



Plasma amino acid and urine organic acid profiles of Filipino patients with maple syrup urine disease (MSUD) and correlation with their neurologic features



Mary Anne D. Chiong, MD, MSc^{a,*}, Marilyn A. Tan^b, Cynthia P. Cordero^c, Esphie Grace D. Fodra^a, Judy S. Manliguia^a, Cristine P. Lopez^a, Leslie Michelle M. Dalmacio^d

^a Institute of Human Genetics, National Institutes of Health, University of the Philippines Manila, Philippines

^b Department of Pediatrics, Section of Neurology, Philippine General Hospital, University of the Philippines Manila, Philippines

^c Department of Clinical Epidemiology, University of the Philippines Manila, Philippines

^d Department of Biochemistry, University of the Philippines Manila, Philippines

ARTICLE INFO

Article history:

Received 1 August 2016

Received in revised form 6 October 2016

Accepted 6 October 2016

Available online 12 October 2016

Keywords:

MSUD

Plasma amino acids

Urine organic acids

Neurologic features

ABSTRACT

Background: Maple syrup urine disease (MSUD) is the most common inborn error of metabolism in the country. The cause of the neuropathology is still not well established although accumulation of branched chain amino acids (BCAA) and alteration in large neutral amino acids (LNAA) as well as energy deprivation are suggested. It is therefore the aim of this study to determine the plasma amino acid and urine organic acid profiles of patients with MSUD and correlate the findings with their neurologic features.

Methodology: Twenty six Filipino patients with MSUD were studied in terms of their plasma amino acid and urine organic acid profiles. Their results were compared with 26 age and sex matched controls. The neurologic features were correlated with the results of the plasma amino acids and urine organic acids.

Results: Majority of the patients with MSUD had developmental delay/intellectual disability (88%), speech delay (69%), and seizures (65%). Their amino acid profiles revealed low glutamine and alanine with high levels of leucine, isoleucine, phenylalanine, threonine and alloisoleucine compared to controls ($p < 0.05$). The urine organic acids showed significantly elevated excretion of the branched chain ketoacids and succinate ($p < 0.05$). However there were no biochemical markers that correlated significantly with the neurologic features.

Conclusion: The findings suggest that there could still be altered LNAA metabolism among patients with MSUD when the BCAAs are elevated. Although the biochemical findings were not significantly correlated with the neurologic features, the study showed that prevention and avoidance of neurologic disturbances may still rely primarily on early diagnosis and prompt institution of treatment, along with strict compliance with the dietary regimen and maintenance of good metabolic control over time.

© 2016 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Maple syrup urine disease (MSUD) is an autosomal recessive amino acid disorder caused by a deficiency in the activity of the mitochondrial enzyme branched chain α -keto acid dehydrogenase (BCKAD) complex. The metabolic block from BCKAD deficiency results in the accumulation of the branched chain amino acids (BCAA) leucine, isoleucine and valine, as well as their corresponding ketoacids [1,2].

The estimated worldwide incidence of MSUD is approximately 1:185,000, however, the prevalence is much higher in certain ethnic

groups [2,3]. In the Philippines, over 150 patients have been diagnosed clinically for 2 decades now and the incidence based on newborn screening is 1:82,354 (Newborn Screening Reference Center Philippines, 2015).

The accumulated metabolites in MSUD primarily cause neurologic toxicity. It is believed that leucine and its α -ketoacid, α -ketoisocaproate (KIC), which accumulates most in the disorder, are the principal neurotoxic agents. However, studies revealed that besides the characteristically elevated plasma concentrations of the BCAA leucine, isoleucine and valine, most of the large neutral amino acids (LNAA), namely tryptophan, tyrosine, methionine and phenylalanine, showed significantly reduced concentration in plasma of patients during metabolic decompensation compared to controls of the same age. It is possible that the decrease in plasma concentrations of LNAA may also explain the neurological dysfunction seen in MSUD [4].

Through the years, it has been a local practice to monitor the branched chain amino acids of patients with MSUD using thin layer

Abbreviations: MSUD, maple syrup urine disease; BCAA, branched chain amino acids; LNAA, large neutral amino acids; BCKAD, branched chain keto acid dehydrogenase.

* Corresponding author at: Institute of Human Genetics, National Institutes of Health, 625 Pedro Gil St, Ermita, Manila, Philippines.

E-mail address: mdchiong1@up.edu.ph (M.A.D. Chiong).

chromatography (TLC). This is a semi-quantitative method that measures combined leucine/isoleucine values, and although it was found to correlate well with the values of branched chain amino acid levels in the plasma [5], this does not analyze quantitatively the full amino acid and or branched chain amino acid profiles of patients. Despite monitoring and provision of the recommended diet to these children, adjustments in the dietary management if based on the TLC levels alone are not ideally done. We surmise that this might be a contributing factor in the poor neurologic outcome observed in our patients with MSUD. It is postulated that the abnormalities in the branched chain amino acid levels along with other amino acids could contribute to the neurodevelopmental features exhibited by patients with MSUD. Likewise, energy deprivation through Krebs cycle disruption is associated with branched chain ketoacid accumulation [6].

This is the first Filipino study that has embarked on the complete plasma amino acid and urine organic acid profiling of patients with MSUD. It primarily aims to compare the levels of plasma branched chain amino acids (BCAA), large neutral amino acids (LNAA) and other amino acids, as well as the metabolites for energy production on urine organic acid analysis between patients with MSUD and controls. The findings on the plasma amino acids and urine organic acids may provide an indication regarding the possible basis of the neurologic and clinical findings seen in patients with MSUD and may consequently have an impact on the treatment and long term management of these patients.

2. Methodology

2.1. Study design

This was a case control study involving the analysis of the plasma amino acid and urine organic acid profiles of MSUD cases and their age and sex matched controls. The study also described the neurologic and clinical profiles of MSUD cases and the correlation of these findings with the plasma and urine amino acid results.

