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A Severe Neonatal Argininosuccinic Aciduria Case Investigated by ^{1}H NMR Spectroscopy

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Abstract: *The NMR urine analysis of a term newborn with severe general deterioration of the clinical state revealed the presence in high concentrations of orotic and argininosuccinic acids. The newborn was suspected for an intoxication-like inborn error of metabolism, and the urine samples were followed up by NMR spectroscopy for several days in order to assess the metabolic pattern. The identified markers led to a definitive biochemical diagnosis of argininosuccinic aciduria.*

Keywords: *urea cycle disorders (UCD), argininosuccinic aciduria (ASA), argininosuccinate lyase (ASL), NMR spectroscopy, inborn errors of metabolism (IEM).*

1.Introduction

Argininosuccinic aciduria (ASA) belongs to the urea cycle disorders that are included in inborn errors of metabolism (IEM). The IEM term was introduced by Sir Archibald Garrod in 1908 in the Croonian Lectures, when he described for the first time four types of genetically conditioned diseases: albinism, alkaptonuria, cystinuria, and pentosuria [1]. He observed that this lifelong duration inherited conditions were caused by a decreased activity (or by a complete absence) of a certain enzyme which may stop a specific metabolic pathway (in cystinuria a membrane transporter is affected). Garrod also introduced the notion of "chemical individuality" [2], but the importance of this ideas was underestimated for many years. Today, it is acknowledged that his ideas provided an important background for a pioneering vision in system biology.

Urea cycle is the main route for ammonia detoxification for most terrestrial animals, also known as ureolitic species; its defects (urea cycle disorders – UCDs) generally cause hyperammonemia [3]. Out of the total of more than 750 IEM currently described, urea cycle defects belong to disorders of intermediary metabolism affecting small molecules. ASA is due to the deficiency of argininosuccinate lyase (ASL), a cytosolic enzyme that cleaves argininosuccinic acid to produce arginine and fumarate in the fourth step of the urea cycle (fig. 1) [3-5].

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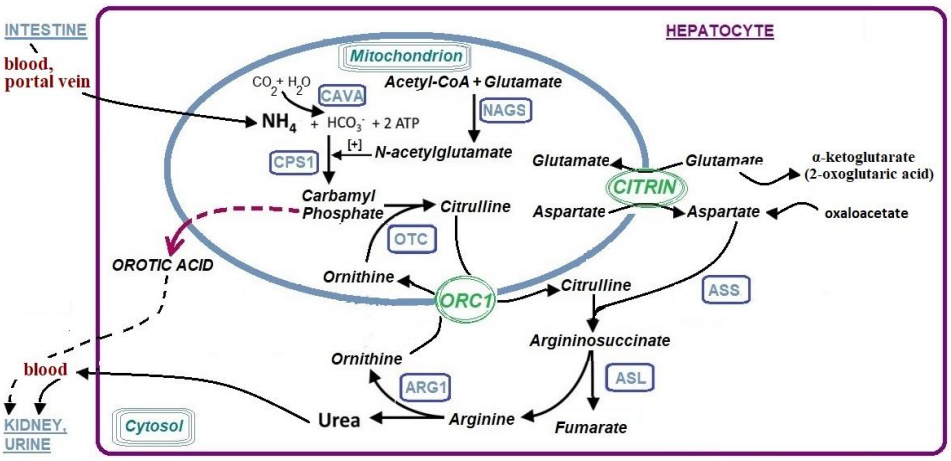

Figure 1. The urea cycle and main associated pathways - image conceived according to literature data [3, 6]. For simplicity, not all the substrate and products of each reaction are shown. ***Enzymes I***: Located into the mitochondrial matrix: CAVA: Carbonic anhydrase Va; CPS1: Carbamoyl phosphate synthetase (the dotted line from carbamoyl phosphate indicates that several metabolic steps in the cytosol are required for orotic acid synthsesis.); NAGS: N-acetylglutamate synthetase; OTC: Ornithine transcarbamolase. ***Enzymes II***: Located into the citosol: ASS: Argininosuccinate synthetase; ASL: Argininosuccinate lyase; ARG1: Arginase 1 (*arginase 2 is extrahepatic, and therefore not shown*). ***Transporters*** *(located into the inner mitochondrial membrane)*: CITRIN (SLC25A13): aspartate/glutamate antiporter; ORC1 (SLC25A15): ornithine/citrulline antiporter.

