RESEARCH ARTICLE

Plasma Acylcarnitine and Urinary Organic Acid Profiling for the diagnosis of Fatty Acid Oxidation Disorder and Organic Acidurias using tandem mass spectrometry (MS/MS) and gas chromatography tandem with mass spectrometry (GC-MS): A Retrospective Study

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ABSTRACT

Introduction: Plasma acylcarnitines (PLAC) and urinary organic acids (UOA) are essential diagnostic markers for some Inborn Errors of Metabolism (IEM) such as fatty acid oxidation disorders (FAODs) and disorders related to organic acids metabolism.

Objective: This study aimed to establish the PLAC and UOA profiles of Filipino newborn babies at risk for IEMs using FIA-MS/MS and GC-MS. Further, this study describes the process of determining the true positive cases of FAODs and some organic acidurias.

Methodology: PLAC and UOA analyses were performed using Waters® MS/MS and Agilent® GC-MS. Results were obtained from databases and IEM registry of the Biochemical Genetics Laboratory from 2015 to 2021. Descriptive statistics was used to evaluate the detection rates of FAODs and Organic Acidurias.

Results: Data from 2015-2021, indicated 176 true positives out of 1642 babies screened at risk for FAODs and organic acidurias. The use of MS/MS and GC-MS yielded a detection rate of 10.6% with 104 Filipino newborn babies with FAODs while 72 with organic acidurias. Medium-chain acyl-CoA dehydrogenase deficiency was reported to be the most common FAOD with 67 cases. Organic acidurias such as glutaric aciduria type 1 and 3-Methylcrotonyl-CoA carboxylase deficiency were found to be common with 34 and 26 cases, respectively.

Conclusion: The PLAC and UOA profiles of Filipino newborn babies with FAODs and organic acidurias were presented in this paper. This study emphasizes the importance of conducting confirmatory testing to establish the true positive cases. Thus, this study warrants further studies on the validation of analytical methodologies for targeted measurements of biomarkers of IEMs.

Keywords: *organic acidurias, acylcarnitines, plasma, fatty acid oxidation disorders, inborn error of metabolism, tandem mass spectrometry, GC-MS*

Introduction

Fatty-acid oxidation disorders (FAOD) are a group of inborn errors of metabolism (IEM) caused by missing or deficiency in enzymes needed to break down fats in the body. FAOD has an estimated combined incidence of 1:9300 in Australia, Germany and the United States of America (USA) while prevalence in Asia is said to be much lower [1]. As of 2021, prevalence of FAOD in the Philippines is 1: 42,391 [2]. Clinical symptoms are mainly induced by catabolism when energy from fatty acid oxidation is needed. Patients with FAOD may be asymptomatic or may have a wide variety of symptoms, such as hepatic encephalopathy with hypoketotic hypoglycemia and coma similar to Reye-like syndrome, cardiomyopathy, arrhythmias, progressive myopathy, fulminant hepatic failure or sudden infant death [3]. If undiagnosed, significant morbidity may occur which for some may result to death during the first episode of metabolic crisis, while others may experience irreversible neurologic condition [4]. However, if recognized before the onset of

symptoms, FAOD appears to be an eminently treatable condition with good clinical outcomes. Management involves prevention of fasting, consumption of carbohydrate-rich food, and carnitine supplements if needed.

Meanwhile, organic acidurias are a more diverse group of IEM which lead to the accumulation of organic acids in the tissues and subsequently excretion in the urine [5]. The clinical presentation and treatment may vary between different organic acid disorders and may depend from person to person condition. Symptoms may include neurological damage and developmental delay associated with slow growth, lethargy, vomiting, hypoglycemia, hypotonia, metabolic acidosis, hyperammonemia, or ketoacidosis [6]. As of 2021, prevalence of organic acidurias in the Philippines is 1: 61,232 [2].