2.2. Study population and recruitment of cases and controls

The study consisted of 26 Filipino patients confirmed to have MSUD by newborn screening or by biochemical assay such as thin layer chromatography or urine high voltage electrophoresis. They were recruited from the Philippine General Hospital (PGH) and or the Institute of Human Genetics-National Institutes of Health (IHG-NIH). They were known patients and are included in the metabolic registry of IHG-NIH. After signing the consent forms, the clinical histories of the patients were reviewed along with their metabolic control and dietary regimen. Specific attention was given to the age of onset, presenting symptoms, age at diagnosis, age at initiation of treatment, metabolic control based on TLC leu/isoleu levels, diet history, current anthropometrics, and presence of the characteristic smell. A pediatric neurologist likewise evaluated their neurologic function and development.

Similarly, 26 controls were included in this study and were recruited from the Sick and Well Baby Clinics of the Philippine General Hospital. It was done randomly by frequency matching in accordance with the age and sex of their MSUD counterparts. The parents and guardians of the children went through the same informed consent process as the cases. Children aged 7 years old and above went through an informed assent process. Only healthy children with normal z scores for height/length and weight, or children with acute mild illnesses who came in for check up and had no symptoms of MSUD were included. Their clinical histories were reviewed and they were physically examined by the principal investigator.

This study was approved by the University of the Philippines Manila Research Ethics Board (UPMREB 2013-019-01/2013-019-P2).

2.3. Data collection procedure

2.3.1. Specimens

Around 3 mL of venous blood from the brachial vein of patients with MSUD and controls were collected and was centrifuged at 2700 rpm for 15 min to separate the plasma. The plasma samples were then kept at -80°C until the day of analysis, with a maximum of one week. At the same time, around 20 ml of urine was also randomly collected in a sterile bottle and was frozen at -20°C immediately after collection. They were stored for not more than a month.

2.3.2. Specimen processing

The blood samples underwent quantitative plasma amino acid analysis using the fully automated Waters Ultra Performance Liquid Chromatography (UPLC). The Waters UPLC Analyzer was set-up according to the MassTrak amino acid analysis instrument method. The amino acid peaks were then integrated and the concentrations were calculated using the Empower software and previously optimized processing method. All standards, solvents and chemicals for MassTrak plasma amino acid analysis were purchased from Waters.

Organic acids were extracted using previously optimized methods and were analyzed using gas chromatography–mass spectrometry (GC–MS) system by Agilent. All organic solvents and other reagents were of analytical grade.

2.4. Statistical analysis

For the demographic data, descriptive statistics were utilized in order to examine the clinical and neurologic characteristics of the MSUD cases. Amino acid values were summarized by means, medians and standard deviations separately among the cases and controls. Independent *t*-test for unequal variances was used to test the hypothesis of equal means between the cases and controls. A discriminant analysis using the Stata 12 statistical software was also done to generate discriminant function scores for cases and controls using identified important amino acids. Fisher's exact test was used to test the hypothesis of similarity of the distribution of organic acid assessments among cases and controls.

All hypothesis testing procedures used in this study were done at 5% level of significance (two-tailed). All statistical analysis was carried out in the statistical package of Stata 12 software and was used for the amino acid analysis, organic acid analysis and the correlation studies.

3. Results

A total of 26 MSUD cases and 26 controls (chosen by age-sex frequency matching) participated in the study. The average age of participants was 4.9 years, the youngest was a little more than a month (1.2 months) and the oldest was 16 years. Of the 52 participants, there were more males than females (34 males, 65% vs. 18 females, 35%).

3.1. General disease profile

Based on the data gathered during the review of the patients' histories, the average age of onset of symptoms was 15 days (23.8 SD). The onset ranged from 3 to 90 days. The mean age at diagnosis was 42.2 days (105 SD, range 3–485 days). The mean was influenced by three patients who were diagnosed at 120, 270 and 485 days. The median was much lower at 9 days. Sixty five percent of patients were diagnosed when they were 10 days old or younger.

The most common presenting symptoms at the time of diagnosis were seizures (13/26) and sleepiness (13/26), followed by poor suck/cry (12/26) and the characteristic burnt sugar smell of MSUD (9/26). One patient was asymptomatic at birth but was otherwise treated as a case of MSUD even before leucine values were taken because a previous sibling died of MSUD. Most of the patients were admitted to the hospital

at the time of diagnosis (23/26; 88.46%) and almost half of them (12/26; 46.15%) underwent dialysis as a means to get rid of the accumulating branched chain amino acids. Those with neurologic symptoms were usually seen by pediatric neurologists for the management of seizures and acute encephalopathy.

The initial leucine levels at the age of diagnosis averaged at 1944.9 $\mu\text{mol/L}$, the lowest was 500 $\mu\text{mol/L}$ and the highest was 4500 $\mu\text{mol/L}$ ($\text{NV} < 300 \mu\text{mol/L}$). For the monitoring of leucine levels during the long term course of the disease, the average minimum leucine level was 269.6 $\mu\text{mol/L}$ while the average maximum was 850.8 $\mu\text{mol/L}$. The majority of the patients (25/26; 96%) strictly complied with the prescribed diet of low protein and BCAA free formula. Table 1 shows the average natural protein in grams being taken daily by patients with MSUD and the leucine intake in mg/kg for most patients were within the recommended values for age [7].

Seventy three percent (19/26; 73%) had fair metabolic control (leucine > 300 – $1000 \mu\text{mol/L}$), 19% (5/26) had good control (leucine below 300 $\mu\text{mol/L}$) and 7.6% (2/26) had poor control with levels that were always above 1000 $\mu\text{mol/L}$.

3.2. Neurologic evaluation

During the neurologic evaluation done at the time of study by a pediatric neurologist, the most common neurologic abnormality seen among the cases was developmental delay or intellectual disability (22/26). There were only 3 patients who were developmentally at par with age. Among those with developmental delay/intellectual disability, 18 patients had speech delay. The majority also had seizures and spasticity (Table 2). Of the 26 patients, only 15 had electroencephalograms (EEG) done, 5 patients had normal results and 10 had epileptiform discharges.

