

ORIGINAL ARTICLE

Argininosuccinic aciduria: Clinical and biochemical phenotype findings in Malaysian children

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Abstract

Argininosuccinic aciduria is an inborn error of the urea cycle caused by deficiency of argininosuccinate lyase (ASL). ASL-deficient patients present with progressive intoxication due to accumulation of ammonia in the body. Early diagnosis and treatment of hyperammonemia are necessary to improve survival and prevent long-term handicap. Two clinical phenotypes have been recognized – neonatal acute and milder late-onset form. We investigated patients with hyperammonemia by a stepwise approach in which quantitative amino acids analysis was the core diagnostic procedure. Here, we describe the clinical phenotypes and biochemical characteristics in diagnosing this group of patients. We have identified 13 patients with argininosuccinic aciduria from 2003 till 2009. Ten patients who presented with acute neonatal hyperammonemic encephalopathy had markedly elevated blood ammonia ($>430 \mu\text{mol/L}$) within the first few days of life. Three patients with late-onset disease had more subtle clinical presentations and they developed hyperammonemia only during the acute catabolic state at two to twelve months of age. Their blood ammonia was mild to moderately elevated ($>75\text{--}265 \mu\text{mol/L}$). The diagnosis was confirmed by detection of excessive levels of argininosuccinate in the urine and/or plasma. They also have moderately increased levels of citrulline and, low levels of arginine and ornithine in their plasma. Two patients succumbed to the disease. To date, eleven patients remained well on a dietary protein restriction, oral ammonia scavenging drugs and arginine supplementation. The majority of them have a reasonable good neurological outcome.

Keywords: Argininosuccinic aciduria, argininosuccinate lyase deficiency, hyperammonemia, urea cycle disorders, quantitative amino acid analysis

INTRODUCTION

Argininosuccinic aciduria (ASA, #MIM608310) is a rare autosomal recessive disorder caused by the deficiency of argininosuccinate lyase. It is one of the six enzymes in the urea cycle pathway that converts the toxic ammonium nitrogen into urea before being excreted in the urine (Figure 1). The gene for ASL deficiency is located on chromosome 7 and has been mapped to locus 7q11.2.^{1,2,3} The estimated worldwide incidence among general population is 1: 70,000.⁴ Two clinical phenotypes have been recognized – a neonatal acute form (the classical form) and a milder late-onset form. Patients with neonatal-onset disease present with severe hyperammonemic coma within the first few days of life. They usually have an overwhelming

illness that rapidly progresses from poor feeding, vomiting, lethargy or irritability and tachypnea to seizure, coma and respiratory arrest. Early clinical recognition and laboratory diagnosis, and urgent treatment to control hyperammonemia are crucial in order to prevent death and severe neurological handicap.⁵ Patients with late-onset disease may present at any age outside of the newborn period. Their clinical manifestations are generally less acute and more subtle than the neonatal-onset variant, and often are precipitated by stress such as infection and anesthesia.^{6,7} Their symptoms may include anorexia, recurrent vomiting, failure to thrive, epilepsy, developmental delay and behavioral problem.^{1,2,3} We report here our experience in diagnosing and treating a cohort of 13 children with argininosuccinic aciduria.

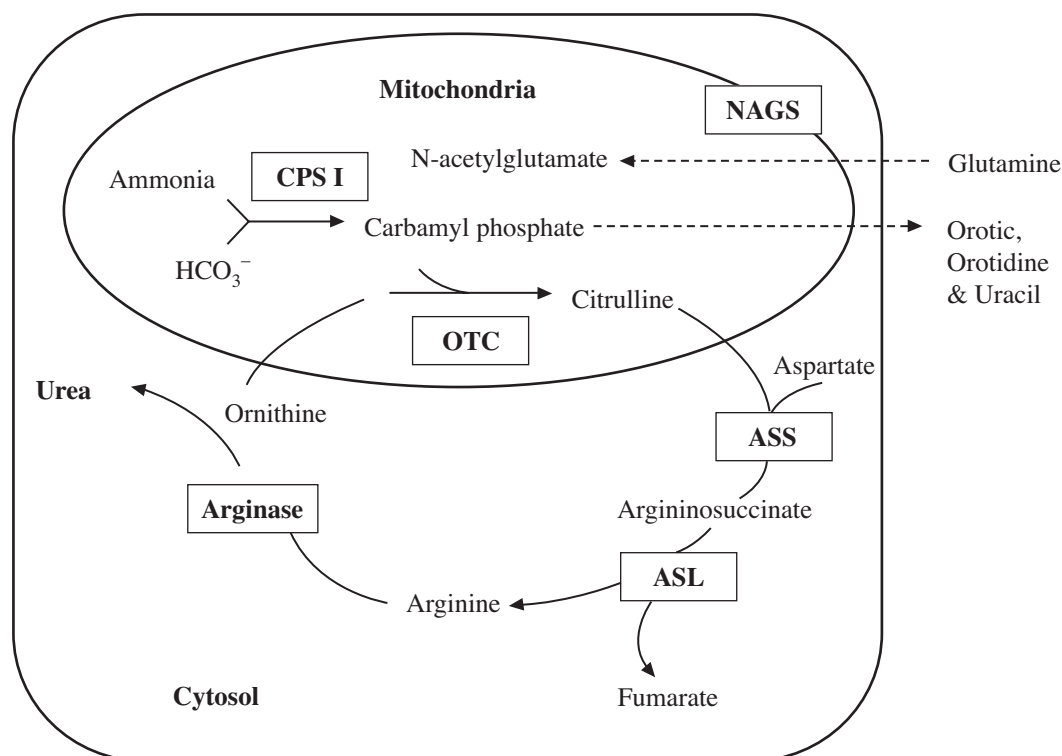


FIG. 1: Urea cycle pathway. The Urea cycle comprises of six enzymes: N-acetyl-glutamate synthase (NAGS), Carbamyl-Phosphate-Synthetase-I (CPS I), Ornithine Transcarbamylase (OTC), Argininosuccinate Synthetase (ASS), Argininosuccinate Lyase (ASL), and Arginase.