Patients with ASA cannot convert argininosuccinate into arginine, which leads to increased amounts of precursors in the pathway detoxifying ammonia. This build-up can lead to increased ammonia levels in the blood and other tissues, and may cause brain damage. This deficiency occurs in approximately 1 in 70,000 newborns [7], and represents the second most frequent defect among the UCDs (after OTC deficiency). It presents two forms: a severe type, with neonatal onset (that is clinically indistinguishable from other proximal urea cycle disorders) and a late onset form that is less severe [8, 9].

Our NMR expertise and interest in metabolism in general [10-13] and in IEM in particular [14-16] prompted us to attempt an NMR based diagnosis in a neonatal case suspected for a severe genetic metabolic disorder.

2.Material and methods

The urine samples were collected in sterile containers with tight-fitting covers as individual points for each excretion moment, and as 24 h mixtures. The urine samples were frozen and stored at -20 °C until ^{1}H NMR analysis.

The NMR spectra were recorded on a Bruker Avance Neo 400 MHz spectrometer, using a 5 mm inverse detection multinuclear probe equipped with gradients on the z-axis. The samples were run in 5 mm Wilmad 507 NMR tubes. Before NMR analysis, the samples were allowed to reach room temperature (typically one hour) and centrifuged at 7,000 rpm for 10 min. To 0.9 mL urine, 0.1 mL of a stock solution of 5mM sodium 3-(trimethylsilyl)-[2,2,3,3-d$_4$]-1-propionate (TSP) (Aldrich) in KH_2PO_4/KOH/D_2O buffer (Aldrich) was added. The pH was not adjusted. The chemical shifts are reported as δ values (ppm) referenced to TSP as internal standard. The ^{1}H NMR spectra were recorded with water presaturation. The pulse sequence used 32 scans, a 90° pulse, 30 s relaxation delay, 3 s CW irradiation and 4 s acquisition time as previously described [10-13].

Case presentation

A Romanian child (boy, birth weight: 3230g) born by caesarian section at term (38 weeks of gestation), with Apgar score 9/10, was presenting clinical deterioration after the first 3 days of breast feeding. The newborn had a progressive deterioration of clinical state, starting with axial hypotonia (first identified after about eight hours of protein intake), then lethargy, neurologic signs as unusual movements of the limbs/seizures, and hiccups. These were interpreted as signs of progressing encephalopathy and, being suspected for a genetic metabolic disorder, the protein intake was replaced, the nutritional support being ensured by carbohydrate substrate (Duocal and glucose). Before the restricted protein intake, the blood samples were collected for general biochemical investigations, and for special metabolic analyses, i.e. blood ammonia, amino acids for thin layer chromatography, and several dot blood spots (DBS) for acyl-carnitines analyzed through mass spectrometry. The clinical picture was suggestive for an intoxication-type of disorder, like amino acid disturbances, organic aciduria or a urea cycle disorder. Several urine samples (collected as individual samples during the first days of life) have been sent for NMR spectroscopy analyses. The routine biochemical investigations have shown modified serum parameters suggesting severe hepatic disturbances with high transaminases, low plasma proteins: 4.2 g/dL, metabolic acidosis with HCO_3: 14.5 mmol/L (n.v. 22-26 mmol/L), lactate: 5.4 mmol/L (n.v. 0.5-2 mmol/L), anion gap: 21.9 mmol/L (n.v. 7-16 mmol/L). The blood urea level was close to the inferior limit (4 mg/dl), normal values for newborns in the third day of life being 3.0-34.4 mg/dL [17].

3.Results and discussions

The relevant regions of the urine ^{1}H NMR spectrum are presented in Figures 2 and 3.