3.3. Plasma amino acid analysis

The mean levels of isoleucine and leucine were higher among cases than controls. The level of valine was normal in patients with MSUD and was slightly higher in the controls but this was not statistically significant. In addition, the LNAAs phenylalanine, threonine, isoleucine and its derivative alloisoleucine were significantly higher in cases than in controls ($p < 0.05$). Alloisoleucine was not found in any of the controls. The average glutamine and alanine levels were lower among cases than the controls. Other amino acids such as hydroxyproline, taurine, lysine, and sarcosine were likewise significantly higher in the cases than in the controls ($p < 0.05$) (Table 3).

The seven amino acids that were both clinically and statistically significant (glutamine, alanine, leucine, isoleucine, phenylalanine, threonine and allo-isoleucine) were used for discriminant analysis as variables to generate a coefficient score that could distinguish between a case and a control. The most important amino acid was threonine (highest coefficient), followed by leucine. The least important was allo-isoleucine (Table 4). The D (discriminant) scores were also lower

Table 1
Protein and leucine intake per age group.

| Age group | Average daily protein intake (g/kg) ^a | Average weight (kg) | Average daily leucine intake (mg) | Recommended daily leucine intake (mg/kg) |
|-------------|--|---------------------|-----------------------------------|--|
| 0–6 months | 0.97 | 9.1 | 60 | 40–100 |
| 1–3 years | 0.8 | 12.2 | 48 | 40–70 |
| 4–8 years | 0.59 | 17 | 35 | 35–65 |
| 9–13 years | 0.38 | 26 | 23 | 30–60 |
| 14–18 years | 0.54 | 35 | 32 | 15–50 |

^a 1 g of protein = 60 mg leucine [8].

Table 2
Frequency of neurologic signs among the 26 cases.

| Neurologic signs | Number | Percent (%) |
|--|--------|-------------|
| Developmental delay/intellectual disability | 22 | 84.62 |
| Speech delay | 18 | 69.23 |
| Seizures | 17 | 65.38 |
| Long tract signs (clonus, Babinski, hyperreflexia) | 11 | 42.31 |
| Spasticity | 11 | 42.31 |
| Hypotonia | 6 | 23.07 |
| No visual threat/dazzle | 5 | 19.23 |
| Hyperactivity | 5 | 19.23 |
| Microcephaly | 4 | 15.38 |
| Drooling | 2 | 7.69 |
| Esotropia | 1 | 3.85 |
| No reaction to sound | 1 | 3.85 |
| Jitteriness | 1 | 3.85 |
| Weakness (dragging leg) | 1 | 3.85 |
| Spastic quadriplegia | 1 | 3.85 |

among the controls. All the cases scored positive except for three patients (patient numbers 5, 9 and 19 scored -1.79). These were also the cases identified as controls by discriminant analysis but were actually cases.

All controls scored negative (Fig. 1). There was a more positive D score value for the cases (mean 1.57; 1.29 SD; range -1.79 to 4.12) for the seven amino acids mentioned above compared to the controls (mean -1.57 ; 0.58 SD; range -2.94 to -0.53). From a statistical standpoint, the low glutamine and alanine and the high leucine, isoleucine, phenylalanine, threonine and alloisoleucine, when seen altogether in an amino acid profile distinguishes an MSUD case from that which is not in this series, and that this model explains 72% (canonical correlation 0.8483) of the variation seen among the cases and controls. In Fig. 1, this distinct separation between a case and a control using discriminant analysis is shown.

3.4. Organic acid analysis

The difference in the distribution of cases and controls was only statistically significant for 2-hydroxyisovalerate (2HIV), 2-ketoisocaproate (2KIC), 2-keto 3-methylvalerate (KMV) and succinate (Table 5).

3.5. Correlation studies between neurologic features and biochemical findings

3.5.1. Neurologic features and amino acids

There were statistically significant differences in the distribution of some amino acids including derived amino acids (i.e. citrulline, ornithine) between the presence and absence of developmental delay, seizures, speech delay and spasticity between cases and controls. There were no statistically significant amino acids that differ between those cases with and without long tract signs. For developmental delay, valine was statistically significantly higher in those with developmental delay than those without; for speech delay, valine and ornithine were also significantly higher in those with speech delay than those without; for seizures, citrulline was lower in those presenting with seizures than those without; and for spasticity, hydroxyproline and sarcosine were lower, and histidine and citrulline were higher in those with spasticity than those without. All values were significant at $p < 0.05$ (Appendix A).

3.5.2. Neurologic features and organic acids

There were statistically significant results for seizures after admission and 2-ketoisocaproate, citrate, 2-hydroxybutyrate and 3-hydroxybutyrate. The absence or less than trace amounts of the above compounds were seen more predominantly in those cases with seizures. For speech delay, the absence or less than trace amounts of

Table 3
Amino acid levels of cases and controls.

