Circadian gene expression and impact of iduronate-2-sulfatase treatment in human fibroblasts from Hunter syndrome” discusses the circadian clock controlled gene expression changes present within fibroblasts derived from patients with Hunter syndrome. The findings are indeed very intriguing, especially given the findings that idursulfase has an effect in these altered fibroblasts, and that several key networks linked with the circadian rhythm are associated with Hunter syndrome. The manuscript is well thought out, reads well, and has logic to the experimental development. There are however, in this reviewer’s opinion, that should make the manuscript more clear for the interested readers. The suggestions, all being “minor essential revisions” include the following:

1) Please explain how the authors came to the conclusion that three time points from idursulfase treatment (that is, before treatment, at 24 hours and 144 hours) would be sufficient for your analysis.

THERE IS EVIDENCE THAT THE ENZYME IS TOTALLY ABSORBED BY CELLS AFTER 4-6 HOURS, THE PEAK LEVEL OF INTRACELLULAR CONCENTRATION IS REACHED AFTER 24 HOURS, AND THEN IT GRADUALLY DECREASES. AFTER 6 DAYS INTRACELLULAR CONCENTRATION IS EXTREMELY REDUCED AND FOR THIS REASON PATIENTS ARE TREATED AT WEEKLY INTERVALS.

2) Please reference or explain why the authors decided that 62.5 nM idursulfase exposure was the concentration that was necessary to perform on the serum-shock induced synchronization of Hunter syndrome derived fibroblasts?

THIS CONCENTRATION WAS CHOSEN AFTER EXPERIMENTAL EVALUATIONS OF DIFFERENT DOSES AND CONCENTRATIONS

3) Please note what version of MATLAB was used for the statistical analyses.

MATLAB 6.5

4) Do the authors intend to share with the public their raw expression data? If so, how and where will this be hosted?

THESE EVALUATIONS ARE PART OF A WHOLE TRANSCRIPTOME ANALYSIS AND WE ARE ANALYSING RAW DATA FOR ANOTHER STUDY. THEY WILL BE SHARED WITH THE PUBLIC AS SOON AS POSSIBLE

5) Under “Focus,” please correct spelling for (s)ynrome.

CORRECTED

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:

I declare that I have no competing interests
Reviewer's report
Title: Circadian gene expression and impact of iduronate-2-sulfatase treatment in human fibroblasts from Hunter syndrome
Version: 1 Date: 2 July 2013
Reviewer: Kun-Ruey Shieh

Reviewer's report:

Major Compulsory Revisions
In this study, Mazzoccoli and colleagues have studied that circadian gene expression in human fibroblasts from Hunter syndrome with iduronate-2-sulfatase (elaprase) treatment for 24 or 144 hours. According to the functions of detective gene expression, the authors found 5 clusters of genes are related to the impacts of Hunter syndrome and iduronate-2-sulfatase treatment. These findings are interesting; however, there are some concerns to be answered or revised by the authors.

Major Comments:
1. Figure 1 and 2 were the same. This is unpredictable and unprofessional mistake.

THE FIGURES WERE UPLOADED CORRECTLY, AND THE SUBMITTED PROVISIONAL PDF WAS CORRECT AND CORRECTLY APPROVED. THE MISTAKEN VERSION IS RELATED TO TECHNICAL PROBLEMS NOT IMPUTABLE TO THE AUTHORS

2. Some important rationales were still needed to provide. Especially the specimens were fibroblasts. For example, why or how the changes of gene expression of hypoxia-inducible factor in the cardiovascular system (cluster 1) are important? Why or how the changes of gene expression of liver development (cluster 2) are important? Why or how the changes of gene expression of adipogenesis pathway (cluster 5) are important?