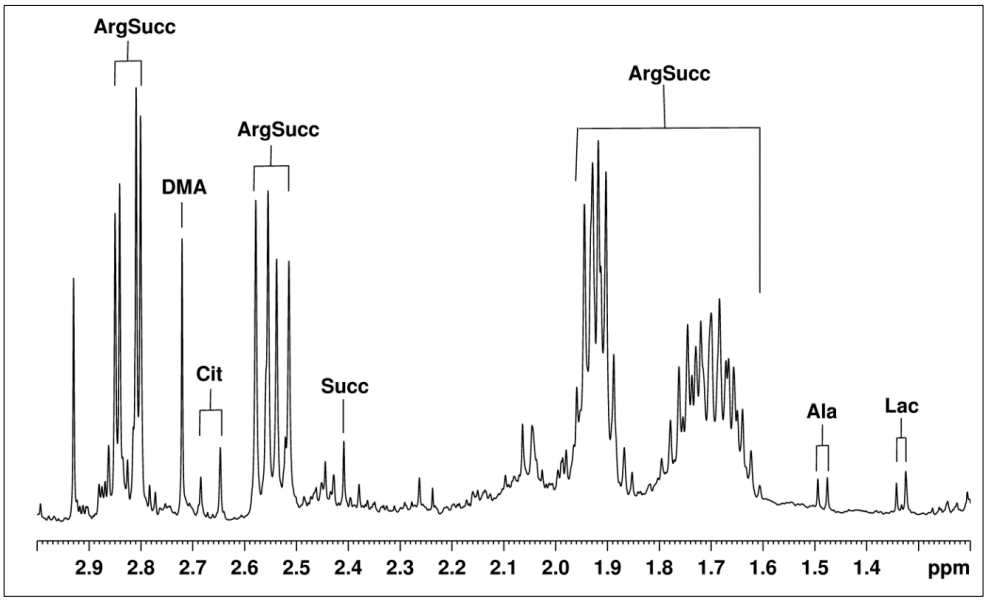

Figure 2. Region 1.2-3.0 ppm in the ^{1}H NMR spectrum of a urine sample with assignments of metabolites

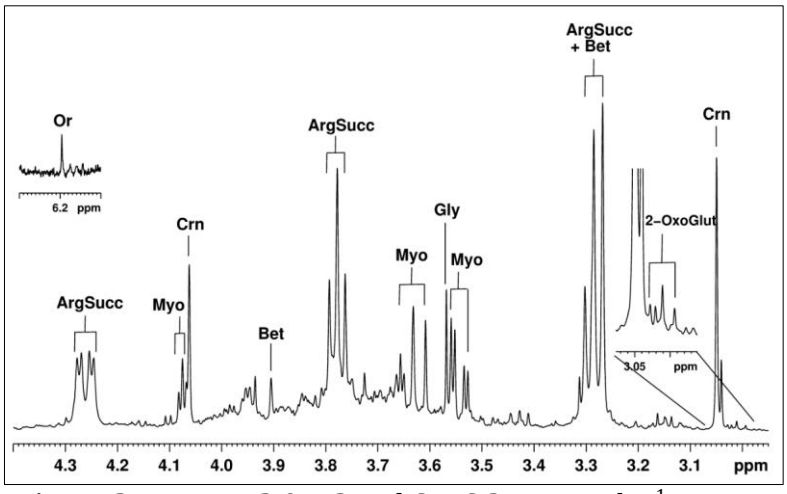

Figure 3. Regions 3.0-4.3 and 6.1-6.3 ppm in the ^{1}H NMR spectrum of a urine sample with assignments of metabolites

The concentrations for several metabolites obtained from the NMR spectra are presented in table 1.