| Amino acid | Cases | | | | Controls | | | | p value |
|------------------|-------|------------------------|--------------------------|--------|----------|------------------------|--------------------------|-------|-------------------|
| | No. | Mean $\mu\text{mol/L}$ | Median $\mu\text{mol/L}$ | SD | No. | Mean $\mu\text{mol/L}$ | Median $\mu\text{mol/L}$ | SD | |
| BCAAs | | | | | | | | | |
| Valine | 26 | 235 | 212.23 | 131.25 | 26 | 244.46 | 236.21 | 123.4 | 0.7892 |
| Isoleucine | 26 | 172.6 | 163.35 | 97.61 | 26 | 63.49 | 61.22 | 21.87 | <0.0001 |
| Leucine | 26 | 815.5 | 733.15 | 468.61 | 26 | 112.83 | 109.63 | 32.21 | <0.0001 |
| Alloisoleucine | 25 | 113.4 | 89.20 | 69.39 | 26 | 0 | 0 | 0 | |
| LNAAs | | | | | | | | | |
| Tyrosine | 26 | 82.9 | 77.25 | 44.4 | 26 | 64.13 | 59.41 | 20.61 | 0.0586 |
| Methionine | 26 | 21.47 | 20.53 | 10.8 | 26 | 18.18 | 18.43 | 6.56 | 0.1912 |
| Histidine | 26 | 138.1 | 89.97 | 108.67 | 26 | 132.71 | 102.37 | 62.13 | 0.828 |
| Phenylalanine | 26 | 83.88 | 81.31 | 29.84 | 26 | 67.74 | 68.59 | 24.23 | 0.0374 |
| Threonine | 26 | 155.9 | 158.19 | 60.74 | 26 | 115.03 | 111.44 | 36.34 | 0.0053 |
| Other AAs | | | | | | | | | |
| Glutamine | 26 | 444.6 | 437.55 | 113.4 | 26 | 596.33 | 575.49 | 127.1 | <0.0001 |
| Alanine | 26 | 200.8 | 176.84 | 120.1 | 26 | 354.18 | 328.44 | 123.3 | <0.0001 |
| Hydroxypro-line | 26 | 12.6 | 10.35 | 8.85 | 26 | 20.26 | 18.34 | 10.67 | 0.0070 |
| Taurine | 26 | 59.56 | 67.56 | 28.77 | 26 | 43.5 | 46.51 | 20.45 | 0.0250 |
| Lysine | 26 | 223.2 | 201.51 | 116.64 | 26 | 159.25 | 157.14 | 54.09 | 0.0158 |
| Sarcosine | 20 | 85.22 | 70.44 | 53.43 | 15 | 44.33 | 26.72 | 35.20 | 0.0149 |

Bold values indicate significance at $p < 0.05$.

lactate was also statistically significant in cases who presented with speech delay. The rest of the neurologic features did not correlate significantly with the results of the organic acids (Appendix B).

4. Discussion

Although there is no sex predilection seen in MSUD, most of the patients in this study were males. The ages ranged from 1 month to >10 years because some were diagnosed earlier by newborn screening while some were not, thus the diagnosis was done at a later age. Most of the patients included in the study manifested with symptoms early during the neonatal period, hence, they belonged to the classical type of MSUD [2]. It was beyond the scope of this study to correlate this phenotype with mutation analysis of the patients. However, in a study done among Filipino patients with MSUD, a novel deletion in the E2 component of the *BCKAD* gene spanning 4.1 kb of intron 10 and 601 bp of exon 11, caused by non-homologous recombination was found in most patients presenting with the classical phenotype [9].

Despite the early presentation among the patients in this cohort, most of them had late diagnosis with a mean age of 42 days. This mean was affected by those patients who did not undergo newborn screening for MSUD. It took an average of 27 days from the time of symptom presentation to confirmation of diagnosis. This late diagnosis which was previously observed in a local study could be due to the lack of awareness of physicians leading to the late referral of cases [10]. Another reason could be the late inclusion of this disease in the newborn screening program in the country thus, most of the patients especially those whose ages were 3 years old and above, did not have the

opportunity to be screened early, as MSUD was only included in the panel during the latter part of 2012. Studies have shown that newborn screening for MSUD provided a favorable effect on the neonatal course of the disease as patients detected on newborn screening had lowering of the plasma leucine values at an earlier age that led to less severe clinical symptoms [11]. But that this favorable effect was only achieved with immediate transfer of the neonate to a metabolic center for adequate treatment in case of a positive screening result.

The most common presenting symptoms of seizures, increasing sleepiness, poor cry and suck along with the characteristic MSUD smell were consistent with what has been described in the literature [12]. These symptoms were primarily attributed to the strong neurotoxic effects of leucine and its ketoacid, 2-ketoisocaproic leading to acute and chronic brain dysfunction [13]. The leucine levels of the cases in this study had an average of 1944.9 $\mu\text{mol/L}$ (NV < 300 $\mu\text{mol/L}$) at the time of diagnosis, explaining the neurologic manifestations. Almost half of the cases underwent peritoneal dialysis to reduce the levels of

Table 4
Discriminant analysis of the 7 clinically and statistically significant AAs.

| Amino acid | Standardized coefficient |
|----------------|--------------------------|
| Glutamine | -0.2740460 |
| Alanine | -0.4569194 |
| Isoleucine | 0.2054675 |
| Leucine | 0.5336659 |
| Alloisoleucine | 0.0423058 |
| Threonine | 0.6737449 |
| Phenylalanine | 0.2258760 |

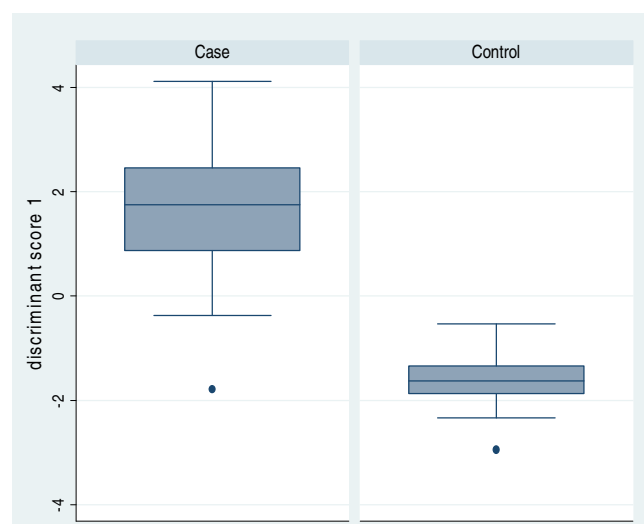
**Fig. 1.** Discriminant scores of the cases and controls showing distinct separation.