Acylcarnitines are esters of L-carnitine and fatty acids. Similar to fatty acids, acylcarnitines vary depending on the length of the acyl groups, often categorized as short (C0 to C5), medium (C6 to C12) and long-chain acylcarnitines (C14 to C18) (simply marked as SCACs, MCACs and LCACs, respectively). Acylcarnitines play an important role in cell physiological activities like regulating the balance of intracellular sugar and lipid metabolism [7]. They serve as carriers to transport activated long chain fatty acids (LCFAs) to the mitochondria for subsequent β-oxidation, necessary in supplying energy for various cell activities.

Defects or atypical expression of enzymes involved in the metabolism of acylcarnitines may result in accumulation of acyl-CoA species, thus, this condition may lead to some inborn errors of metabolism (IEMs). IEM can manifest at any age, frequently leading to serious health problems or life-threatening episodes of metabolic decompensation. For this reason, early comprehensive neonatal screening is used to detect abnormalities of acylcarnitines to avoid major physical and neurological effects [8].

In 2004, Republic Act (R.A.) 9288 otherwise known as the Newborn Screening (NBS) Act was decreed to ensure that every infant born in the Philippines shall undergo genetic screening to promote early diagnosis and intervention from IEMs that may lead to mental retardation and death if undetected and untreated. This NBS program covers the conduct of screening tests to identify the population at high risk of IEM to include 28 disorders such as fatty acid oxidation disorders and organic acidurias. In case of positive screens, newborn screening centers (NSCs) located in various regions in the Philippines, send plasma and urine samples to Biochemical Genetics Laboratory (BGL) at the National Institutes of Health (NIH), University of the Philippines Manila (UPM) for confirmatory testing. BGL performs biochemical confirmatory tests using various analytical methodologies that will either confirm or rule out IEM in newborns with an out-of-range(positive) ENBS screening result.

Electron Ionization Spray (ESI)-Flow Injection Analysis with tandem mass spectrometry (FIA-MS/MS) and single quadrupole gas chromatography tandem with mass spectrometry (GC-MS) technologies were employed for metabolic profiling of acylcarnitines and organic acids in plasma and urine samples of Filipino newborn babies, respectively. Results were correlated with patterns/profiles that appeared consistent with FAODs and Organic Acidurias such as identifying and quantifying biomarkers specific to an inborn error of metabolism (IEM). Here we summarized the frequency, pattern and main laboratory findings of IEMs encountered at BGL over a period of six years, that is, from 2015 to 2021. This study was registered with the Research Grants Administration Office (RGAO) of the University of the Philippines-Manila and since it is a retrospective study and all investigations performed were for diagnosis/confirmatory purpose, no informed consent was required from patients.

Methodology

Subjects

From 2001 to 2003, BGL mainly relied on Urine Metabolic Screening (UMS) via High Voltage Electrophoresis (HVE) for laboratory investigation of babies suspected of having an amino acidopathy, organic aciduria or fatty acid oxidation disorder if presented mainly with seizures (n= 839), developmental delay (n = 713), sepsis (n= 227), epilepsy (n = 166) or hypotonia (n = 115). However, it was reported that the technique yielded low overall diagnostic yield of 1.3% [9]. From 2001 to 2021, there were 2927 samples referred to BGL for UMS. In 2003, single quadrupole gas chromatography in tandem with mass spectrometry (GC-MS) technology was introduced by BGL to identify the key metabolic intermediates present in urine. Meanwhile, ultrahigh performance liquid chromatography (UPLC) was deployed for diagnosis of amino acidopathy via plasma amino acid profiling in 2013. BGL has catered to 2378 urine samples for GC-MS analysis and 1601 plasma samples for amino acid analysis. Then in 2015, BGL acquired the liquid chromatograph MS/MS for plasma acylcarnitine profiling. BGL has already analyzed 1609 plasma samples using tandem mass spectrometric technique from 2015 to 2021. From 2015 to 2021, there were 4203 newborn babies and pay patients referred to BGL for biochemical confirmatory testing. These

samples typically from various locations in the Philippines through the different NSCs located in key regions such as Northern Luzon, Central Luzon, Southern Luzon, Western Visayas (Iloilo), Central Visayas (Cebu), Mindanao and the National Capital Region. In addition, there were also samples from pay patients referred to by various private hospitals and clinics in the Philippines for biochemical testing.