Table 1. NMR derived metabolites concentrations in the urine samples

Metabolite (mmol/mol Creatinine)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6 (after protein intake restriction)	*References values for age <1 month* (mmol/mol Creatinine)
Orotic acid (Oro)	622.2 (↑)	19.0 (↑)	8.3 (↑)	12.2 (↑)	545.5 (↑)	366.9 (↑)	<3.4 [6, 18]
Arginino-succinic acid (Asa)	2338.1 (↑)	3130.0 (↑)	3337.0 (↑)	5532.9 (↑)	4426.2 (↑)	4067.6 (↑)	<0.5 [6, 18]
Lactic acid (Lac)	177.8	100.4	104.2	107.9	1393.9 (↑)	381.7 (↑)	51-156 [19]
Alanine (Ala)	118.5	96.2	100.0	104.8	272.7 (↑)	187.7	75-244 [19]
2-oxoglutaric acid (Oglu)	4044.4 (↑)	158.6	158.3	138.9	431.8	349.5	22-567 [19]
Succinic acid (Suc)	222.2	74.5	86.5	70.2	90.9	114.7	35-547 [19]
Glycine (Gly)	400.0	713.5	868.8	815.3	1386.4 (↑)	1075.4	283-1097 [19]

Urinary creatinine (Crn) concentrations (mmol/L): 0.52 (Sample 1); 2.13 (Sample 2); 1.73 (Sample 3); 2.40 (Sample 4); 0.58 (Sample 5); 1.40 (Sample 6).

The first clinical symptoms in urea cycle disorders or organic acidurias presenting hyperammonemia are generally poor feeding and lethargy, followed by irritability and other neurological signs; in these cases, often the differential diagnosis should include sepsis but, in our case, several specific markers, including white blood cells number or C-reactive protein (CRP), were not abnormal. This diagnosis (sepsis) is less likely to be taken into account when there are no risk factors. In our case, the first laboratory results available were analyses showing a general deterioration of acid-base status and of hepatic functions. The urine amino acids analysis by thin layer chromatography (TLC) showed high Asa and Asa anhydride levels (the spots are well visible, and do not co-elute with leucine or isoleucine - as in liquid chromatography). The concentrations for argininosuccinic acid and orotic acid detected by NMR spectroscopy (table 1) are abnormally high, this being a strong indication for diagnosis of argininosuccinic aciduria. This diagnostic was supported by results received later for ammonia levels, i.e. 641 µmol/L (n.v. 16-60 µmol/L) and for mass spectrometry on dot blood spots that found only

abnormal high levels of citrulline. The concentrations of the other metabolites quantified in the first four urine samples by NMR spectroscopy (Lac, Ala, Suc, Gly in table 1) were not reported as being abnormal. The first urine sample has a very high concentration of 2-oxoglutaric acid (or α-ketoglutarate - that is a key intermediate in Krebs cycle, suggesting a mitochondrial dysfunction). However, we can comment that for Sample 5 there is a significant increase in three metabolites, namely Lac, Ala and Gly. Lactic acid is increased in the last two sample analyzed and is linked with the secondary involvement of mitochondria with the impossibility to maintain the acid-basic status. Although for the first urine samples the urine lactate concentrations are still close to the upper limit previously reported as normal range, considering the concentrations in the other samples, we can comment that for this particular subject the concentrations are abnormally high. In the era of personalized medicine when there are opportunities for monitoring many metabolic parameters, one can more accurately define normal ranges for each individual organism and can trigger warnings for unusual behaviors even when such behaviors are not, or not yet, associated with any pathological condition, assuming that samples are collected on a regularly basis.

Another sign, suggesting abnormal urea cycle reactions was the low blood urea levels i.e. 4 mg/dl, normal values for term newborns being between 3.0 and 34.4 [17]. On the other hand, the high levels of blood ammonia (10 fold higher than normal values, due to the urea cycle dysfunction) explained the clinical picture that was rapidly deteriorating resulting in neurologic involvement. The life-threatening metabolic crises have indicated a severe form of an UCD. In all these cases, immediate decrease in ammonia level is essential even before knowing the exact molecular defect, and then it is important to continue the metabolic investigations to elucidate the underlying defect [5].

In our case, the results obtained through TLC and ^{1}H NMR spectroscopy indicated the particular enzyme deficiency, due to the association of high urinary levels of orotic acid and Asa. As all urinary samples collected over 6 days were received in the NMR laboratory in the same time, one can look into historical perspective, and it is interesting to note the following: although for all samples collected, both Oro and Asa are well above normal values, with Asa having constantly very high values, increased urinary concentrations for Asa and Oro did not go perfectly parallel. Asa and Oro in urine were identified in very high levels in the first day of life (explained by the defect in UCD and the catabolic state). To ensure the positive sample with diagnostic marker (in blood and/or urine), the child was breastfed for a limited period of time. Sample 6 was collected after milk restriction (decreasing in this manner the protein intake), and the analysis has shown a decreased levels in urinary Asa and Oro. Other important increases were observed for lactate, 2-oxoglutaric acid and succinate due to the secondary metabolic disturbances linked with metabolic acidosis. Nevertheless, higher excretion of glycine was identified in only one sample (before the breastfeeding restriction) being most likely a reversible tubular defect similar to iminoglycinuria often identified in newborns.

According to a simple classification, deficiencies in urea cycle enzymes (located into the hepatocytes) include three types of defects [3, 5]: (*i*) deficiencies of one of urea cycle enzymes located within the hepatocyte's mitochondrial matrix (CPS1, OTC or NAGS), (*ii*) deficiencies of the urea cycle enzymes located into the hepatocyte's cytosol (ASS, ASL or arginase, respectively), and (*iii*) defects of one of the mitochondrial transporters (ORC1 or Citrin), these being very rare.

The ASL deficiency presents two types of diseases: a severe one, with neonatal onset, and a late onset form. ***The severe neonatal onset type*** is indistinguishable from ASS or mitochondrial matrix UCDs, and is characterized by hyperammonemia within the first few days after birth accompanied by vomiting, lethargy, poor feeding, and hyperammonemic encephalopathy; in the absence of treatment, lethargy, seizures, and coma worsen, resulting in death [8, 9]. In contrast, the patients with ***late onset type*** of disorder may presents different clinical picture, ranging from episodic (intermittent) hyperammonemia triggered by acute infection or stress, to cognitive impairment, behavioral abnormalities, and/or learning disabilities in the absence of any documented episodes of hyperammonemia. Manifestations of ASL deficiency that appear to be unrelated to the severity or

duration of hyperammonemic episodes include: (*i*) neurocognitive deficiencies (attention deficit hyperactivity disorder [ADHD], developmental disability, seizures, and learning disability); (*ii*) liver disease (hepatitis, cirrhosis); (*iii*) trichorrhexis nodosa (coarse brittle hair that breaks easily); and (*iv*) systemic hypertension [3, 7, 20].

Regarding the classical laboratory diagnosis in ASL deficiency, elevated plasma ammonia concentration (sometimes up to ≥2000-3000 µmol/L), elevated plasma citrulline level (usually 200-300 µmol/L), and elevated argininosuccinic acid in the plasma or urine establish the diagnosis of ASL deficiency; the arginine level in ASA patients is always deficient. High blood citrulline levels is helpful to differentiate the extra-mitochondrial from mitochondrial UCDs. The high renal clearance of argininosuccinate explains the relative modest elevation of this amino acid in plasma in ASL defect, than of citrulline (that more frequent is about 1000-fold increased) in ASS defect [3, 21]. While patients with ASA share the acute clinical phenotype of hyperammonemia, encephalopathy, and respiratory alkalosis common to other UCD, they also present with unique chronic complications most likely caused by a combination of tissue specific deficiency of arginine and/or elevation of argininosuccinic acid.

Using ^{1}H NMR spectroscopy method, that are not using laborious pre-analytical steps, the anhydride forms are not occurring. Moreover, it was shown that in liquid chromatography, the argininosuccinate chromatographic peak might co-elute with leucine or isoleucine, resulting in an apparent increase in one of these amino acids [20].

^{1}H NMR spectroscopy of body fluids has several advantages, and high reproducibility, requiring minimal or no sample pre-treatment, avoiding potential destruction of metabolites structure, it is less prone to experimental artifacts than chromatography or mass spectrometry, and ensures wide coverage of chemical classes. The resulting spectra show the majority of proton-containing compounds, and provide an overall view on metabolism, and this holistic view makes NMR spectroscopy a cornerstone of metabolomics. In the diagnostics of hereditary metabolic diseases, this is a major advantage compared to other techniques [22-24].