MATERIALS AND METHODS

We received samples and referral from paediatricians nationwide for the diagnosis of urea cycle disorders (UCD) in children with hyperammonemia. We followed a stepwise diagnostic protocol as shown in Figure 2. Quantitative amino acid analysis in plasma and/ or urine (for patients suspected of having argininosuccinic aciduria) is the most important diagnostic tool in the evaluation for UCD. Presence of argininosuccinate in plasma or urine was mandatory in order to make a diagnosis of ASL deficiency/argininosuccinic aciduria. We reviewed retrospectively the clinical records and laboratory data of more than 360 children from 8270 samples (4.35%) received who were evaluated for hyperammonemia in our centre over a seven-year period (2003 – 2009).

Samples

Blood and urine samples were collected from acutely ill children when the basic metabolic screen showed significant hyperammonemia. Blood (1-2 mL) was collected in a heparin tube

and the plasma was separated from the blood cells immediately by centrifugation. A minimum of 2 mL urine was collected in a sterile container. Plasma and urine were frozen at -20°C if they could not be analyzed immediately. Samples were transported in an ice box and arrived frozen in the laboratory.

Chemicals

Argininosuccinic acid, 5-sulphosalicylic acid (SSA), and physiological standard A and B were purchased from Sigma. The ultra physiological fluid chemical kit was purchased from Biochrom Ltd., (Cambridge, UK).

Instrument

Amino acids were quantified by ion-exchange chromatography using a dedicated amino acids analyzer (Biochrom 30) and post column detection. In principle, the instrument system works by pumping buffers of varying pH and ionic strength through a column of cation-exchange resin to separate the various amino acids. The column eluent is mixed with the ninhydrin reagent, and the mixture is then passed

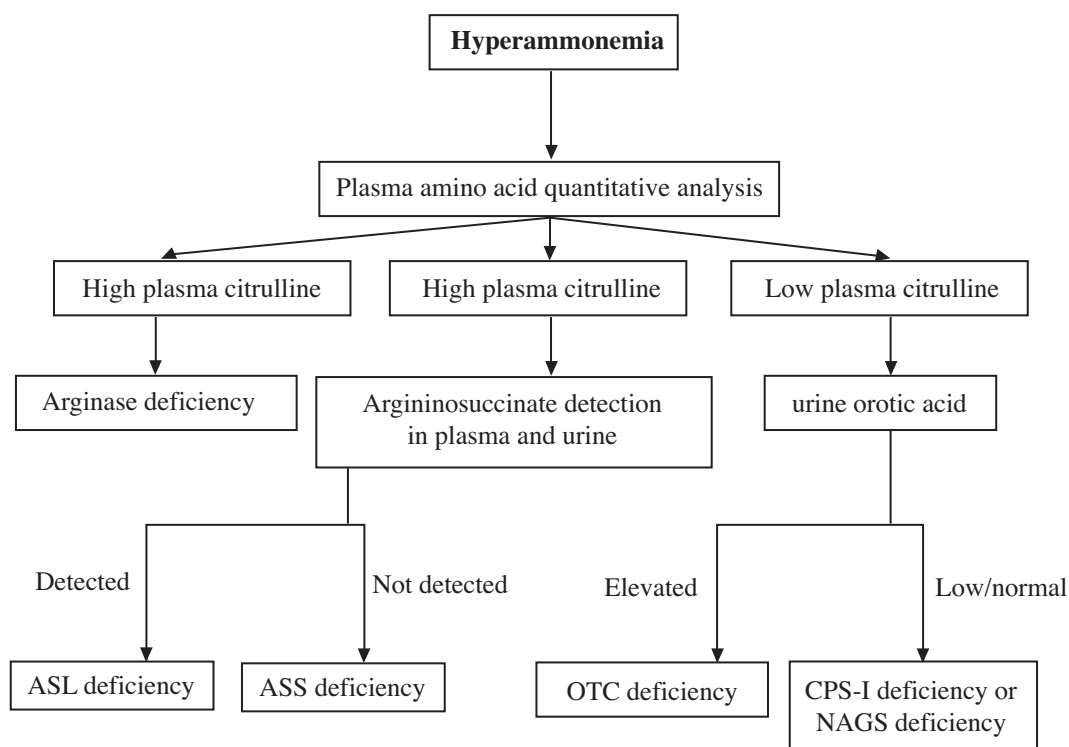


FIG. 2: Stepwise diagnostic protocol for the investigation of hyperammonemia. ASL: Argininosuccinate Lyase; ASS: Argininosuccinate Synthetase; CPS-I: Carbamyl-Phosphate-Synthetase-I; NAGS: N-Acetyl-Glutamate Synthase; OTC: Ornithine Transcarbamylase

through a high temperature reaction coil. In the reaction coil, the ninhydrin reacts with the amino acid to form a coloured compound, and the amount of coloured compound produced is directly proportional to the quantity of amino acid present. The absorbance is measured by wavelengths at 570 nm and 440 nm. The whole system is computer-controlled.

Plasma amino acids analysis

Plasma (100 μ L) was pipetted into an eppendorf tube and 100 μ L of 10% SSA solution was then added. The tube was capped, agitated for a few seconds, and allowed to stand for 1 hour at 4°C. It was then centrifuged at 10,000 rpm for 5 minutes. The supernatant was filtered through a 0.2 μ m membrane to remove any remaining particulate materials prior to analysis. The filtrate was transferred into a vial and loaded into an autosampler. The filtrate (20 μ L) was then injected into the amino acid analyzer. A running time of 2 hours was required for each sample.

Urine amino acids analysis

About 2mL of urine was required and the method for sample processing was similar to that of plasma. About 20 μ L of filtrate was injected into the amino acid analyzer. The running time takes 2 hours for each sample. Creatinine concentration was determined in the urine sample by the modified Jaffe (alkaline-picric) using Modular Biochemistry Roche Analyser and Roche reagents prior the analysis of amino acids.