Table 5
Frequency distribution of organic acids that showed statistically significant differences between cases and controls.

| Organic acid | Cases | | | | | Controls | | | | | Fishers exact p value |
|--------------|-------|-----------------|-------|-------------|----------|----------|-----------------|-------|-------------|----------|-----------------------|
| | Neg | Less than trace | Trace | Slight inc. | Mod inc. | Neg | Less than trace | Trace | Slight inc. | Mod inc. | |
| 2HIV | 8 | 8 | 5 | 4 | 1 | 26 | 0 | 0 | 0 | 0 | <0.0001 |
| 2KIC | 6 | 11 | 3 | 4 | 2 | 26 | 0 | 0 | 0 | 0 | <0.0001 |
| KMV | 7 | 10 | 3 | 4 | 2 | 24 | 2 | 0 | 0 | 0 | <0.0001 |
| Succinate | 2 | 8 | 9 | 7 | 0 | 1 | 18 | 5 | 2 | 0 | 0.032 |

the toxic metabolites rapidly during the acute crisis upon diagnosis. For the long term management, most of the patients while maintained on this regimen of low protein diet with special amino acid supplement had fair metabolic control with leucine levels that ranged from below 300 $\mu\text{mol/L}$ to 850 $\mu\text{mol/L}$. Although most of their leucine intake were in the recommended allowance for age in individuals with MSUD when well [7], what they were actually getting might indeed be high for classical patients with MSUD. In a study done on classical patients who underwent elective liver transplantation, the average leucine tolerance was only 15–37 mg/kg/day pre transplant [13]. This amount was much lower compared to what our patients actually get. To achieve appropriate BCAA blood concentrations, it has been recommended that a leucine concentration of 75–200 $\mu\text{mol/L}$ for infants and children < 5 years old and between 75 and 300 $\mu\text{mol/L}$ for individuals > 5 years of age be maintained to achieve a favorable cognitive outcome [7]. Therefore it can be said that in our patients with MSUD, the control of leucine values over time was not satisfactory and this can be explained by the following reasons: 1) leucine levels can be affected by inappropriate amounts of leucine, concurrent illness, protein imbalance, low valine and isoleucine, inadequate calories, or any form of catabolic stress [14] and 2) social factors such as lax diet supervision of parents or incompletely understood feeding protocols may also influence the levels of leucine over time [15].

There is evidence that in patients with classic MSUD, the intellectual outcome is inversely related to the duration of high plasma leucine during the neonatal illness and inversely related to the longitudinal plasma leucine concentrations in childhood, being an indicator of the quality and compliance with the dietary treatment [15]. The patients in this study given the early time of presentation but late diagnosis and treatment could have suffered significant brain insult during the neonatal period [16–18]. Likewise the cohort's unsatisfactory metabolic leucine control over time could have contributed to the neurologic features being seen among the patients studied [19]. This was especially true in one patient who was diagnosed early because a previous sibling died of MSUD and did not have neurologic symptoms initially but consequently developed intellectual disability due to poor metabolic control.

Alloisoleucine was not found in any of the controls. This metabolite has a delayed clearance and high levels persist in plasma for several days following an episode of decompensation. It is detectable in classic MSUD patients at all times [2]. The presence of allo-isoleucine in 25/26 patients gave an overall picture of how the isoleucine and indirectly the other branched chain amino acid levels behaved over a period of time. With it being present in all patients except in one case whose isoleucine was also normal at the time of blood extraction, verified our finding that the metabolic control of our patients has not been optimal despite the dietary intervention.

The significantly low levels of alanine seen in our cohort can be consistently found when branched chain amino acids are high and may be due to the overconsumption of alanine to reaminate the increased branched chain ketoacids in the blood [2]. In a study done in hyperleucinemic rats, the plasma alanine levels were likewise found to be reduced and were attributed to the activation of insulin secretion and protein synthesis by leucine with consequent utilization of the

amino acids by peripheral tissues especially skeletal muscles. Insulin stimulates the A membrane carrier system in the muscle which activated the transport of especially small neutral amino acids and could explain the diminution of alanine [20]. The significantly lower levels of alanine together with glutamine could also be consistent with the finding that there is reduced availability of gluconeogenic substrates in the liver caused by increased ketone body utilization [6] during a catabolic stress.

In the literature, the neurotoxicity of leucine stems in part from its ability to interfere with the transport of other large neutral amino acids (LNAA) across the blood brain barrier reducing the brain's supply of phenylalanine, tryptophan, methionine, isoleucine, tyrosine, histidine, valine and threonine. The transport of these amino acids in the brain and peripheral tissues is carried out by large neutral amino acid transporter (LAT1) which has a low Km for leucine and is inhibited by other neutral amino acids including glutamine, histidine, methionine, phenylalanine, serine, threonine, tryptophan and tyrosine [21]. In the previous studies, significant increases in the plasma concentration of leucine were accompanied by concomitant reduction in the plasma concentrations of the LNAAs phenylalanine, tyrosine, isoleucine, valine, methionine as well as alanine, serine and histidine [1,4,20]. Among the possibilities that were given to explain the above phenomena were: 1) stimulation of muscle protein synthesis by leucine and insulin 2) activation of glutamate dehydrogenase activity in the liver and skeletal muscle by leucine which could result in the increased amino acid utilization by these tissues resulting to influx of these amino acids in to the peripheral tissues and a fall in their plasma concentrations and 3) accelerated catabolism of the amino acids appearing at low concentrations in the plasma of MSUD patients through LNAA competition with leucine for the efflux from the tissues through this LAT1 membrane carrier system [4]. However the above findings were not verified in this study and the reverse effect was seen in the LNAAs in that they all appeared higher compared to controls when the BCAAs were also elevated. Our study showed statistically significant differences in the levels of two large neutral amino acids apart from leucine and isoleucine namely threonine and phenylalanine. One possibility for this is that this LNAA transporter in the blood brain barrier was also likened to a "revolving door" [22]. Because this transporter is almost fully saturated at normal plasma LNAA concentrations and is competitive, the uptake of each LNAA into the brain will be affected not only by its own concentration in plasma but also by that of its competitors. In this study, it is postulated that the high levels of leucine in our cohort of patients may have saturated the LAT1 (large neutral amino acid transporter) transporter in the blood brain barrier and blocked the uptake of threonine and phenylalanine into the brain capillaries causing them to revert back to the systemic circulation and result to higher plasma values compared to controls. Another possibility why threonine was elevated in the plasma is the fact that in the order of affinity for the LAT1 transporter, threonine has a decreased affinity thus, it was one of those LNAAs that reverted back to the blood compared to other LNAAs [1]. The mean plasma values of the other LNAAs methionine, tyrosine and histidine were likewise higher among cases compared to controls, however this was not statistically significant. The amino acids hydroxyproline, sarcosine, taurine and lysine were statistically significantly higher in the cases ($p < 0.05$),

however these amino acids were not clinically significant and the increase may be diet related.