The limitations in our territory are related to the difficulty to have rapid results for urinary organic acids and/or acylcarnitines from DBS corresponding to extended newborn screening. Beside this, in our case, the result for blood investigations through mass spectrometry was available later, and has shown just "*high citrulline levels in plasma*", without pointing the diagnostic. However, high citrulline in plasma is found in *citrullinaemia type I, citrullinaemia type II,* in *argininosuccinate lyase deficiency* and in *Pyruvate Carboxylase deficiency type B,* but for rapid differential diagnosis are necessary evaluations of amino acids and organic acids, including urinary orotic acid and orotidine [25, 26].

As the literature outlines, even in countries where the expanded tandem mass spectrometry newborn screening has been introduced, plasma ammonia should be measured in all newborns and infants with unexplained signs and symptoms such as lethargy, poor feeding, irritability and vomiting. Outlining the practical aspects, for a reliable determination of blood ammonia, it is important to collect the blood without tourniquet, to store and immediate transport the sample on ice toward the lab for a rapid ammonia evaluation [5]. Regarding the suspicion for late onset of ASL deficiency, the ammonia should be measured (but preferably during a period of clinical deterioration) in all elder children, adolescents or in adults with unexplained (sometimes episodic) encephalopathy [5]. In a reported case of less severe ASL [9], during newborn screening evaluations, the amino acids elevations were too subtle to provoke metabolic referral, and the diagnosis was delayed.

Molecular genetic testing of *ASL* (the only gene in which mutation is known to be causative) and assay of ASL enzyme activity may be helpful when the biochemical findings are equivocal, i.e. in the case published by Ganetsky, that has two known pathogenic mutations – one with no residual activity and one with reported 10% residual activity [8, 9].

Prevention of primary manifestations: Dietary restriction of protein and dietary supplementation with arginine are the mainstays in long-term management; for those not responsive to these measures, oral nitrogen scavenging therapy can be considered. Orthotopic liver transplantation (OLT) is considered

only in patients with recurrent hyperammonemia or metabolic decompensations resistant to conventional medical therapy. Moreover, a large study done on more than 450 patients with UCDs (E-IMD patient registry) has shown that non-interventional variables of disease severity, such as age at disease onset and peak ammonium level of the initial hyperammonemic crisis (cut-off level: 500 μmol/L) best predicted the neurological outcome [8, 18, 27].

4.Conclusions

The heterogeneity of IEM cases is nowadays well known, and often helped in understanding normal function of different organs. Our experience with ^{1}H NMR spectroscopy showed that this approach is robust and potentially useful as a screening tool for several IEMs, avoiding thus multiple, time-consuming biochemical assays which would cause delay in clinical management. Besides, in modern societies there is an ever increased pressure to apply the current medical knowledge for personalized diagnosis.

Moreover in countries where economical constrains have delayed the expansion of national screenings for rare metabolic diseases, a few IEM specialized NMR laboratories can successfully become the main tool for diagnosing or narrowing the hypotheses in suspected cases. Thus, physicians should be aware about these rare disorders and about the important advantage of a multi-metabolite rapid analysis in cases with clinical suspicion for a genetic metabolic disorder.

We reported on a severe UCD case, and depicted the successful use of urinary NMR spectroscopy for urine analyses, showing high levels of Asa in several urine samples collected from the newborn breastfed and after the protein intake restriction. Also, the method was very suitable for quantification of orotic acid – as a biomarker easily identified in other cases of UCDs diagnosed by us - OTC deficiencies. In this severe ASA case, future implications are linked to the specific treatment of the child and genetic counseling for this family. We outline the necessity of expanding the availability of rapid metabolic investigations and the national newborn screening in our country.

Given the reliable and fast results provided by NMR spectroscopy, this is a highly valuable diagnosis method for ASA and other IEM; this method has an increased value for differential diagnosis mainly in the cases investigated in emergency departments with changes in acid-base homeostasis. NMR based metabolomics is not only an effective tool for diagnosing known IEM, but also a very powerful tool for discovering new IEM and for supporting hypothesis for biological mechanisms of diseases.

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