RESULTS

We identified 13 patients (from 12 families) with argininosuccinic aciduria (four boys and nine girls). This is 0.16% of 8270 patients referred to our centre for investigation of possible inborn errors of metabolism over seven year period. Table 1 and 2 summarize the clinical presentations and laboratory data of our cohort respectively. Eleven out of thirteen patients were Malay. Parental consanguinity was noted in two families.

TABLE 1: Summary of the clinical phenotypes of 13 patients with argininosuccinic aciduria

	Gender	Ethnic	Parental consanguinity	Age at onset	Clinical symptoms	Age at diagnosis	Acute treatment	Current age	Recurrent crises	Current status
1 [#]	B	M	no	3d	feeding refusal, vomiting, lethargy, seizure, coma	15d	a,c	-	no	Died
2 [#]	B	M	no	3d	feeding refusal, vomiting, lethargy	4d	a,b	3y	no	1,2,3
3	G	M	yes	3d	feeding refusal, vomiting, lethargy, coma	10d	c	3.5y	no	1,2,4
4	B	M	no	2d	feeding refusal, vomiting, lethargy, coma, respiratory distress	7d	a,b,c	5y	yes	1,2,5
5	G	C	no	10d	feeding refusal, vomiting, lethargy, coma	21d	b,c	2y	no	1,2,4
6	G	M	no	2d	feeding refusal, vomiting, lethargy, coma	23d	c	1.5y	no	1,2,3
7	G	M	no	3d	feeding refusal, vomiting, lethargy, seizure, coma	5d	b,c	3m	no	1,2,3
8	G	I	no	13d	feeding refusal, vomiting, lethargy, coma	20d	a,b,c	7y	no	1,2,3
9	G	M	no	3d	feeding refusal, vomiting, hyperirritability, coma	5d	a,b,c	2y	no	1,2,4
10	G	M	yes	5 d	feeding refusal, vomiting, lethargy, seizure, coma	8 d	a,c	-	no	Died
11	B	M	no	8m	feeding refusal, vomiting, lethargy, coma (following febrile illness)	14m	c	4.5y	no	1,2,4
12	G	M	no	1y	developmental delay, epilepsy, brittle hair	6y	-	12y	no	1,2,4
13	G	M	yes	2m	developmental delay, epilepsy, recurrent vomiting, growth failure	5y	-	11y	yes*	1,2,5

B: boy; G: girl; M: Malay; C: Chinese; I: Indian; d: day, m: month; y: year; acute treatment: a, acute dialysis b, intravenous ammonia scavenger drugs; c, ventilator support; current status: 1, protein restricted diet; 2, oral ammonia scavenger drugs; 3, normal development; 4, mild mental retardation; 5, moderate mental retardation. #: Patient 1 and 2 are siblings. *: until five years old.

TABLE 2: Biochemical phenotypes of 13 patients with argininosuccinic aciduria

PATIENT	Plasma		Urine		Plasma Amino Acid				
	ammonia	orotic	Arginino succinate	Arginino succinate	Arginino succinate	Citrulline	Arginine	Glutamine	Alanine
1	1,586	not done	1,084	570	259	2	1,993	3,397	
2	430	14.6	1,570	263	152	12	1,093	692	
3	1,172	not done	1,600	640	324	17	1,183	593	
4	1,035	13	4,970	406	160	22	711	530	
5	693	not done	598	110	55	15	1,999	619	
6	521	118	1,879	403	358	28	1,408	576	
7	1,205	117	2,862	379	265	29	1,719	1,878	
8	572	19.2	1,600*	229*	147	39	3,913	854	
9	1,848	not done	2,357	187	247	56	800	600	
10	780	elevated	5,448	576	160	20	1,101	1246	
11	262	36	184	not detected	202	28	406	241	
12	264	26.4	1,716	88	172	25	2,646	1,126	
13	175	elevated	1,725	335	419	70	1,065	985	
Reference range	Neonate: <110µmol/L, older child 50-80µmol/L	1.0-3.2 mmol/mol creatinine	absent (µmol/mmol of creatinine)	absent	3-36 µmol/L	17-119 µmol/L	<700 µmol/L	132-455 µmol/L	

*Argininosuccinic acid was detected after protein challenge

Ten patients (Patient 1 to 10) had the acute neonatal form of the disease, with symptoms of hyperammonemia appearing between the second and thirteenth day after birth. The blood ammonia level ranged from $430\mu\text{mol/L}$ to $1,848\mu\text{mol/L}$. Nine of the ten patients had argininosuccinic acid detected in blood (Fig. 3a) during the acute episode. Argininosuccinate was detected in the blood of Patient 8 only after a protein challenge. Argininosuccinate found in the urine was two to ten times higher in the plasma levels. Plasma glutamine and citrulline levels were elevated in all patients, whereas arginine and ornithine levels were low. Urine orotic acid was measured in five patients and; all of them had raised orotic acids levels, three to thirty times the normal limit.

Three patients presented later (between the age of two months and twelve months) with milder clinical symptoms. Late-onset patients excreted significantly less argininosuccinate compared to the neonatal-onset group. In one of the patient, argininosuccinic acid was detected only in urine.

Two patients (Patient 1 and 10) with neonatal-onset disease died at the age of 12 days and 4 months when they had a recurrent hyperammonemic coma. However, nine patients survived with a reasonably good neurological outcome; four patients have normal developmental status and five have mild delayed development. Two patients (patient 4 and 13) have severe neurological disabilities as a consequence of recurrent hyperammonemic episodes.

DISCUSSION

Argininosuccinic aciduria is the second most common disorder of inborn errors of the urea cycle in European countries and the United States. The reported incidence is about 1 in 70,000 live births in the United States.⁸ Our study shows a prevalence of 0.16% (13 positive) from 8270 patients referred to our centre for investigation of various inborn errors of metabolism disorder. It is also considered to be the most common disorder of urea cycle diagnosed in our country.

