Author’s response to reviews

Title: Novel variants in a patient with late-onset hyperprolinemia type II: diagnostic key for status epilepticus and lactic acidosis

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Author’s response to reviews:

Point by Point response

Reviewer reports:
Bjørnar Hassel (Reviewer 1): Motte et al "A case report: Novel mutations in a late-onset hyperprolinemia type II: diagnostic key by status epilepticus and lactic acidosis"

This is a description of a patient whose epileptiform seizures may have been caused by hyperprolinemia, leading to P5C accumulation, which in turn may quench pyridoxine and cause pyridoxine-responsive seizures. The authors detected very high levels of proline and hydroxyproline in blood, urine, and CSF, but not P5C, in this elderly woman during an episode characterized by metabolic decompensation. Treatment with vitamin B6 (pyridoxine) appears to have helped. The paper is of potential interest, both because of the late onset nature of the patient's epilepsy and because of the genetic findings in the ALDH4A1 gene. However, the paper has been submitted prematurely, as the language leaves much to be desired and because the discussion appears to ignore the genetic findings that need discussion. The authors should follow the outline of the abstract through the rest of their communication.
Some examples of language problems:
Case presentation:
"…due to sudden difficulties in with (delete "in", insert "with") swallowing and (delete comma, insert "and") speech, slight vertical and horizontal eye movement disorder
Changed.

(Please specify: restricted movement? Ophtalmoplegia?),
Changed.

dysesthesia of the limbs ("pins and needles" Please specify distribution: glove and stocking?), and
(insert "and") generalized areflexia.
The neurological examination, documented in an admission note from 2006, describes an ophthalmoparesis with a vertical and horizontal eye movement disorder. A follow-up in 2008 also describes a residual more vertical ophtalmoparesis. So the slight vertical eye movement disorder in December 2017 could also be residual. We discuss this point below.

Muscle strength was normal but without any paresis." Changed in: Muscle strength was normal.

Please use the full name: Miller Fisher syndrome.
Changed.

Cerebral MRI showed no pathological findings, (comma) and a (delete "a") therapy with levetiracetam was started." Changed.

"accompanied from a vertical eye movement disorder with by vertical eye movement paresis (Ophtalmoplegia or gaze paresis?), a lactic acidosis…"
Changed.

Lactate levels should be given with the same unit in serum and CSF, preferably in mmol/L. Divide mg/dL values by 11.2 to obtain mmol/L. (Lactate has mw 112, and you have to correct for the dL vs L). Thank you for this helpful hint. We take a molar mass of lactat with 89,08g/mol (C3H5O3), exact factor is then 0,1122586439. So the new values were calculated.

"…no signs for of (delete "for", insert "of")a Wolff-Parkinson-White syndrome.
Changed.

Correct "hyperkaliemia» to «hyperkalemia»
Changed.

Discussion/Conclusion
The discussion should be re-written. As it reads now, the authors are looking for metabolic explanations for the disease and avoid discussing the genetic alterations they have identified. The discussion should focus on whether these are indeed mutations and whether they are pathogenic.
Thank you for this comment. We discussed these again with our experts for genetic and co-authors. We have no doubt that the two identified trans ALDH4A1 mutations, are causally responsible for the
disease. However, the results of the variants will presumably directly influence the metabolism of the mitochondria in particular.

To substantiate our opinion, we have updated the assessment of the two variants:

**Variant 1:**
ALDH4A1 gene (NM_003748.3) Intron 1: c.62+1G>A heterozygous

This mutation affects a canonical nucleotide of the splice donor site of intron 1 and is therefore thought to lead to aberrant splicing.

The variant is
- Not listed in HGMD® (Human Gene Mutation Database Professional 2018.2, full access) and in ClinVar.
- Listed in the NCBI SNP Database with SNP rs1395544074.
- Did not occur among the control populations in the Exome Sequencing Project (ESP), ExAC (Exome Aggregation Consortium) and gnomAD (Genome Aggregation Database).

In summary of all results, we classify the variant according to the Plon et al. adapted 5-step classification in class 4 (probably pathogenic).

**Update 09.12.2019:** nothing new, that means not even in the current HGMD version Professional 2019.3 and ClinVar. No published allele frequencies, although well covered, that means extremely rare.

**Variant 2:**
ALDH4A1 gene (NM_003748.3) Exon 5 c.349G>C, p.(Asp117His) heterozygous

This missense mutation affects a highly conserved residue in the dehydrogenase domain of the protein. Furthermore, three out of four in silico predictions applied (SIFT, MutationTaster, Polyphen-2) support the role of this sequence alteration as a pathogenic mutation.

