Author's response to reviews

Title: A new mutation in the gene encoding mitochondrial Seryl-tRNA Synthetase as a cause of HUPRA syndrome.

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Author's response to reviews: see over
3rd July 2013

Editorial
BMC Nephrology

Dear Dr A.J. McKnight,

I am enclosing a revised copy of the manuscript entitled “A new mutation in the gene encoding mitochondrial Seryl-tRNA Synthetase as a cause of HUPRA syndrome.” (Manuscript ID 1268650317988287), in which we have taken into consideration all the comments of the reviewers. Thus, complying with the reviewers' requests, we have introduced a new figure showing the abnormal mitochondrial morphology in kidney tubule. We have introduced new information in “Case presentation” section and we have rewritten part of the “Conclusions” section, by introducing new comments following the reviewers' suggestion. Finally, we have explained other minor points. The point-by-point responses to the concerns are described below this letter. Additionally, three new authors have been introduced which were not included initially by mistake.

We sincerely hope that you will find satisfactory the changes that we have introduced into the manuscript in response to the reviewers' constructive criticisms, which we believe serve to strengthen it substantially and that the paper is now in a form suitable for publication in BMC Nephrology as “Case report”.

Sincerely yours,

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Response to Reviewer 1: Shamima Rahman

Major Compulsory Revisions

1. It is of note that respiratory chain enzyme activities were found to be normal in skeletal muscle in both cases, which may make it difficult to diagnose this condition. Do the authors consider that this disorder may be under-recognised? Abnormal respiratory chain enzyme activities in skin fibroblasts in the context of normal activities in skeletal muscle is an unusual finding, and worthy of comment. However I am not convinced that the fib activities are truly 'abnormal' (see my comment re Table below). Please can the authors provide more details about the respiratory chain enzymology method performed in kidney, since this is not widely available. How was the renal reference range derived? How many control samples were assayed? And how were these obtained?

This disorder, from genetic point of view may be underdiagnosed. However, because the phenotype (HUPRA syndrome) is very clear and it has been associated with a mutation in the SARS2 gene, it is likely that from now on is not.

Although it is not usual to show normal values in mitochondrial respiratory chain (MRC) from skeletal muscle and complexes deficiencies in skin fibroblasts, being the other way round what most frequently happens due to the different oxidative/glycolytic metabolic requirements of each these tissues, it is not impossible. Thus, there are several publications were this apparently inconsistent situation was reported (Oglesbee et al. 2006, Loeffen et al. 2000). However, another explanation for this issue could rely on methodology. As these are retrospective family cases, MRC activities from muscle and fibroblasts were measured using slightly different methods at distinct times of the study. In cultured skin fibroblast we used a standardized respiratory chain spectrophometric method partially modified of that reported by Medja (Medja et al. 2009); particularly, complex I activity assay greatly improved its performance respect to the method previously used in our lab which was used for measuring MRC activities in muscle homogenate in these patients.

The following paragraphs have been included in the text:

- page 6, section “Biochemical studies”: “These two methodologies are slightly different being the methodology used in fibroblasts more sensitive and reproducible.”

- page 8, section “Conclusion”: “The patients II-1 and II-2, unlike the Palestinian patients, had a more severe anemia, a milder developmental delay, normal levels of blood lactate, and normal COX histochemistry and mitochondrial respiratory chain activities in muscle. However, normal respiratory chain activities and morphology in muscle does not rule out the diagnosis of mitochondrial dysfunction. Thus, similar situations have been described before where respiratory chain activities in fibroblasts showed deficiencies while respiratory chain activities and histochemical analysis in muscle were normal [14].”

After reviewing respiratory chain activities data from kidney we have decided to remove these values from the Table 1 and any mention in the text by the following reasons:

- the complexes activities from the kidney was compared with an unique control value in the same run, and
2. The presentation of the data in the Table needs to be improved. What does ‘Level’ mean? If this is the control reference range then please label these columns as such. In the Kidney section what does ‘C’ refer to? If this is the value obtained in a single control, then this should be explicitly stated, since that greatly reduces the significance of the apparently low respiratory chain enzyme activities observed in the patient. Furthermore, asserting that there were combined complex I and IV deficiencies in the patient’s fibroblasts is an overstatement, since the activities are only just below the control range. I think the chevrons may be pointing in the wrong direction in all the control columns – surely normal is greater (>) than a certain value not less (<)?