The clinical presentation of argininosuccinic aciduria is rather non-specific, just like other urea cycle disorders. Neonatal disease resembles a neonatal infection whereas late-onset disease can mimic many other neurological disorders.^{1,2} As such the recognition of argininosuccinic aciduria heavily relies on biochemical laboratory testing. The first clue to alert the clinician and

laboratory scientist that he/she may be dealing with argininosuccinic aciduria or a urea cycle disorder in a sick child is raised blood ammonia. It is, therefore, essential to measure ammonia early in every sick child without a clear diagnosis. After excluding false hyperammonemia such as improper sample collection and transportation, struggling or a haemolysed blood sample, blood ammonia more than $200\mu\text{mol/L}$ in a previously healthy term newborn or more than $150\mu\text{mol/L}$ in an older child is strongly suggestive of an underlying urea cycle disorders such as argininosuccinic aciduria.^{2,9} This should prompt the clinician to contact the diagnostic laboratory for urgent plasma and urine amino acids analysis.

Plasma quantitative amino acid analysis is necessary to confirm a specific diagnosis of urea cycle disorder. Argininosuccinic aciduria is one of the 3 urea cycle disorders (the other two are citrullinemia and arginase deficiency) in which changes in amino acids are usually diagnostic without the need for further enzymatic or molecular testing.^{2,9,10} Presence of argininosuccinate is the characteristic marker for diagnosis of argininosuccinic aciduria, which is usually not detected in a normal person.¹¹ Other significant amino acids are citrulline and orotic acid. In patients with argininosuccinic aciduria, the plasma citrulline is usually elevated to levels of 150 to $250\mu\text{mol/L}$. Hyperglutaminemia and hyperalaninemia are also often present. Elevated glutamine signifies a hyperammonemic state as glutamine is an ammonia scavenger. Raised plasma alanine is a non specific finding. Under normal circumstances, arginine is produced from argininosuccinate. Hypoargininemia will, therefore, be expected and is a common finding in argininosuccinic aciduria.¹¹

Although plasma amino acid quantification is diagnostic, potential pitfalls in amino acid analysis need to be recognized. Firstly, argininosuccinic acid is not one of the usual amino acids routinely detected in an amino acids analysis and can easily be misidentified, because it may co-elute with other amino acids especially leucine (Fig 3b).¹² Secondly, argininosuccinate acid is highly soluble and rapidly cleared from blood. Therefore, the amount present may be too little to be detected. As such urinary amino acid analysis is helpful in confirming argininosuccinic aciduria because of the marked excretion of argininosuccinate acid in urine.¹¹ In addition, urine samples treated with heat or barium precipitation prior

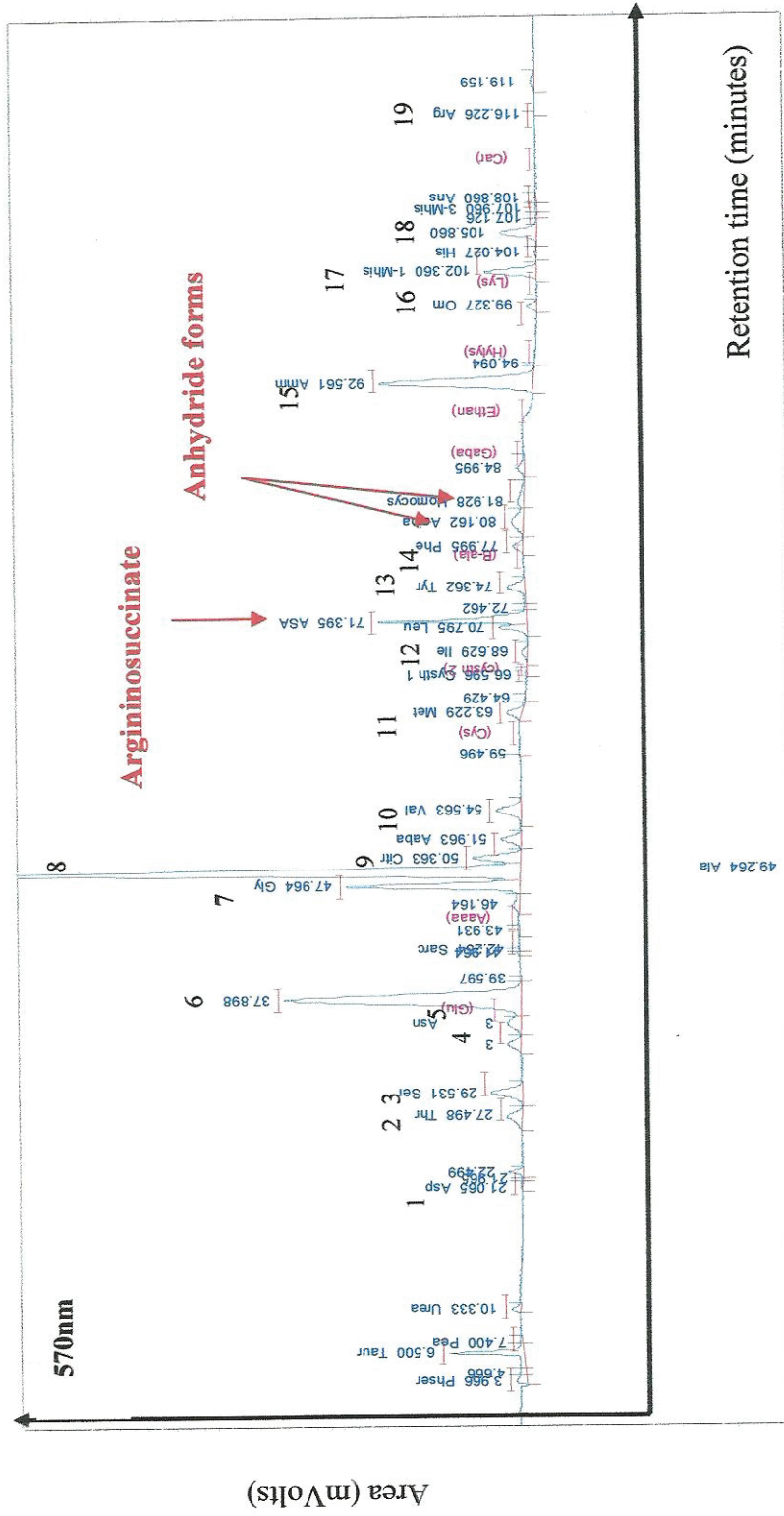


FIG. 3a: Plasma Amino Acid Chromatogram for Patient 1 with Argininosuccinate Lyase Deficiency

This chromatogram was obtained at 570nm. The amino acids can be separated because of different pKa and hence eluted with different retention times. In this chromatogram, argininosuccinate was eluted immediately after leucine.