REASONS FOR CHOOSING FIBROBLAST AS MAIN SPECIMEN ARE DRIVEN BY WELL-KNOWN AND CONSOLIDATED CLINIC PRACTICES ABOUT THIS DISEASE. DEFINITIVE DIAGNOSIS IS ESTABLISHED BY ENZYME ASSAY IN LEUKOCYTES AND PLASMA, TOGETHER WITH FIBROBLASTS. THROUGH BIOINFORMATICS ANALYSIS, WE CONFIRMED THOSE BIOLOGICAL PROCESSES WHICH ARE IMPORTANTLY DEREGRULATED BY THIS DISEASE, AND THEN MEASURED THE ROLES OF CLOCK AND CLOCK-CONTROLLED GENES WITHIN THESE PROCESSES. THUS, ALL THE PROCESSES COMING FROM THE CLUSTERING ANALYSIS ARE USUALLY SIGNIFICANTLY DEREGRULATED IN DISEASED PATIENTS. WHY CARDIOVASCULAR, LIVER DEVELOPMENT AND LYSOSOMAL STORAGE PROBLEMS ARE IMPORTANT FOR THE DEVELOPMENT OF THIS DISEASE ARE WELL-DISCUSSED IN THE CITED REFERENCES: WE HAVE MADE THIS POINT CLEARER THROUGHOUT THE TEXT. WE WOULD LIKE TO POINT OUT THAT THIS BIOINFORMATICS ANALYSIS AIMED AT TRAVERSING THE GO GRAPH, THROUGH THE FAT LEVEL, IN SEARCH OF THE CLOSER PROCESSES THAT MIGHT BE QUALITATIVELY OVER-REPRESENTED BY OUR GENES. THE BIAS OF NOT HAVING CONSIDERED THE "HOW" OF THE REFEREE'S QUESTION, NAMELY THE MAGNITUDE OF THE EXPRESSION OF THESE GENES AT THIS STAGE, WAS OVERCOME WITH THE CONSTRUCTION OF THE WEIGHTED SEMANTIC NETWORK, WHICH FINALLY TOOK THIS QUANTITATIVE ASPECT INTO PROPER CONSIDERATION”.

3. Due to the effects of iduronate-2-sulfatase were temporary and fade, the authors should provide more information about the conditions of two subjects
with Hunter syndrome. For example, did these subjects take the iduronate-2-sulfatase (elaprase) treatments? Were good or ill for these subjects? Could these changes of gene expression as the predictable tools for therapeutical or curing indices?

HUMAN FIBROBLASTS FROM SKIN BIOPSY OF FIVE HS PAEDIATRIC PATIENTS CARRYING DIFFERENT MUTATION IN IDS GENE WERE OBTAINED FROM “CELL LINE AND DNA BANK FROM PATIENTS AFFECTED BY GENETIC DISEASES”, GASLINI INSTITUTE (GENOVA, ITALY). AS HEALTHY CONTROLS HUMAN FIBROBLASTS FROM FOUR CHILDREN’S CIRCUMCISION WERE USED; THEY WERE OBTAINED FROM THE HISTOLOGY UNIT OF THE DEPARTMENT OF HISTOLOGY, MICROBIOLOGY AND MEDICAL BIOTECHNOLOGY (UNIVERSITY OF PADOVA, IT). WRITTEN INFORMED CONSENTS WERE OBTAINED FROM PATIENTS AT THE TIME OF BIOPSY AND THE STUDY WAS APPROVED BY THE ETHICS COMMITTEE OF THE UNIVERSITY OF PADUA, PADUA, ITALY. ALL CELLS WERE ANONYMously OBTAINED. THE SUBJECTS WERE NOT PREVIOUSLY TREATED WITH IDURSULFASE. CHANGES OF CLOCK GENE EXPRESSION SHOW THAT THE EFFECTS ARE EVIDENT BUT TEMPORARY, AND SUGGEST THAT ENZYME REPLACEMENT THERAPY IS NOT SUFFICIENT FOR COMPLETE FUNCTIONAL RESTITUTION AND ADDITIONAL THERAPEUTIC STRATEGIES ARE NECESSARY

Minor Comments:
1. Please indicate the full name at the first time before using the abbreviation. For example, NGS.
CORRECTED

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: