Author’s response to reviews

Title: Assessment of cellular cobalamin metabolism in Gaucher disease

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Version: 1 Date: 01 Aug 2019

Author’s response to reviews:

August 1st, 2019.

To
Editor-in-Chief
BMC Medical Genetics

Dear Editor,

As suggested by the BMC Medical Genetics reviewers, we are submitting the changes that have been made to our paper entitled: Assessment of cellular cobalamin metabolism in Gaucher disease for publication in BMC Medical Genetics.

We hope to have addressed all points raised by the reviewers and look forward to the publication of our manuscript.

We would like to thank the reviewers for their time evaluating our manuscript and for providing us with their valuable comments which definitively improved this manuscript. We would also like to thank you for the attention that has been given to the evaluation of our paper.

Sincerely,
Reviewers reports:

Reviewer #1: The authors address a question that is clinically relevant. Patients with Gaucher disease have abnormalities in plasma transport of vitamin B12 that are not explained at the cellular level. Vitamin B12 is decreased along with a paradoxical increase in holo-TC.

To answer this question, the authors carried out a study in patient fibroblasts, which shows very interestingly the absence of dramatic modification of the key substrates of the remethylation pathway. They do not observe a difference in detection of cellular transcobalamin, in western blot.

However, several remarks can be made in relation to the question asked:

a) Manifestations of GD involve the liver, the spleen and the reticuloendothelial system. The metabolism of vitamin B12 has specificities in each of these organs and tissues, with a prominent role of the liver in B12 storage and transport. It is not sure that fibroblasts are the most appropriate model for this study.

RESPONSE: Thank you for bringing up this important aspect, which we now address explicitly in the first paragraph of new manuscript section ‘Limitations of this study’, as follows: ‘Our studies were performed with skin fibroblasts. Skin fibroblasts have been used extensively to investigate various aspects of GD, including β-glucosidase activity [22-28] and is the cell type of choice for the study of inborn errors of cobalamin metabolism. However, clinical presentations in patients indicate that the most relevant cell types in the pathogenesis of GD are macrophages and hepatocytes. It would be of great interest to recreate our studies in these cell types, shall they become readily available for research purposes.’

Some studies suggested that fibroblasts can be a model for GD. Because skin fibroblasts from patients and controls can be cultured under the same closely defined conditions, thereby minimizing the possibility of individual differences occurring as a secondary consequence of the
disease process, they are a particularly useful source of the enzyme for comparative studies of the GD. Cultured human fibroblasts have been found to be increasingly useful in the diagnosis of metabolic diseases. Beutler et al. (1971) indicated that Gaucher’s disease and its carrier state can be added to the list of disorders which can be diagnosed in this way. This study demonstrated a severe deficiency of β-glucosidase activity in fibroblasts of patients with GD. Furthermore, fibroblasts can easily be obtained from patients and transported from one laboratory to another for assay. Certainly, the use of macrophage as a model for GD can be the most appropriate model for our study, but it is more difficult to obtain from GD patients and we had available only fibroblast cells for this study. Fuller et al. (2008) demonstrated that glucocerebroside was not confined to the lysosome but increased throughout the cell. The sequelae of extra-lysosomal accumulation may have implications in the pathogenic mechanisms of GD by interaction with biochemical and metabolic pathways located outside the lysosome. An additional motivation for the use of skin fibroblasts in this study emerges from the fact that the vast majority of cobalamin metabolism studies in the field of inborn errors have indeed, been carried out using skin fibroblasts. Functional cobalamin deficiency in canonical errors of cobalamin metabolism have been studied with success by assessing marker metabolites and cobalamin-binding proteins. By using skin fibroblasts, we have the opportunity to compare the ‘B12’ phenotype of GD cells with respect to those with authentic defects in cobalamin metabolism in the framework of a cell line that retains the lack of β-glucosidase activity as a major marker of GD.

References:


b) The mass spectrometry assays are very convincing. However, cell incorporation tests of radiolabelled vitamin B12 should also be carried out to study its subcellular distribution and its possible accumulation in lysosomes.

RESPONSE: We agree with the reviewer that this would be an interesting experiment to perform if there was strong evidence of metabolic disruption of vitamin B12 pathways. Our in-depth examination of hallmark biomarkers of vitamin B12 status in cultured cells with and without hydroxocobalamin supplementation showed no differences between healthy control cells and GD cells. Had we had marked changes in Hcy, MMA and/or Met, we would have pursued the examination further to include subcellular distribution of cobalamin using radioactive vitamin B12. Our data of normal intra- and extracellular Hcy and MMA indicates that the two vitamin B12 dependent enzymes methionine synthase and methylmalonyl-CoA mutase are operating in a comparative way in healthy controls and GD; therefore, it is improbable that significant cobalamin trapping or similar disruption occurs in the lysosomal compartment.

c) Moreover, it is essential to look for potential abnormal intracellular distribution of the lysosomal transporters of vitamin B12, LMBRD1 and ABCD4 and also to check whether these two proteins have interactions that are preserved.

RESPONSE: We fully agree with the reviewer that this is an important aspect for further look. We have added this thoughtful suggestion as the closing paragraph of our Discussion, as follows: ‘While we did not find evidence of disturbed cobalamin metabolism in skin fibroblasts, a possibility exists that abnormal lysosomal storage in other cell-types alters their cobalamin transporters and metabolism in the lysosome, including: (a) the retention and efflux of cobalamin in the organelle over time, and (b) the distribution and interactions of lysosomal cobalamin transportes LMBRD1 and ABCD4. This distinct possibility awaits further investigation.’

d) It would also be desirable to have additional data on plasma transport in patients for whom these cell studies were made, including the saturation percentage of vitamin B12 on haptocorrin and the saturation percentage of vitamin B12 on transcobalamin.

RESPONSE: We are working in another study that evaluates B12 metabolism in plasma samples from patients with GD and this is part of another manuscript in preparation. We have measured serum tHcy, MMA, vitamin B12 and holo-TC in a cohort of 15 patients from Brazil comprising GD I, GD II and GD III. We identified no signs of functional cobalamin deficiency as judged by tHcy, MMA and vitamin B12 with respect to diagnostic reference ranges. Elevated holo-TC was
observed in the majority of patients, in line with previous literature. We hypothesize that elevated circulating holo-TC may be a secondary effect of dysfunctional liver and/or macrophage activation. This would require, again, performing studies with macrophages, which we are currently trying to recruit, but which proves difficult to attain.

Reviewer #2:

Major Comments:

1 - Methods, line 157: Preparation of whole cell lysates. Is this the lysate that was used both for enzymic studies as well as for transcobalamin studies? If yes what is the role of protease inhibitors in the former and the use of the detergents in the later dissolve any membranes?)?

General comment small number of cell lines tested.

RESPONSE: Yes, the same whole cell lysate was utilized to measure enzyme activities and the content of total intracellular holo-TC by western blot. The protease inhibitor cocktail is added to prevent proteolytic degradation, thereby preserving TC, β-glucosidase and α-glucosidase and intracellular proteins overall. The detergents Triton and taurodeoxycholate are added to solubilize extracellular and intracellular membrane structures, such as the lysosomal membrane. The protease inhibitor cocktail and detergents are added to the lysis buffer freshly and simultaneously. The composition of the lysis buffer was adapted from a published procedure to meet the conditions necessary for enzymatic determination (Chamoles et al., 2002), and is part of the diagnostic portfolio of the Metabolic Center Freiburg, Freiburg, Germany. Whole cell lysates were prepared freshly on the same day of enzymatic assay testing. We have now added this information and the citation from the literature to our methods section, to depict this clearly to the reader, as follows:

‘Whole cell lysates were prepared freshly on the same day of enzymatic assay testing. The composition of the lysis buffer for the preparation of whole cell lysates was adapted from a published procedure [18] and is part of the diagnostic portfolio of the Metabolic Center Freiburg, Freiburg, Germany.’

Concerning the small number of cells tested, this limitation of the study is presented explicitly in new manuscript section ‘Limitations of the study’, third sentence, as follows: ‘Another limitation of our study is the low number of GD cell lines examined with respect of the variability of clinical manifestations of patients with GD I, GD II and GD III. Further research with a greater number of samples may reveal so far unrecognized associations between GD and vitamin B12 metabolism.’

Reference:

2 - Methods, lines 173-174: As a control of lysosomal integrity, we measured α-glucosidase enzymatic activity assay using the substrate 4-methylumbelliferyl…… There is no way that they could evaluate lysosomal integrity by measuring the activity of another lysosomal enzyme. What is achieved by this assay is to show that the β-glucosidase deficiency assayed is not due to the quality of the specimen. The method employed for assaying of α-glucosidase activity should be given.

RESPONSE: The conditions of the assay for α-glucosidase were given in the original version of our manuscript. The method employed for α-glucosidase activity assay was mentioned between lines 183-185: “The assay conditions were exactly as described herein for β-glucosidase activity, except that 4-methylumbelliferyl-α-D-glucopyranoside was used as the substrate.”

We agree with the reviewer that this control experiment only examines quality of specimen preparation, and we have now modified our manuscript to clearly specify this both in Methods and Results, as follows:

Methods: ‘As a control of quality of the specimen, we measured α-glucosidase enzymatic activity assay using the substrate 4-methylumbelliferyl-α-D-glucopyranoside (Sigma-Aldrich).’