We have improved the data in the Table 1. The data from kidney have been removed by the reason explained above. The term “Level” has been changed by “C (mean±SD)” and the total number of measurements have been included (N=11 for skeletal muscle and N=6 for fibroblasts). The activities of the complex I and IV are below the level mean-1SD, and then they are considered as deficiency. However, it is true that they are only just below the level of decision, and then the following paragraph has been included in the text:

-en page 6, section “Patient II-2”:
“Enzyme analysis of respiratory chain complexes in cultured fibroblasts from a skin biopsy at 17 months revealed mild reduced activity of complexes I and IV (Table 1).”
-en page 6, section “Biochemical studies”:
“However, the patient II-2 showed a mild deficiency of complex I and complex IV activities in cultured skin fibroblasts (Table 1), with residual activities being 71% and 87% of the mean control values (less than one standard deviation (1SD) away from the mean value).”
-en page 8, section “Conclusion”:
“Additionally, one of them also showed a mild deficiency of the mitochondrial respiratory chain in fibroblasts (Table 1).”

3. Why was SARS2 sequenced in these patients? The diagnostic rationale needs to be carefully explained. Was this the only gene sequenced in their patients? Was the mitochondrial DNA studied? Were any other nuclear genes sequenced? Or are they asserting that the HUPRA phenotype is so specific that the SARS2 gene should be targeted as a first line investigation? Have they screened any other patients for SARS2 mutations? Can they estimate how frequently SARS2 mutations may cause infantile onset renal disease?

These patients died without a definitive diagnosis. They were comprehensively investigated for common etiologies of anemia, uric acid metabolism and mitochondrial metabolism, including nuclear genes sequencing (HNF1, UMOD, CoQ2 and PDSS2) and partial sequencing of the mtDNA, but no underlying cause was found. Then, after the results reported by Belostotsky (Belostotsky et al. 2011), linking mutation in the gene SARS2 with the phenotype HUPRA, and because of the symptoms of our patients were very similar to that published, we decided to sequence the SARS2 gene, identifying the mutation described here. We have introduced the following paragraph to explain this fact:
The patients were comprehensively investigated for common etiologies of anemia, uric acid metabolism and mitochondrial metabolism, including sequencing of several genes (HNF1, UMOD, CoQ2, PDSS2 and partially the mtDNA) but no underlying cause was identified.

Now, there are five patients reported with similar phenotype (in our patients the anemia was more severe and the developmental delay milder) associated with mutations in the SARS2 gene, so we can assert that HUPRA phenotype is so specific that the SARS2 gene could be targeted as a first line of diagnostics.

We have introduced the following paragraph to explain this:

Finally, we consider that the SARS2 gene must be the first line of investigation when the HUPRA phenotype is present.

At the moment we have not screened other patients for SARS2 mutations. We cannot estimate how frequently SARS2 mutations may cause infantile onset renal diseases because we work in a metabolic unit and we do not receive all of the patients with infantile onset renal disease. However, we are one of the reference centers in Spain for mitochondrial diseases since many years ago, and we have seen other patients with progressive renal disease (older than the two patients described in the paper) who presented with other phenotypes: corticosteroid-resistant nephrotic syndrome, tubulo-interstitial nephritis, Fanconi syndrome, etc. It is the first time that we have seen cases like that.


4. My strongest criticism of this study is that only in silico evidence of pathogenicity is provided. This study would be far more convincing with some functional data to demonstrate mutation pathogenicity.

The reviewer is right, and we are planning further studies to confirm the pathogenetic mechanism. However, we consider of critical importance to show the results to the medical-scientific community for diagnostic approaches as soon as possible, while we perform the functional characterization of the reported mutation.

5. This manuscript would benefit from a more detailed discussion of the function of SARS2, with respect to disease mechanisms in HUPRA. The authors should also postulate reasons for the observed tissue specificity despite a global impairment of mitochondrial translation.