Sample Collection

Plasma samples were isolated via centrifugation from blood samples extracted from newborn babies who were screened to be at high risk for FAODs or organic acidurias. Blood samples were collected in lithium heparin tubes while the separated plasma samples were stored in plain tubes. Urine samples were collected randomly within the 24-hour period to yield at least 20 milliliters (mL) for testing. Both samples were immediately transported to the laboratory packed in ice or cold gel to avoid metabolite degradation. All plasma samples are best kept frozen at -80°C in an an ultralow freezer while urine samples were kept at -20°C until analysis. BGL adopts established criteria for sample rejection as well as for documenting receipts of test specimens following the ISO 9001 standards. It is important that clinical abstracts such as those results of Newborn Screening (NBS) should also be submitted along with the specimen for appropriate interpretation of plasma acylcarnitine (PLAC) and urinary organic acids (UOA) test results. Acylcarnitine profiling using MS/MS for confirmation of FAODs or organic acidurias requires the use of plasma while determination of organic acids via GC-MS employs urine samples.

Tandem mass spectrometric analysis

Reagents and Solvents

Standards of L-carnitine (C0) and acylcarnitine hydrochlorides were purchased from Amsterdam University Medical Center, The Netherlands. Acylcarnitines standards include acetyl- (C2), propionyl- (C3), butyryl- (C4), isovaleryl- (C5), hexanoyl- (C6), octanoyl- (C8), decanoyl- (C10), dodecanoyl- (C12), tetradecanoyl- (C14) and palmitoyl- (C16). Stable-isotope labeled carnitine and acylcarnitine internal standards (NSK-B) were acquired from Cambridge Isotope Laboratories (Andover, MA, USA). LC-MS-grade acetonitrile and ethanol were obtained from J.T. Baker (Poland). Formic acid was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water (18.2 MΩ resistivity) from Barnstead Smart2Pure Pro Water Purification system was used throughout the analysis. Internal Quality Control (IQC) Acylcarnitines (CAR-02.1 and CAR-02.2) with two different concentration ranges (low and high) were obtained from MCA Laboratory-Queen Beatrix Hospital, The Netherlands.

Sample Preparation

Plasma samples, including Quality Control (QC) samples, to which known concentrations of stable isotope-labeled internal standards (IS) (d_9-carnitine, d_3-acetylcarnitine, d_3-propionylcarnitine, d_3-butyrylcarnitine, d_9-isovalerylcarnitine, d_3-octanoylcarnitine, d_9-myristoylcarnitine and d_3-palmitoylcarnitine) have been added, were deproteinized using ethanol. The ethanol extracts (supernatant) were then dried under a stream of nitrogen gas with heating at 60°C. Each dried extract was subsequently derivatized with the addition of butanolic hydrochloric acid (HCl) and was heated at 60°C to form butyl esters of the –COOH group of the acylcarnitines. After which, the excess butanolic HCl was evaporated off under nitrogen air and all the butylated extracts were reconstituted with acetonitrile/water and 0.1 % formate for subsequent injection into the FIA-MS/MS. IQC samples (CAR-02.1 and CAR-02.2) were reconstituted in ultrapure water and were treated in the same manner as performed on to patients' plasma samples.

Preparation of calibration materials.