Results: ‘To evaluate the quality of the specimen, the lysosomal α-glucosidase activity was measured and revealed that healthy individuals and GD fibroblasts exhibit an intact α-glucosidase activity (Fig. 1b).’

3 – Results, lines 259-261: It is well known that carriers of GD and most of the metabolic diseases, cannot be diagnosed through assaying of enzyme activity since there is a great overlap between carrier and control values. Furthermore, they should not draw any conclusions from one cell line.

RESPONSE: We agree fully with the reviewer and we revised the manuscript once again to ensure that there are no such claims. Our manuscript did not and does not discuss the diagnostic value of compensation or lack of compensation in asymptomatic carriers; we only presented the experimental result for the asymptomatic carrier as is. This asymptomatic carrier of GD was of interest because she is the clinically unaffected mother of GD patient GM00877.

4 – Results, lines 266-268: These results exclude the occurrence of pleiotropic effects induced by mutations in the GBA1 gene as well as unwanted damage of lysosomal components during the sample preparation protocols. What do the authors mean, pleiotropic effects induced by mutations in GBA1? Is this all based on normal α-glucosidase activity? See comment Lines 173-174.
RESPONSE: Thanks for pointing this out. We have removed this unnecessary statement to avoid confusion and overinterpretation of the results. This section now reads as follows: ‘These results exclude the occurrence of unwanted damage of relevant enzymatic lysosomal components during sample preparation.’

5 – Results, Fig1: (b) All examined subjects presented comparable α-glucosidase activity (nmol/mLxh), suggesting preserved activity of lysosomal components not associated with the GBA1 mutation. See comments lines 173-174; give abbreviations. It appears at least in some cell lines α-glucosidase activity is either or lower when the medium was supplemented with α-glucosidase activity. Could they comment on that? It would be helpful to have the individual values for the enzyme activities.

RESPONSE: We agree with the reviewer that there appears to be a trend where alpha-glucosidase activity is slightly higher in the presence of supplementation with HOCbl in GD II and GD III cell extracts. However, statistical analysis comparing healthy human controls versus GD (all cell lines together) retrieved no statistically significance to support these differences. The values for enzymatic activities are now provided in new Table 2.

6 – Results, lines 286-287: These data only refer to fibroblasts when the cell that really suffers in GD is the macrophage, the disturbed function of which underlies many of the pathologies of the disease. So, the statement that: previously reported abnormalities in plasma holo-TC in GD patients [7] do not seem to arise from abnormal biosynthesis/turnover of this protein in GD cells is not valid and should be rephrased. Also, the present work does not deal with nerve cells.

RESPONSE: Firstly, we are in agreement with the reviewer that macrophage is perhaps the most disease-relevant cell type in GD, and therefore, the use of skin fibroblasts presents as a limitation. We have now added this limitation in a new section of the manuscript entitled ‘Limitations of the study’, first sentence, as follows: ‘Our studies were performed with skin fibroblasts. Skin fibroblasts have been used extensively to investigate various aspects of GD, including β-glucosidase activity [22-28], and is the cell type of choice for the study of inborn errors of cobalamin metabolism. However, clinical presentations in patients indicate that the most relevant cell types in the pathogenesis of GD are macrophages and hepatocytes. It would be of great interest to recreate our studies in these cell types, shall they become readily available for research purposes.’

Secondly, the objective of this work was not to elucidate GD mechanisms, but instead, to investigate vitamin B12 metabolism in this disease that affects the lysosome, a compartment that is also important for intracellular utilization of vitamin B12. In particular, the cell content of TC is critical, as this is the protein that transports vitamin B12 from the extracellular milieu into the lysosome. A revision from the literature shows that TC is expressed in a broad variety of cell types, including skin fibroblasts. We have added this important literature to the paper, and provide clarification of the value of skin fibroblasts as a model cell type to investigate vitamin B12 metabolism. Indeed, the vast majority of canonical inborn errors of cobalamin metabolism
have been investigated and diagnosed using skin fibroblasts, which allow direct comparison with the results obtained herein.

As per the valuable suggestions from the reviewer, we have entirely rephrased the section on TC as follows:

‘Content of transcobalamin, the cellular transporter of cobalamin

RESPONSE: Because cobalamin reaches all cells in the body bound to transporter protein TC, and once inside the cell, release of cobalamin for downstream use is preceded by lysosomal degradation of TC, we examined whether abnormal lysosomal storage brought about by mutations in GBA1 affect the TC in cells. Expression of TC has been demonstrated in a variety of human cell types [19, 29-36], including fibroblasts [37, 38]. Western blot analysis of whole cell lysates indicated comparable content of TC in healthy individuals and GD fibroblasts (Fig. 2a). A slightly decreased content of TC was documented in GD cells but this difference was not statistically significant (Fig. 2b). Given the widespread expression of TC in a number of different cell types and the results obtained herein, the previously reported abnormalities in plasma holo-TC in GD patients [9] do not seem to arise from abnormal biosynthesis/turnover of this protein in GD cells.’