The variant is
- Did not occur among the control populations in the Exome Sequencing Project (ESP), ExAC (Exome Aggregation Consortium) and gnomAD (Genome Aggregation Database).

Assessment of the sequence variant with different in silico prediction methods:
- MutationTaster: disease causing with a probability of 1,000 out of 1,000 (Schwarz et al., Mutation Button Evaluates Disease-causing Potential of Sequence Alterations, Nat Methods., 2010; 7 (8): 575-6.)
- Alamut revealed:
  - highly conserved nucleotide (phyloP 5.29, phastCons 1.00)
  - Moderately conserved amino acid residue (considering 13 species)
  - AGVGD: C0 (Function restriction unlikely)
  - SIFT: "non-tolerated amino acid exchange", exchange probability 0.04
  - PolyPhen-2: probably harmful variant, "probabilistic score" 1.000 (probability that this calculation is false positive: 0%, probability that this calculation is really positive: 100%)
  - splicing behavior: no indication of aberrant splicing

Thus, two of the three Alamut prediction programs for missense mutations (SIFT and PolyPhen-2) as well as mutation probes indicate a functional relevance of this sequence change.

**Update 09.12.2019:** nothing new, that means not even in the current HGMD version Professional...
The first variant of the ALDH4A1 gene affects a canonical nucleotide of the splice donor site of intron 1 and is therefore thought to lead to aberrant splicing. The first variant is according to the Clon 5-step classification a class 4 variant (probably pathogenic).

The second variant of the ALDH4A1 gene is a missense variation and affects a highly conserved residue in the dehydrogenase domain of the protein. Furthermore, three out of four in silico predictions applied (SIFT, MutationTaster, Polyphen-2) support the role of this sequence alteration as a pathogenic variation. This variant is according to the Clon 5-step classification a class 3 variant (possibly pathogenic). In summary, it is highly probable that the two identified trans-ALDH4A1 variants are causally responsible for the disease.”

Table 1. (Amino acids in blood, urine and CSF).
Please provide a legend that includes units, presumably µmol/L.
The table was revised. We added the units and reviewed the values and reference values with our laboratory. Unfortunately the values for CSF are given in mg/dl as this is common in our country.

The reference value for glutamate in blood ("glutamine acid") is clearly wrong.
Thank you for this correction, you are absolutely right. It was a typing error.

Specify also if these values are obtained in the fasted state or not.
Values were obtained in a fasted state in the morning. We added this in the table legend.

Most values are not relevant; proline and hydroxyproline are the relevant ones and could be given in the text, provided they are correct: Was U-proline actually >45 mmol/L?
Yes, urine-proline was increased to 46531 µmol/L. You are right, the most relevant values are proline and hydroxyproline. We have highlighted these values by formatting in bold. To exclude other relevant pathologies all measured amino acids were shown. We added a comment to the manuscript text.

Figure 1: The authors should expand the evidence in favor of these genetic findings being mutations and pathogenic. This should be done in the discussion section.
Please see our corrections of the discussion part above. We pointed out the role of the genetic variants as disease cause.

Saadet Andrews (Reviewer 2):
Reviewer comments

Title: A case report: Novel mutations in a late-onset hyperprolinemia type II: diagnostic key by status epilepticus and lactic acidosis

Journal: BMC Neurology

Manuscript number: NURL-D-19-00715

Manuscript type: Case Report
Authors: Motte J. et al.

Motte et al report an adult case with hyperprolinemia type II who presented with refractory seizures and treated successfully with pyridoxine.

Abstract

1. Pyridoxine dependent epilepsy refers to genetic disorders including ALDH7A1, PNPO and PLPBP deficiencies. Authors should use “disorders of vitamin B6 metabolism”. This should be applied throughout the manuscript. Thank you for this correction. We changed ‘pyridoxine-deficiency epilepsy’ to ‘epilepsy associated to disorder of vit b6 metabolism’ in our manuscript.

2. “mutation” is an old terminology and should be replaced with “Variant” throughout the manuscript. Thank you for this correction, we replaced the word mutation with variant in the manuscript

3. Please provide both variants in the abstract. Due to word limit, we have not been able to describe the variants in the abstract so far. We have now added it.

4. What is abdominalgia? What kind of neuritis is this? The word abdominalgia was used as technical term for 'abdominal pain', not for neuritis. This formulation may lead to confusion, so we have reworded it to ‘abdominal pain’.