Stock solutions of both free (C0) and acyl carnitine standard solutions (C2–C12) were prepared in ultrapure water, whereas stock solutions of long-chain acylcarnitine standards (C14 and C16) were prepared in ethanol. The solution of internal standard was prepared by reconstituting NSK-B with 10 mL ethanol. Calibration curves were generated from at least 5 to 6 increasing concentrations (points), prepared from each of the eleven (11) standards containing C0-, C2-, C3-, C4, C5-, C6-, C8-, C10-, C12-, C14-, and C16-carnitine and were used for quantification. For acylcarnitines without available pure standards such as glutaryl carnitine, malonyl carnitine, tigylycarnitine, tetradecanoylcarnitine, hexanoylcarnitine and others, concentrations were calculated semi-quantitatively by considering that the response factors of these acylcarnitines and the selected internal standards were identical. For instance, response of tiglylcarnitine was assumed similar to the response of d_3-isovalerylcarnitine internal standard while the response of glutarylcarnitine was compared against the response of d_3-myristoylcarnitine internal standard.

MS/MS instrument conditions

Concentrations of free carnitine and 21 acylcarnitines from plasma were measured on a Waters Acquity® ultrahigh Performance Liquid Chromatograph (UPLC) coupled with triple quadrupole (TQD) mass spectrometer (MS/MS) operated in

electrospray ionization (ESI) positive mode. Waters Acquity ESI UPLC- tandem MS (1)/MS (2) coupled with multiple reaction monitoring (MRM) was used for the selective measurement of a particular acylcarnitine. The mobile phase consisted of 0.1% formic acid in acetonitrile (mobile phase A) with flow rate of 0.05mL/min (isocratic elution) and running time of 1.6 minutes per injection. Extracts were delivered into the column via flow injection analysis.

Detection and Data Acquisition

All data files were then processed using the MassLynx software of the MS/MS equipment, and results of the ratio of analyte to IS were calculated and plotted against varying concentrations of eleven acylcarnitines including free carnitine to generate 11 different calibration curves.

Interpretation and Documentation

Reference ranges for all reported acylcarnitines are age matched. The reference ranges were adopted from NSW Biochemical Genetic Service in Sydney, Australia, from which experimentally measured acylcarnitine values were compared. The reference ranges give the minimum and maximum concentrations of each acylcarnitine and carnitine in patients with normal profile. Any deviation is then tagged as "outside normal values" and will then be correlated with GC-MS test results. Age dependent reference ranges specifically derived from Filipino population are yet to be established as of this writing.

GC-MS analysis

Metabolic disorders such as fatty acid oxidation disorders may also bring about variations in the urine organic acids, thus, the patients' urine samples were also analyzed for the presence of organic acids using GC-MS for correlation with plasma acylcarnitine results. For example, in the diagnosis of propionic acidemia, in addition to obtaining the propionylcarnitine level in plasma, GC-MS analysis is also conducted in urine to determine the presence of propionylglycine, as another key intermediate metabolite for propionic acidemia. This is an important step in the interpretation of results to ensure consistency in the biochemical test results. This is also consistent with the algorithm employed by NSCs in which closing of FAOD related disorders will be based on two (2) second-tier methods. It was only in 2021 that the ENBS program through the Newborn Screening Reference Center (NSRC) has included gene testing for FAOD cases confirmation. Hence, from 2015 to 2021, confirmation of FAOD and organic acidurias would rely on the results generated from urinary organic acids and plasma acylcarnitine analyses, as second-tier methods.

GC-MS Sample preparation

Prior to GC-MS analysis, urine samples are sent to UP-Philippine General Hospital (PGH) for creatinine testing. The aliquot of urine samples to be taken for GC-MS analysis is calculated based on the creatinine value.

Urine samples were subjected to acidification using hydroxylamine-hydrochloric acid in pyridine. This was followed by extraction with ethyl acetate. The supernatant was then evaporated to dryness in a sample concentrator under nitrogen gas stream. Residue obtained after drying was subjected to pre-column derivatization by N,O,-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) for 10 minutes at 60-65°C. Each derivatized solution was injected using Agilent 7693 Autosampler into the Agilent 7890A Gas chromatograph system equipped with Agilent 5975C inert XL MSD with Triple Axis Detector.