We have introduced the following paragraphs to discuss the possible mechanism and tissue specificity:

Recently, mutations in genes encoding mitochondrial aminoacyl-tRNA synthetases (mtARSSs) have emerged as a new cause of human disease, resulting in surprisingly tissue-specific phenotypes, although they are all expected to impair mitochondrial protein synthesis and thus affect the OXPHOS system. However, the molecular mechanisms behind the selective tissue involvement are not currently understood. Several possibilities have been pointed out to explain the tissue-specificity phenotype of mtARS mutation [19]: (i) the remaining residual activity of mtARS is sufficient to maintain mitochondrial translation in most cell types but not in the specific tissues; (ii) tissue-specific differences in mitochondrial
chaperone activities may play a role in determining the stability of some mutants; (iii) tissue-specific levels of particular uncharged tRNAs and amino acids; and (iv) mtARS could also have additional functions that are indispensable in specific cell types or developmental stages.

The principal affected tissue in HUPRA syndrome by SARS2 mutations is the kidney. Mutations in SARS2 resulting in decreased aminoacylation of one tRNA isoacceptor (tRNA^{Ser}_{AGY} or tRNA^{Ser}_{UCN}) by the serine amino acid are expected to adversely affect mitochondrial translation systems and lead to derangements in the synthesis of mitochondrial proteins and consequently in energy supply. Reduced energy production may account for impaired tubular function, which is known to be especially vulnerable to mitochondrial dysfunction since it has very high energy requirements [6].”

**Minor Essential Revisions**

**Case Presentation**

p4, line 5: please provide more details regarding the nature of the anaemia

The anaemia was normocytic, normochromic, non-regenerative, with normal leucocytes, thrombocytes and reticulocytes. Her bone marrow aspiration was normal; she had neither ringed sideroblasts nor vacuolization of hematopoietic precursors.

It has been introduced the anemia type, page 4, section “Case presentation”:

“Blood test revealed a normocytic normochromic anemia (Hb 8.4 g/dL (>12), VCM 75 fL (70-115), HCM 27 pg (23-35), with normal leucocytes, thrombocytes and reticulocytes)”

“Her bone marrow aspiration was normal and she had neither ringed sideroblasts nor vacuolization of hematopoietic precursors.”

p4, line 10: HCO¯ not ¯HCO#

-This mistake has been corrected in the manuscript.
Response to Reviewer 2: Daniel Gale
Minor essential revisions
1. Table 1 is difficult to interpret. The authors should explain what is meant by “Level” and “C” in the header and exactly what the significance is of the values that appear in red.

We have improved the data in the Table 1. After reviewing respiratory chain activities data from kidney we have decided to remove these values from the Table 1 and any mention in the text by the following reasons:

- the complexes activities from the kidney was compared with a unique control value in the same run, and
- the control used was a biopsy from an adult kidney.

The term “Level” has been changed by “C (mean±SD)” and the total number of measurements have been included (N=11 for skeletal muscle and N=6 for fibroblasts). The activities of the complex I and IV are below the level mean-1SD, and then they are considered as deficiency and the values appear in bold (in red in old version of the manuscript).

The following paragraph has been included in the text:

- page 6, section “Patient II-2”:
  “Enzyme analysis of respiratory chain complexes in cultured fibroblasts from a skin biopsy at 17 months revealed mild reduced activity of complexes I and IV (Table 1).”
- page 6, section “Biochemical studies”:
  “These two methodologies are slightly different being the methodology used in fibroblasts more sensitive and reproducible.”
  “However, the patient II-2 showed a mild deficiency of complex I and complex IV activities in cultured skin fibroblasts (Table 1), with residual activities being 71% and 87% of the mean control values (less than one standard deviation (1SD) away from the mean value).”
- page 8, section “Conclusion”:
  “Additionally, one of them also showed a mild deficiency of the mitochondrial respiratory chain in fibroblasts (Table 1).”

2. Lactic acid levels were normal in both patients described here. Elevated blood lactate levels are described in all previous cases of HUPRA and in patients with other defects of mitochondrial aminoacyl-tRNA synthetases and also other mitochondrial disorders. Its absence in this report is somewhat surprising and should be discussed.