References:


Regec AL, Quadros EV, Rothenberg SP. Transcobalamin II expression is regulated by transcription factor(s) binding to a hexameric sequence (TGGTCC) in the promoter region of the gene. Arch Biochem Biophys. 2002; 407:202–208.


Results, lines 292-293: Under our experimental conditions, no differences were identified between healthy and GD cells, suggesting normal expression of transport protein transcobalamin. This is not the impression I get looking at Fig2. Did they scan their blots? Some type of quantitative assessment of this figure is needed. Also the term expression not appropriate.

RESPONSE: We thank the reviewer for pointing out this omission on our part. We have now added the quantification for the western blot, as Fig 2b. We noted slightly lower content of TC in GD cells compared to healthy human controls, however, the differences do not hold statistical significance. TC content responded to hydroxocobalamin supplementation comparably in healthy human controls and GD cells, a finding that is mirrored in the response of marker metabolites to hydroxocobalamin.

We replaced the term ‘expression’ with the term ‘content’.

Discussion: The investigators have used fibroblasts. They have neither looked at the plasma of GD patients nor at other types of cells including macrophages and neuronal cells. Since we are not dealing with patients with primary defects in cobalamin metabolism but, if at all, with secondary defects caused by the primary disorder it should be taken into account the physiology of different cell types can be affected differently. This should be taken into account when discussing the results. Furthermore, small numbers of cell lines /type hinders any definite conclusions. The discussion should be shorter and more precise.

RESPONSE: We fully agree with the reviewer and have now addressed this as requested. We shortened the discussion section and introduced a comment on the limitation of examining only one cell type, skin fibroblasts. The limitations associated with the use of skin fibroblasts are again explicitly mentioned under ‘Limitations of the study’. We also mentioned the fact that cobalamin metabolism is however highly conserved across cell types, and in this specific respect, skin fibroblasts are an adequate model to investigate vitamin B12 metabolism.

Conclusions: The conclusion that: ‘The presence of a normal α-glucosidase activity confirmed that under our cell culture and sample preparation conditions, the lysosomal compartment is overall preserved in the presence of mutations in the GBA1 locus, is not valid. Although this could be a potentially interesting study it should not be accepted in its present form.

RESPONSE: We agree and corrected this as requested. The mention to alpha-glucosidase activity in ‘Conclusions’ was unnecessary, and therefore, we removed the statement.

Minor Comments:
Abstract, line 39-40: Gaucher disease (GD), one of the most prevalent lysosomal diseases, is caused by biallelic pathogenic mutations in the GBA1 gene that encodes beta-glucosidase (GCase). This is not always so. In rare cases GD is caused by mutations in the Saposin C gene.

RESPONSE: Thank you for pointing us to this omission. This information was now added between lines 40 and 41.

Abstract, line 41-42: Clinically, GD manifests with heterogeneous multiorgan involvement mainly affecting hematological, hepatic and neurological axes. Mention of the different types in relation to clinical manifestations should be included.

RESPONSE: The following text was included between lines 43 and 45: “This disorder is divided into three types, based on the absence (type I) or presence and severity (types II and III) of involvement of the central nervous system.”

Abstract, line 48: Dermal fibroblasts isolated from healthy subjects. The correct term is Skin Fibroblasts and they are cultured not isolated. The term should be changed in all places it appears.

RESPONSE: Thanks, we changed this.

Background, line 94: In rare cases GD is caused by mutations in the Saposin C gene. This should be included together with relevant reference.

RESPONSE: Ok done.

Background, line 115: Reference no.7 only refers to elevated holo-transcobalamin findings. Need to include references for reduced plasma Cbl.

RESPONSE: Ok done.

Background, line 62: Intracellular expression of transcobalamin. The use of the term expression is not justified on the basis of their experimental protocol.

RESPONSE: We changed the term ‘expression’ for the term ‘content’ throughout the manuscript.

Methods, line 142: Healthy human should be changed to Control human.

RESPONSE: Ok done. We now use ‘healthy human control’.
- Methods, line 187: Cultured fibroblasts were isolated by… Cultured fibroblasts were harvested by...

RESPONSE: Ok done.

- Results, line 254: Asymptomatic heterozygous carrier should be changed to asymptomatic carrier of GD.

RESPONSE: Ok done. We changed this wording throughout the manuscript.

- Results, line 280: Expression of Transcobalamin... I do not think that the term Expression reflects what they have done. It should be corrected everywhere it appears.

RESPONSE: Ok done. We have changed the term ‘expression’ for the term ‘content’.