In this study, the discriminant function analysis of the amino acids was able to define the boundary between a case and a control in terms of their amino acid characteristics. Strictly speaking, alloisoleucine should not have been included as a variable because it was only present in the cases and not in the controls. However, the discriminant score might be lower and this will also not be reflective of the true variation between a case and a control because presence of alloisoleucine in the cases and not in the controls indicated already a significant variation. As a group, this set of plasma amino acid metabolites consisting of low glutamine and alanine, and high leucine, threonine, isoleucine, phenylalanine and alloisoleucine has not yet been reported in the literature as a means that would define a case of MSUD or not, although this metabolic profile may have also been highly influenced by diet.

The organic acid profiles of the cases showed statistically significant increase in the excretion of the branched chain keto acids (BCKAs) more than the controls. Accumulation of 2 ketoisocaproic acid has been found to contribute greatly to the neurotoxicity seen in MSUD in by depletion of glutamate via bidirectional transaminase reaction [1]. An increase in α KG/glutamate ratios may inhibit the malate/aspartate shuttle resulting in increased NADH/NAD⁺ ratios preventing conversion of lactate to pyruvate. Alternatively, at the mitochondrial level, accumulation of α KIC has been previously shown to inhibit oxidative metabolism through inhibition of pyruvate dehydrogenase (PDH) and α -ketoglutarate dehydrogenase (α KGDH) resulting in Krebs cycle dysfunction. In MSUD mice given high protein diet exposure, it was found that alpha ketoglutarate levels were elevated by 25%, pyruvate was decreased by >50% and lactate doubled [6]. In our study, only succinate was found to be present in statistically significant amounts compared to controls. There were no statistically significant differences found in the levels of lactate, pyruvate and alpha ketoglutarate or other Krebs cycle metabolites between cases and controls. This slight elevation of succinate in the cases could still be normal since no other Krebs cycle metabolites were deranged. An increase in succinate could be due to bacterial contamination, ketosis, tissue ischemia and 2 ketoglutarate degradation [23]. Although in a previous study succinic acid was also found to be transiently elevated in an MSUD patient who underwent multiple exchange transfusion during a crisis and they attributed this to two possible minor pathways: 1) 2-hydroxyisovaleric acid was produced from 2 ketoisocaproic acid directly by 2 ketoisocaproic oxidase and 2) isobutyryl CoA, isovaleryl CoA and alpha methylbutyryl CoA were produced beyond the metabolic block. But these metabolites were found to be in small amounts and the pathways were thought to be not significant [24].

When the neurologic features (developmental delay, seizures, speech delay, spasticity and long tract signs) were correlated with the amino acid results, no specific pattern was deduced. There were statistically significant differences seen between some amino acids and neurologic features such as: valine and developmental delay; ornithine and valine and speech delay; citrulline and seizures; hydroxyproline, histidine, citrulline, sarcosine and spasticity. However the means and standard deviations of the above mentioned amino acids were very variable and widely distributed even if their p value was <0.05. This could indicate a low statistical power due to the limited number of samples. Similarly, the amino acids that gave significant results for the neurologic features were not found to be the clinically and statistically significant markers in the classification of the disease, thus these amino acid results would not be very suggestive of a possible correlation with the neurologic features. In the organic acid analysis, there were equally certain organic acids that were found to be statistically significant with two neurologic features namely lactate and speech delay and 2 ketoisocaproic acid, citrate, 2 hydroxybutyrate and 3 hydroxybutyrate and seizures. But the results showed that the mentioned organic and ketoacids were statistically found to be more

negative in those presenting with speech delay and seizures. Even if the results were statistically significant, the trends were not consistent with what was expected, in that, those presenting with neurologic features should have had the presence of the offending metabolites such as lactate or 2 ketoisocaproic acid. These results did not factor in other confounding variables that could have given rise to these findings such as state of illness and or metabolic control over time. Maybe the metabolites for energy depletion like lactate would occur only if the patient is in a severe metabolic decompensation or if the leucine levels are too high. Moreover, the plasma and urine metabolites may also not entirely reflect the levels of these amino acids and organic acids in the brain [25]. Given all these, the explanation for the neurologic features seen in our patients in this study could not be presumed to be secondary to the presence or absence of a specific amino acid and or organic acid metabolite. Therefore with respect to neurologic outcome among MSUD patients, these findings highlight the importance of early disease detection and metabolic control as the ones that might have a substantial impact.

5. Conclusions

This study showed that most of the patients with MSUD presented with symptoms at > 10 days of age and that there was a delay of almost 4 weeks from the time of presentation to confirmation of diagnosis. The elevated initial leucine levels contributed to the presenting neurologic symptoms. In general, most patients had fair metabolic control over time which likewise contributed to the current neurologic status of the patients. The majority of them had developmental delay, intellectual disability, speech delay, seizures, long tract signs and spasticity.

In the plasma amino acid analysis, the characteristic profile of the cases showed low levels of glutamine and alanine together with high leucine, threonine, isoleucine, phenylalanine and alloisoleucine compared to the controls. Although these results were not similar to what has been described in the literature in terms of the relationship of the BCAA elevation and the LNAA levels, the competition at the level of the blood brain barrier may still be operating. However, more evidence to strongly suggest this still needs to be obtained.