5. What are the lactate levels? We have added the values to the abstract.

6. What type of seizures? Due to the limited word count seizures were described as ‘generalized epileptic seizures’ in the abstract. One seizure was documented by video at intensive care unit, 1 hour after extubation. The patient lost her consciousness to a somnolent to stuporous level. She showed arrhythmic, tonic-clonic contraction of all limbs. She pinched her eyes and partly responded to pain stimuli by pain-defense and opening the eyes for a short time. A therapy with Lorazepam showed a decrease of clonic contractions. Due to increasing lactate and increasing respiratory failure, the patient was re-intubated after approximately 15 minutes. Further lactic acidosis with vegetative derailment occurred during the first weaning phase.

7. Do authors think that lactic acidosis was associated with vertical gaze palsy? That is a very important question and our manuscript was not clear on this point. The patient suffered from a Miller Fisher syndrome in 2006. The neurological examination, documented in an admission note from 2006, describes an ophthalmoparesis with a vertical and horizontal eye movement disorder. A follow-up examination in 2008 also describes a residual more vertical ophtalmoparesis. So, the slight vertical eye movement disorder in December 2017 could be residual. We clarified this in the manuscript.

8. This is not a pyridoxine dependent epilepsy, it is hyperprolinemia type II causing secondary pyridoxine deficiency and seizures. Please correct.
Thank you for this correction. The part was changed in the manuscript.

9. What are other clinical features of this patient?
You are right, the whole spectrum of clinical features of HP Type II are not described in the abstract and the manuscript. Please note that this manuscript is a case report and not a review work and limited through word count and the scope of a case report form.

All patients described in literature, had neonatal symptoms or developed symptoms in early infancy to early childhood. They suffered from generalized seizures, intellectual deficit up to mental retardation. HPII was also described in adults, but all these patients enjoyed normal health [1].
In children, seizures due to HPII are often triggered by fever and are considered as benign [2]. Until now, only single case reports about epilepsy in HPII have been described [2].


10. All genes are “italic”, please correct throughout the manuscript.
Changed.

Introduction

11. Please correct deficit/mental retardation to intellectual disability.
Corrected.

Case presentation

12. Authors describe in introduction that the majority of cases present in the neonatal period. Their patient’s history starts from 52 years of age. Was the patient’s history until 52 years completely normal?
As described in the manuscript, the patient suffered from a neuritis, diagnosed as Miller Fisher syndrome 10 years before the onset of seizures. Whether this is in pathophysiological connection with hyperprolinaemia remains unclear.
The patient did also suffer from abdominal pain with unclear reason since childhood. The mental status was normal, she had a degree as a chef.

13. Again please clarify abdominalgia.
Please see explanation above. Abdominal pain was meant. We changed it.

14. Why did authors decide to supplement vitamin B6 and what stage did they supplement? What was the dose?
The patient's symptoms led to several differential diagnoses of seizures, as well as vegetative and metabolic syndromes, so we performed thorough diagnostics including vitamin status. As we detected the vitamin B6 deficit we started supplementation. At this timepoint, the patient was no spontaneously breathing, but supplemented with artificial nutrition for several weeks containing all vitamins and trace elements. We specifically supplemented vitamin B6 with 200mg/d. We added the indication of the dosage in manuscript text (l 146).

15. How did authors exclude porphyria and MERRF? Please include.
Biochemical analysis of blood, urine and feces regarding signs of porphyria in two independent
laboratories were negative. Also consultation of Porphyria Specialist Center of the European Porphyria Network (University Hospital Düsseldorf, Germany) revealed no persistence of a porphyria. MERRF was excluded by muscle biopsy and histological staining by the Institute of Neuropathology of the University Hospital Essen, Germany. Please see the part in the manuscript ll 135-138.

16. Why did authors measure vitamin B6?
Please see the explanation above. We performed a whole diagnostic work-up because of the severe and life threatening disease course and as lactic acidosis indicated a possible metabolic disorder.

Discussion
17. What are the proline levels in malnutrition and liver disease?
This is a very interesting question in context to the pathophysiology of hyperprolinemia.

“Hyperprolinemia can occur with conditions, such as malnutrition or liver disease. In particular, individuals with conditions that cause elevated levels of lactic acid in the blood (lactic acidemia) may have hyperprolinemia as well, because lactic acid inhibits the breakdown of proline.” (Lister Hill National Center for Biomedical Communications U.S. National Library of Medicine National Institutes of Health Department of Health & Human Services)

Other authors described hyperprolinemia as special part for alcoholic liver diseases:
Mata et al. investigated patients with malnutrition and liver disease. “In patients with malnutrition, and especially in severely ill patients, the proline values were always below the normal limit. Patients with nonalcoholic cirrhosis or chronic active liver disease had serum proline and hydroxyproline values similar to those of normal subjects. However, the patients with alcoholic liver cirrhosis had proline and hydroxyproline values significantly higher than the normal group. Furthermore, in patients with alcoholic hepatitis the serum free proline values were significantly higher than in the other groups. The results suggest that alcohol might have a direct effect on proline metabolism or facilitate its release from the liver cell.”