3. In previous cases of HUPRA muscle biopsy showed COX deficiency which was absent in the current case. The authors should comment on the significance of this discrepancy.

4. The authors should provide an explanation for the normal mitochondrial metabolic profile of skeletal muscle compared with those of kidney and skin cells.

Lactic acid elevation in blood is an important, although non-specific and non-sensitive, marker of mitochondrial disease. Thus, despite an elevated blood lactate levels were previously reported in cases of HUPRA, as well as in patients with other mitochondrial disorders (including most of the patients with defects of mtARS), many patients with mitochondrial diseases consistently have normal lactic acid levels (Scaglia et al. 2004).
Furthermore, mutations in the DARS2 gene that causes LBSL (leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation) only an elevated lactate is observed in the affected white matter and not in blood or cerebrospinal fluid (CSF) (Scheper et al. 2007).

Normal morphology and/or mitochondrial respiratory chain activities in muscle does not rule out the diagnosis of mitochondrial dysfunction. Although is not usual to have normal values in mitochondrial respiratory chain (MRC) from skeletal muscle, COX-positive muscle fibers and MRC deficiencies in skin fibroblasts, there are several publications were this situation was reported (Oglesbee et al. 2006, Loeffen et al. 2000). However, another explanation for this issue could rely on methodology. As these are retrospective family cases, MRC activities from muscle and fibroblasts were measured using slightly different methods at distinct times of the study. In cultured skin fibroblast we used a standardized respiratory chain spectrophotometric method partially modified of that reported by Medja (Medja et al. 2009); particularly, complex I activity assay greatly improved its performance respect to the method previously used in our lab which was used for measuring MRC activities in muscle homogenate in these patients.

The following paragraph has been included in the text:

- pages 8 and 9, section “Conclusion”:

“The patients II-1 and II-2, unlike the Palestinian patients, had a more severe anemia, a milder developmental delay, normal levels of blood lactate, and normal COX histochemistry and mitochondrial respiratory chain activities in muscle. However, normal respiratory chain activities and morphology in muscle does not rule out the diagnosis of mitochondrial dysfunction. Thus, similar situations have been described before where respiratory chain activities in fibroblasts showed deficiencies while respiratory chain activities and histochemical analysis in muscle were normal [14]. On the other hand, lactic acid elevation in blood is an important, although non-specific and non-sensitive, marker of mitochondrial disease. Many patients with mitochondrial diseases consistently have normal lactic acid levels even it has been described pathogenic mutations in DARS2 where elevated lactate was only observed in the affected tissue (white matter) and not in blood or cerebrospinal fluid [15]. Additionally, from a cohort of 113 pediatric patients with mitochondrial disease, a significant respiratory chain defect was found in 71% of the patients, focal absence of COX activity was found only in 13% of patients and elevation of plasma lactic acid in 60% of them [16]. Thus, reaching a diagnosis of mitochondrial disease in pediatric patients can be challenging due to it can be accompanied by normal muscle morphology, normal plasma lactate, normal mitochondrial enzymes in skeletal muscle, normal mtDNA mutation screening, and a non-classical clinical presentation.


5. “Cardiomyopathy” should replace “myocardiopathy” on page 5 para 1.

-The mistake has been corrected.

6. “Heterozygosity” should replace “heterozygous” on page 7 line 1.
-The mistake has been corrected.

**Discretionary revisions**

1. If available the authors should report the magnesium levels. Hypomagnesaemia was observed in all 3 previous patients with HUPRA syndrome and implicated dysfunction of the thick ascending limb.

   The magnesium levels in case II-2 were 1.7 mg/dL (N, 1.5-2.30). However we don’t have FeMg data.
   
   It has been introduced in page 5, section “Patient II-2”:
   “Serum magnesium levels were 1.7 mg/dL (1.5-2.3).”

2. An electron micrograph of a kidney tubule showing the abnormal mitochondrial morphology alluded to in the text would be of interest.

   The reviewer is right, and we have introduced a new figure (now Figure 1) with the electron micrograph of a kidney from the patient II-2.