Another salient finding in this study was the discriminant function analysis done between cases and controls wherein a set of 7 amino acids and their amounts clearly defined a case versus a control, but this could have been highly influenced by the dietary manipulation given to patients with MSUD.

The metabolites of interest in the plasma and urine did not show significant relationship when they were correlated with the neurologic features observed in our patients. Thus, the prevention and avoidance of neurologic disturbances may still rely primarily on early diagnosis and prompt institution of treatment along with strict compliance with the dietary regimen, and maintenance of normal leucine values over time. However, the limited number of patients could have contributed to the inconsistent and insignificant findings done in the plasma and urine and the correlation studies with their neurologic features. Further studies are therefore recommended such as longitudinal analysis of amino acids and organic acids in states of good health and illness paralleled with periodic neurological evaluation so that more accurate correlation between the neurological outcome of the patients and the corresponding metabolites in the plasma and urine may achieve more robust results. Likewise, aggressive implementation of the newborn screening program for MSUD should be promulgated, and emphasis should be placed on strict timing of collection of newborn samples so that early diagnosis and adherence to proper dietary management can be suitably provided.

Acknowledgements

The authors would like to thank the Newborn Screening Reference Center of the Philippines for funding this project (NSRC-2013-004).

Appendix A

Table A.1

Comparison of the distribution of the significant amino acids among those with and without developmental delay, speech delay, seizures and spasticity.

| Amino acid | With developmental delay | | | Without developmental delay | | | p value |
|----------------|--------------------------|---------------|--------|-----------------------------|---------------|-------|---------|
| | No. | Mean (μmol/L) | SD | No. | Mean (μmol/L) | SD | |
| Valine | 23 | 250.05 | 131.62 | 3 | 119.24 | 45.51 | 0.0090 |
| Amino acid | With speech delay | | | Without speech delay | | | p value |
| | No. | Mean (μmol/L) | SD | No. | Mean (μmol/L) | SD | |
| Ornithine | 18 | 85.22 | 38.93 | 8 | 55.05 | 25.77 | 0.0302 |
| Valine | 18 | 267.78 | 136.67 | 8 | 161.09 | 84.6 | 0.0243 |
| Amino acid | With seizures | | | Without seizures | | | p value |
| | No. | Mean (μmol/L) | SD | No. | Mean (μmol/L) | SD | |
| Citrulline | 17 | 26.78 | 7.99 | 9 | 34.93 | 6.40 | 0.0105 |
| Amino acid | With spasticity | | | Without spasticity | | | p value |
| | No. | Mean (μmol/L) | SD | No. | Mean (μmol/L) | SD | |
| Hydroxyproline | 11 | 7.91 | 5.33 | 15 | 16.03 | 9.46 | 0.0108 |
| Histidine | 11 | 195.83 | 29.78 | 15 | 95.67 | 63.77 | 0.0364 |
| Citrulline | 11 | 33.26 | 3.79 | 15 | 26.90 | 9.78 | 0.0334 |
| Sarcosine | 11 | 62.80 | 38.95 | 9 | 112.60 | 57.85 | 0.0452 |

Appendix B

Table B.1

Comparison of the distribution of organic acids among those with and without seizures and speech delay.

| Organic acid | With seizures | | | | | Without seizures | | | | | Fishers exact p value |
|--------------------|-------------------|-----------------|-------|-------------|----------|----------------------|-----------------|-------|-------------|----------|-----------------------|
| | Neg | Less than trace | Trace | Slight inc. | Mod inc. | Neg | Less than trace | Trace | Slight inc. | Mod inc. | |
| 2-Ketoiso-caproate | 5 | 8 | 2 | 0 | 2 | 1 | 3 | 1 | 4 | 0 | 0.045 |
| Citrate | 7 | 10 | 0 | 0 | 0 | 3 | 2 | 4 | 0 | 0 | 0.015 |
| 2 hydroxy-butyrate | 17 | 0 | 0 | 0 | 0 | 5 | 4 | 0 | 0 | 0 | 0.008 |
| 3 hydroxy butyrate | 17 | 0 | 0 | 0 | 0 | 5 | 2 | 1 | 1 | 0 | 0.008 |
| Organic acid | With speech delay | | | | | Without speech delay | | | | | Fishers exact p value |
| | Neg | Less than trace | Trace | Slight inc. | Mod inc. | Neg | Less than trace | Trace | Slight inc. | Mod inc. | |
| Lactate | 12 | 6 | 0 | 0 | 0 | 18 | 0 | 0 | 0 | 0 | 0.002 |

Bold values indicate significance at $p < 0.05$.