Another study from Morgan et al. described the plasma amino-acid patterns in liver diseases. They show a significant reduction of plasma proline levels in these patients. (Mata et al. Serum free proline and free hydroxyproline in patients with chronic liver disease. Gastroenterology. 1975 May;68(5 Pt 1):1265-9 and Morgan MY, Marshall AW, Milsom JP, et al Plasma amino-acid patterns in liver disease. Gut 1982;23:362-370 http://dx.doi.org/10.1136/gut.23.5.362).

Finally, this question cannot be answered, however our patient did not show any signs for a liver disease, malnutrition or alcoholism. Please see the discussion part in the manuscript ll 221 -223: “Despite evidence of the genetic alterations, the exact pathomechanism remains unclear. Malnutrition and liver diseases can be a cause of hyperprolinaemia, however this patient did not show any signs of it.”

18. Summarize adult onset HPII cases and their symptoms as well as their diagnosis, if they were healthy.
As our knowledge only Flynn et al. described 7 healthy adult patients with HPII, the oldest was 36 years old. This study is cited in the manuscript. A detailed description of all these cases again is not possible in the case report form currently proposed by the editor.

19. It is not clear why authors choice to include rat studies, as they do not have any oxidative stress
studies in their patient. Please clarify.
You are right, we did not perform oxidative stress studies in our patient. The cited studies were included in the manuscript to discuss, if anti-oxidative substances like vitamin E or vitamin C should be a supplementary therapy in patients with hyperprolinaemia. Further studies in humans are necessary to give an answer to this question.
Since this aspect is not so relevant for our case description and possibly lead to confusion, we have removed this part from the manuscript.

20. Severe lactic acidosis is not reported in patients with HP type II, what do authors think about markedly elevated lactate levels? Please explain this important point. Was there any lactate during the first 10 years between 52-64 years of age in this patient?
Thank you for this very interesting question. In our opinion it remains unclear why the patient developed this extended lactat acidosis. Lactate tests were not performed in the patient’s history before. As described in our manuscript lactic acid inhibits the catabolism of proline and results in a hyperprolinemia. So, an external lactate acidosis, for example because of hypoxia or a severe illness could exacerbate a latent hyperprolinaemia. On the other side, the variation of ALDH4A1 gene and the resulting defect of the P5C dehydrogenase directly affects a mitochondrial enzyme. So, a lactic acidosis could be a direct result of the gene defect as a result of mitochondrial insufficiency.
In plants and animals and humans external prolin application caused programmed cell death, reactive oxygen species production, and DNA laddering.

This means that proline accumulation, caused by the genetic variation, could damage the mitochondria directly.

Our suggestion is, that the heterozygote patient’s gene variations lead to a latent hyperprolinaemia. Because of abdominal pain and a general deterioration of condition the patient developed an increase of lactate and an increase of proline. Proline accumulation results in oxidative stress (as described in rats and plants) and reduced Na+- K+-ATPase activity which led to a circulus virtuoso with further lead to mitochondrial stress and resulted in increasing lactate levels. Whether the vitamin B6 deficiency, detected in our patient was ultimately responsible for the seizures remains unclear. However, the previous case reports suggest such a pathomechanism, and the supplementation with vitamin B6 led to a seizure-free period.

We clarify this point in our discussion part II 227- 241.

21. What do authors think that why this patient had vitamin B6 deficiency at the age of 64 years leading to epilepsy? What are the other HP II patients’ clinical presentation, please summarize this in a table and discuss in the discussion section including biochemical parameters and lactate levels.
Please see the comment for point 18 and 20. Whether the vitamin B6 deficiency, detected in our patient was ultimately responsible for the seizures remains unclear. Hyperprolinemia type II (HP II) is an inborn error first described in Irish travelers, leading to primary generalized seizures in late infancy or childhood and/or mental retardation (Flynn et al., 1989). Until now, only single case reports have been
described outside the Irish Traveler community (Onenli-Mungan et al., 2004). A detailed description of all HPII cases as review article would be very helpful but is not possible in the case report form currently proposed by the editorial board.