1. Aspartic acid, 2. Threonine, 3. Serine, 4. Asparagine, 5. Glutamic acid, 6. Glutamine, 7. Glycine, 8. Alanine, 9. Citrulline, 10. Valine, 11. Methionine, 12. Leucine, 13. Tyrosine, 14. Phenylalanine, 15. Ammonium, 16. Ornithine, 17. Lysine, 18. Histidine, 19. Arginine.

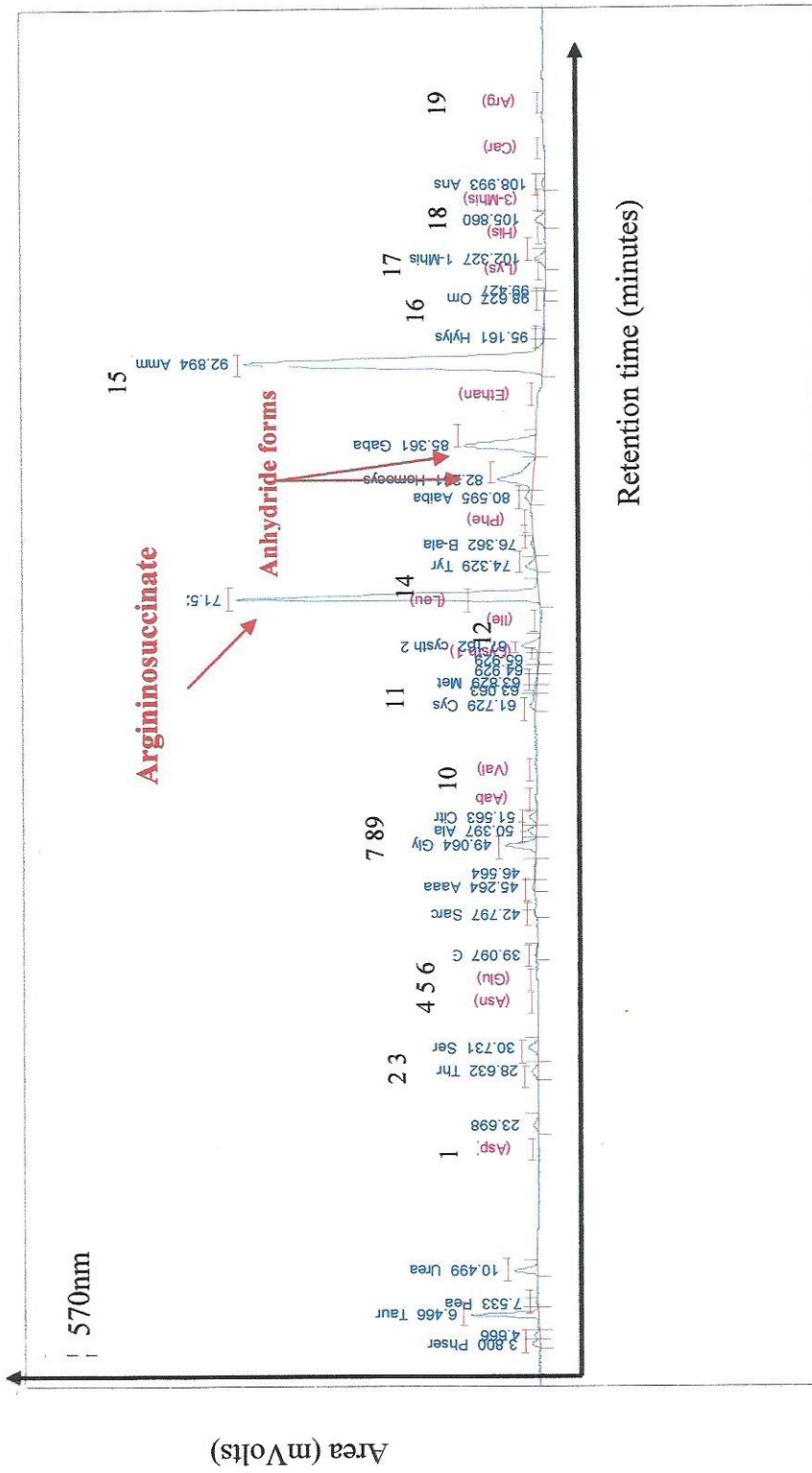


FIG. 3b: Urine Amino Acid Chromatogram for Patient 1 with Argininosuccinate Lyase Deficiency.

In ASL deficiency, argininosuccinate which is the characteristic urinary marker, is excreted in large amount (1084 $\mu\text{mol}/\text{mmol}$ creatinine), and some of these are converted into anhydride forms. In this chromatogram, the two anhydrides of argininosuccinate are eluted at the retention time of homocysteine and gaba peaks, whereas argininosuccinate is eluted closely after the leucine peak.

1. Aspartic acid, 2. Threonine, 3. Serine, 4. Asparagine, 5. Glutamic acid, 6. Glutamine, 7. Glycine, 8. Alanine, 9. Citrulline, 10. Valine, 11. Methionine, 12. Leucine, 13. Tyrosine, 14. Phenylalanine, 15. Ammonium, 16. Ornithine, 17. Lysine, 18. Histidine, 19. Arginine.

to analysis will further improve the sensitivity of detection by converting the argininosuccinic acid into anhydrides.¹² Nevertheless, quantitative analysis of urine amino acids is generally not useful for diagnosis of most amino acid disorders and other urea cycle disorders. This is because urine amino acids concentrations do not reflect the true amino acid concentration in blood due to the effect of renal reabsorption. Urine argininosuccinate quantitative analysis is one of the few exceptions.