References

- [1] K. Strauss, D. Morton, Branched-chain ketoacyl dehydrogenase deficiency: maple syrup urine disease, *Curr. Treat. Options Neurol.* 5 (2003) 329–341.
- [2] D. Chuang, V. Shih, Maple syrup urine disease (branched-chain ketoaciduria), in: C. Scriver, A. Beaudet, W. Sly, D. Valle (Eds.), *The Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, New York, 2001, pp. 1971–2006.
- [3] R.L. Puckett, F. Lorey, P. Rinaldo, M.H. Lipson, D. Matern, M.E. Sowa, S. Levine, R. Chang, R.Y. Wang, J.E. Abdenur, Maple syrup urine disease: further evidence that newborn screening may fail to identify variant forms, *Mol. Genet. Metab.* 100 (2010) 136–142.
- [4] M. Wajner, D.M. Coelho, A.G. Barschak, P.R. Araújo, R.F. Pires, F.L. Lulhier, C.R. Vargas, Reduction of large neutral amino acid concentrations in plasma and CSF of patients with maple syrup urine disease during crises, *J. Inherit. Metab. Dis.* 23 (2000) 505–512.
- [5] J.Y. Lee, M.A. Chiong, M.D. Rana, K.D. Rama, B.C. Cavan, C.P. Cordero, C.D. Padilla, Correlation between dried blood spot thin layer chromatography and plasma high performance liquid chromatography of leucine/isoleucine levels among Filipino patients with maple syrup urine disease seen at the Institute of Human Genetics, National Institutes of Health, *Acta Med. Philipp.* 42 (2008).
- [6] W. Zinnanti, J. Lazovic, K. Griffin, K.J. Skvorak, P. Harbhajan, G. Homanics, M. Bewley, K. Cheng, K. Lanoue, J. Flanagan, Dual mechanism of brain injury and novel treatment strategy in maple syrup urine disease, *Brain* 132 (2009) 903–918.
- [7] D. Frazier, C. Allgeier, C. Homer, B. Marriage, B. Ogata, F. Rohr, P. Splett, A. Stemberger, R. Singh, Nutrition management guideline for maple syrup urine disease: an evidence and consensus based approach, *Mol. Genet. Metab.* 112 (2014) 210–217.
- [8] R. Singh, P. Graham, The lowdown on leucine - grams, milligrams, and exchanges, <http://www.msud-support.org/index.php/articles/news/184> (accessed on 10.04.16).
- [9] C.L. Silao, C.D. Padilla, M. Matsuo, A novel deletion creating a new terminal exon of the dihydrolipoyl transacylase gene is a founder mutation of Filipino maple syrup urine disease, *Mol. Genet. Metab.* 81 (2004) 100–104.
- [10] J. Lee, M.A. Chiong, S. Estrada, E.C. de la Paz, C. Silao, C. Padilla, Maple syrup urine disease (MSUD) - clinical profile of 47 Filipino patients, *J. Inherit. Metab. Dis.* 31 (2008) S281–S285 Suppl.
- [11] E. Simon, R. Fingerhut, J. Baumkotter, V. Konstantopoulou, R. Ratschmann, U. Wendel, Maple syrup urine disease: favourable effects of early diagnosis by newborn screening on the neonatal course of the disease, *J. Inherit. Metab. Dis.* 29 (2006) 532–537.
- [12] K. Strauss, E. Puffenberger, D. Morton, Maple syrup urine disease, <http://www.ncbi.nlm.nih.gov/books/NBK1319/2013> (accessed 10.16.13).
- [13] K.A. Strauss, G.V. Mazariegos, R. Sindhi, R. Squires, D.N. Finegold, G. Vockley, D.L. Robinson, C. Hendrickson, M. Virji, L. Cropcho, E.G. Puffenberger, W. Mcghee, L.M. Seward, D.H. Morton, Elective liver transplantation for the treatment of classical maple syrup urine disease, *Am. J. Transplant.* 6 (2006) 557–564.
- [14] U. Wendel, H. Ogier de Baulny, saudubray, Branched chain organic aciduria, in: F. Fernandes, v. den Berghe, Walter (Eds.), *Inborn Metabolic Diseases*, 4th ed. 2006, pp. 251–253.
- [15] E. Simon, U. Wendel, P. Schadowaldt, Maple syrup urine disease treatment and outcome in patients of Turkish descent in Germany, *Turk. J. Pediatr.* 47 (2005) 8–13.
- [16] S. Herber, I. Schwartz, T. Nalin, C. Netto, J. Junior, M. Santos, E. Ribeiro, L. Schuler-Faccini, C. de Souza, Maple syrup urine disease in Brazil: a panorama of the last two decades, *J. Pediatr.* 91 (2015) 292–298.

- [17] Z. Yunus, D.P. Kamaludin, M. Mamat, Y. Choy, L.H. Ngu, Clinical and biochemical profiles of maple syrup urine disease in Malaysian children, *J. Inherit. Metab. Dis. Reports* 5 (2012) 99–107.
- [18] C. le Roux, E. Myrurphy, P. Hallam, M. Lilburn, D. Orłowska, P. Lee, Neuropsychiatric outcome predictors for adults with maple syrup urine disease, *J. Inherit. Metab. Dis.* 20 (2006) 201–202.
- [19] B. Hoffmann, C. Helbling, P. Schadewaldt, U. Wendel, Impact of longitudinal plasma leucine levels on the intellectual outcome in patients with classic MSUD, *Pediatr. Res.* 59 (2006) 17–20.
- [20] P. Araújo, G. Wassermann, K. Tallini, V. Furlanetto, C. Vargas, C. Wannmacher, C. Dutra-Filho, A. Wyse, M. Wajner, Reduction of large neutral amino acid levels in plasma and brain of hyperleucinemic rats, *Neurochem. Int.* 38 (2001) 529–537.
- [21] R. O'kane, R. Hawkins, Na⁺ dependent transport of large neutral amino acids occurs at the abluminal membrane of the blood brain barrier, *Am. J. Physiol. Endocrinol. Metab.* 285 (2003) E1167–E1173.
- [22] J. Fernstrom, Branched-chain amino acids and brain function, *J. Nutr.* 135 (2005) 1539S–1546S.
- [23] A. Kumps, P. Duez, Y. Mardens, Metabolic, nutritional, iatrogenic and artifactual sources of urinary organic acids: a comprehensive table, *Clin. Chem.* 48 (2002) 708–717.
- [24] Y. Shigematsu, K. Kikuchi, T. Momoi, M. Sudo, Y. Kikawa, K. Nosaka, M. Kuriyama, S. Haruki, K. Sanada, N. Hamano, Organic acids and branched chain amino acids in body fluids before and after multiple exchange transfusions in maple syrup urine disease, *J. Inherit. Metab. Dis.* 6 (1983) 183–189.
- [25] K. Vogel, E. Arning, B. Wasek, S. Mcpherson, T. Bottiglieri, K. Gibson, Brain-blood amino acid correlates following protein restriction in murine maple syrup urine disease, *Orphanet J. Rare Dis.* 9 (2014) 73.