A favourable outcome can be achieved if argininosuccinic aciduria is diagnosed early. Immediate treatment may include acute dialysis to rapidly remove ammonia which is extremely toxic to the brain. Long term treatment will normally include dietary protein restriction, arginine supplementation, use of pharmacological ammonia scavengers such as sodium benzoate and sodium phenylbutyrate.¹³

In conclusion, clinicians should always consider the possibility of a child with unexplained illness, or without a clear explanation, having an inborn error of metabolism such as argininosuccinic aciduria. Close collaboration with the laboratory is potentially life saving.

ACKNOWLEDGEMENT

The authors like to thank all the paediatricians who have referred patients to us, all the patients and their families, and the staff of Metabolic Clinic (Ms Balktiah Mat and Ms Norzawani Che Johari) for assisting in the retrieval of medical records. The authors also wish to thank Dr Keng Wee Teik, Dr. Shanti B, Dr Ch'ng Gaik Siew for their clinical support, and the staff of Biochemical Genetics Unit (Ms Huzaimah bte Sani, Ms Tengku Rosmaliza, Mr Mohd Helmi and Miss Komalam) for their excellent technical assistance.

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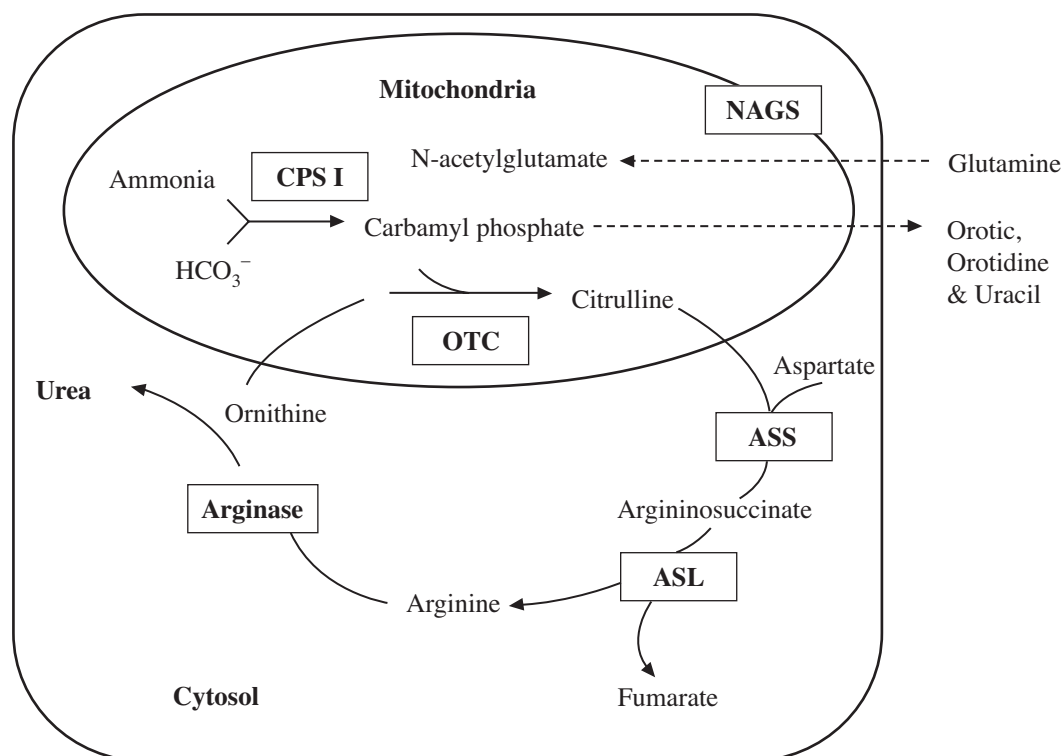


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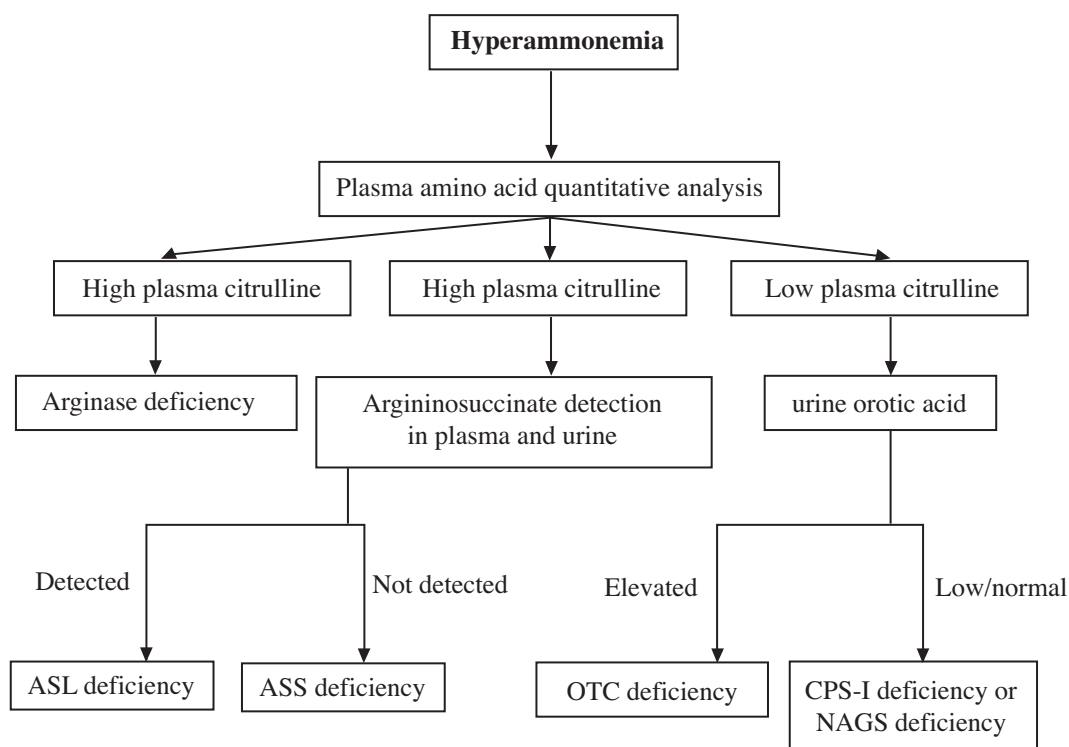


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7	1,205	117	2,862	379	265	29	1,719	1,878	
8	572	19.2	1,600*	229*	147	39	3,913	854	
9	1,848	not done	2,357	187	247	56	800	600	
10	780	elevated	5,448	576	160	20	1,101	1246	
11	262	36	184	not detected	202	28	406	241	
12	264	26.4	1,716	88	172	25	2,646	1,126	
13	175	elevated	1,725	335	419	70	1,065	985	
Reference range	Neonate: <110µmol/L, older child 50-80µmol/L	1.0-3.2 mmol/mol creatinine	absent (µmol/mmol of creatinine)	absent	3-36 µmol/L	17-119 µmol/L	<700 µmol/L	132-455 µmol/L	

*Argininosuccinic acid was detected after protein challenge

Ten patients (Patient 1 to 10) had the acute neonatal form of the disease, with symptoms of hyperammonemia appearing between the second and thirteenth day after birth. The blood ammonia level ranged from $430\mu\text{mol/L}$ to $1,848\mu\text{mol/L}$. Nine of the ten patients had argininosuccinic acid detected in blood (Fig. 3a) during the acute episode. Argininosuccinate was detected in the blood of Patient 8 only after a protein challenge. Argininosuccinate found in the urine was two to ten times higher in the plasma levels. Plasma glutamine and citrulline levels were elevated in all patients, whereas arginine and ornithine levels were low. Urine orotic acid was measured in five patients and; all of them had raised orotic acids levels, three to thirty times the normal limit.

Three patients presented later (between the age of two months and twelve months) with milder clinical symptoms. Late-onset patients excreted significantly less argininosuccinate compared to the neonatal-onset group. In one of the patient, argininosuccinic acid was detected only in urine.

Two patients (Patient 1 and 10) with neonatal-onset disease died at the age of 12 days and 4 months when they had a recurrent hyperammonemic coma. However, nine patients survived with a reasonably good neurological outcome; four patients have normal developmental status and five have mild delayed development. Two patients (patient 4 and 13) have severe neurological disabilities as a consequence of recurrent hyperammonemic episodes.

DISCUSSION

Argininosuccinic aciduria is the second most common disorder of inborn errors of the urea cycle in European countries and the United States. The reported incidence is about 1 in 70,000 live births in the United States.⁸ Our study shows a prevalence of 0.16% (13 positive) from 8270 patients referred to our centre for investigation of various inborn errors of metabolism disorder. It is also considered to be the most common disorder of urea cycle diagnosed in our country.

The clinical presentation of argininosuccinic aciduria is rather non-specific, just like other urea cycle disorders. Neonatal disease resembles a neonatal infection whereas late-onset disease can mimic many other neurological disorders.^{1,2} As such the recognition of argininosuccinic aciduria heavily relies on biochemical laboratory testing. The first clue to alert the clinician and

laboratory scientist that he/she may be dealing with argininosuccinic aciduria or a urea cycle disorder in a sick child is raised blood ammonia. It is, therefore, essential to measure ammonia early in every sick child without a clear diagnosis. After excluding false hyperammonemia such as improper sample collection and transportation, struggling or a haemolysed blood sample, blood ammonia more than $200\mu\text{mol/L}$ in a previously healthy term newborn or more than $150\mu\text{mol/L}$ in an older child is strongly suggestive of an underlying urea cycle disorders such as argininosuccinic aciduria.^{2,9} This should prompt the clinician to contact the diagnostic laboratory for urgent plasma and urine amino acids analysis.

Plasma quantitative amino acid analysis is necessary to confirm a specific diagnosis of urea cycle disorder. Argininosuccinic aciduria is one of the 3 urea cycle disorders (the other two are citrullinemia and arginase deficiency) in which changes in amino acids are usually diagnostic without the need for further enzymatic or molecular testing.^{2,9,10} Presence of argininosuccinate is the characteristic marker for diagnosis of argininosuccinic aciduria, which is usually not detected in a normal person.¹¹ Other significant amino acids are citrulline and orotic acid. In patients with argininosuccinic aciduria, the plasma citrulline is usually elevated to levels of 150 to $250\mu\text{mol/L}$. Hyperglutaminemia and hyperalaninemia are also often present. Elevated glutamine signifies a hyperammonemic state as glutamine is an ammonia scavenger. Raised plasma alanine is a non specific finding. Under normal circumstances, arginine is produced from argininosuccinate. Hypoargininemia will, therefore, be expected and is a common finding in argininosuccinic aciduria.¹¹

Although plasma amino acid quantification is diagnostic, potential pitfalls in amino acid analysis need to be recognized. Firstly, argininosuccinic acid is not one of the usual amino acids routinely detected in an amino acids analysis and can easily be misidentified, because it may co-elute with other amino acids especially leucine (Fig 3b).¹² Secondly, argininosuccinate acid is highly soluble and rapidly cleared from blood. Therefore, the amount present may be too little to be detected. As such urinary amino acid analysis is helpful in confirming argininosuccinic aciduria because of the marked excretion of argininosuccinate acid in urine.¹¹ In addition, urine samples treated with heat or barium precipitation prior

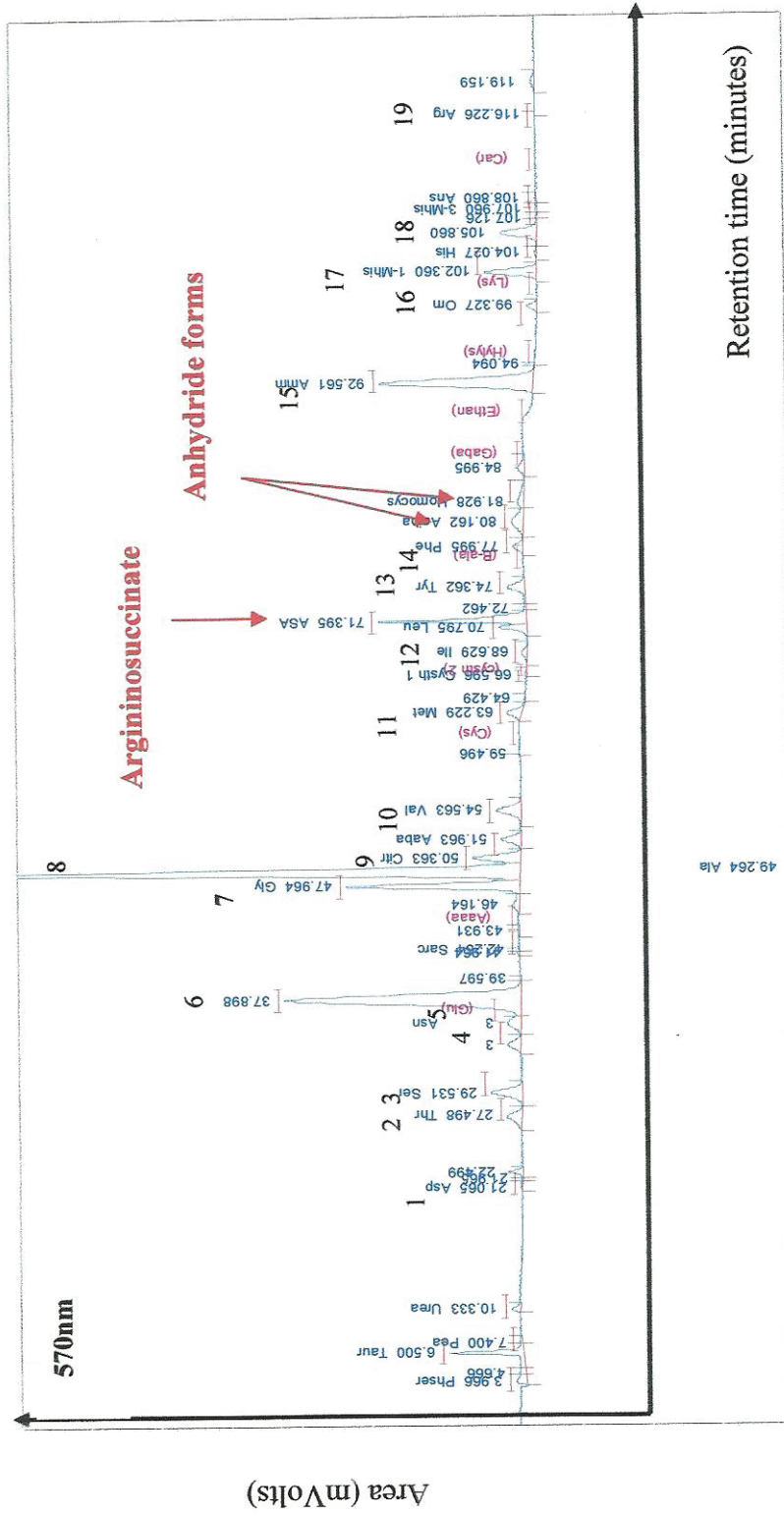


FIG. 3a: Plasma Amino Acid Chromatogram for Patient 1 with Argininosuccinate Lyase Deficiency

This chromatogram was obtained at 570nm. The amino acids can be separated because of different pKa and hence eluted with different retention times. In this chromatogram, argininosuccinate was eluted immediately after leucine.

1. Aspartic acid, 2. Threonine, 3. Serine, 4. Asparagine, 5. Glutamic acid, 6. Glutamine, 7. Glycine, 8. Alanine, 9. Citrulline 10. Valine, 11. Methionine, 12. Leucine, 13. Tyrosine, 14. Phenylalanine, 15. Ammonium, 16. Ornithine, 17. Lysine, 18. Histidine, 19. Arginine.

to analysis will further improve the sensitivity of detection by converting the argininosuccinic acid into anhydrides.¹² Nevertheless, quantitative analysis of urine amino acids is generally not useful for diagnosis of most amino acid disorders and other urea cycle disorders. This is because urine amino acids concentrations do not reflect the true amino acid concentration in blood due to the effect of renal reabsorption. Urine argininosuccinate quantitative analysis is one of the few exceptions.

A favourable outcome can be achieved if argininosuccinic aciduria is diagnosed early. Immediate treatment may include acute dialysis to rapidly remove ammonia which is extremely toxic to the brain. Long term treatment will normally include dietary protein restriction, arginine supplementation, use of pharmacological ammonia scavengers such as sodium benzoate and sodium phenylbutyrate.¹³

In conclusion, clinicians should always consider the possibility of a child with unexplained illness, or without a clear explanation, having an inborn error of metabolism such as argininosuccinic aciduria. Close collaboration with the laboratory is potentially life saving.

ACKNOWLEDGEMENT

The authors like to thank all the paediatricians who have referred patients to us, all the patients and their families, and the staff of Metabolic Clinic (Ms Balktiah Mat and Ms Norzawani Che Johari) for assisting in the retrieval of medical records. The authors also wish to thank Dr Keng Wee Teik, Dr. Shanti B, Dr Ch'ng Gaik Siew for their clinical support, and the staff of Biochemical Genetics Unit (Ms Huzaimah bte Sani, Ms Tengku Rosmaliza, Mr Mohd Helmi and Miss Komalam) for their excellent technical assistance.

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