GC-MS Data acquisition and interpretation

Data acquisition in GC-MS following the scan mode measurements is done with the use of ChemStation software (Agilent®). Compound annotation was performed by comparing the mass fragments and retention time with the mass fragments of reference standards in a laboratory-compiled mass spectral database as well as with NIST® mass spectral database using similarity index of > 70%.

Quality Control in GC-MS analysis

Every sample is spiked with internal standards such as phenylbutyrate and tricarballylic acid. Urine sample collected from an individual with no known IEM is also included in the batch run and spiked with internal standards. Peak abundance of each internal standard spiked on to a normal urine sample is plotted against date of analysis (batch run) to obtain a quality control monitoring chart. Urine organic acid analysis is a semi-quantitative analysis and does not need the use of internal control, given only the abundance of internal standard. Also, 1-2 retained urine samples from previous batch runs are subjected to extraction and measurement to check on method's reproducibility and repeatability [10].

Validation of MS/MS and GC-MS results

BGL participates annually in the following European Research Network for the evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM) External Quality Assurance Schemes (EQAS): 1) qualitative organic acids in urine; 2) quantitative acylcarnitines in serum; 3) quantitative amino acids in serum and 4) amino acid interpretation. BGL's participation in ERNDIM diagnostic proficiency testing enables the laboratory to assess its laboratory performance, and to identify weaknesses in analytical methodologies and other technical issues that could threaten the accuracy of diagnosis.

Documentation

In case of highly suggestive biochemical confirmatory results, BGL endeavors to update the metabolic registry and each case is then endorsed to concerned Newborn Screening Centers for clinical evaluation of the patient.

Results and Discussion

A retrospective review of the results of plasma acylcarnitine was done from year 2015-2021. Data were retrieved from the database maintained by BGL. Those patients registered in IEM registry were identified and the results of their plasma acylcarnitine and urine organic acids were reviewed. It is recognized that the true prevalence of IEMs cannot be solely determined on the basis of clinical suspicion/manifestation. All newborns screened at high risk for IEMs are endorsed to BGL for biochemical confirmatory testing using various analytical techniques such as MS/MS, GC-MS and UPLC. These analytical techniques have significantly improved the efficiency of diagnosing IEMs particularly of FAODs and organic acidurias. There are also cases of amino acidopathies recorded in the Philippines however, these will be reported in a separate publication.

The following are the different classifications of fatty acid oxidation disorders (FAOD) that can be detected by BGL: (1) Disorders of plasma membrane functions (e.g. Carnitine uptake defect (CUD), Long-chain fatty acid transport/binding defect); (2) Disorders of fatty acid transport across the mitochondrial membranes (Carnitine Palmitoyl Transferase (CPT) Deficiency type I, CPT II deficiency); (3) Disorders of long-chain fatty acid β-oxidation such as Very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD); (4) Disorders of medium-chain fatty acid β-oxidation (such as Medium-chain acyl-coenzyme A dehydrogenase (MCAD) deficiency, Medium-chain ketoacyl-CoA thiolase deficiency (MCAT)); and (5) Disorders of short-chain fatty acid β-oxidation: SCAD deficiency) [11]. Figure 1 shows the different cases of FAODs and organic acidurias from 1996 to 2021 in the Philippines.

Analytical performance using FIA-MS/MS

In this paper, we report the significance of conducting acylcarnitine and free carnitine profiling in plasma using ESI tandem MS/MS detection, for further diagnosis and evaluation of these disorders after NBS screening using dried blood samples (DBS). Based on ERNDIM Quantitative Schemes Acylcarnitines in Serum Annual Report covering the periods 2018 to 2021, overall linearity achieved by the laboratory was 0.971 while precision was 16.7%. Meanwhile, recoveries range

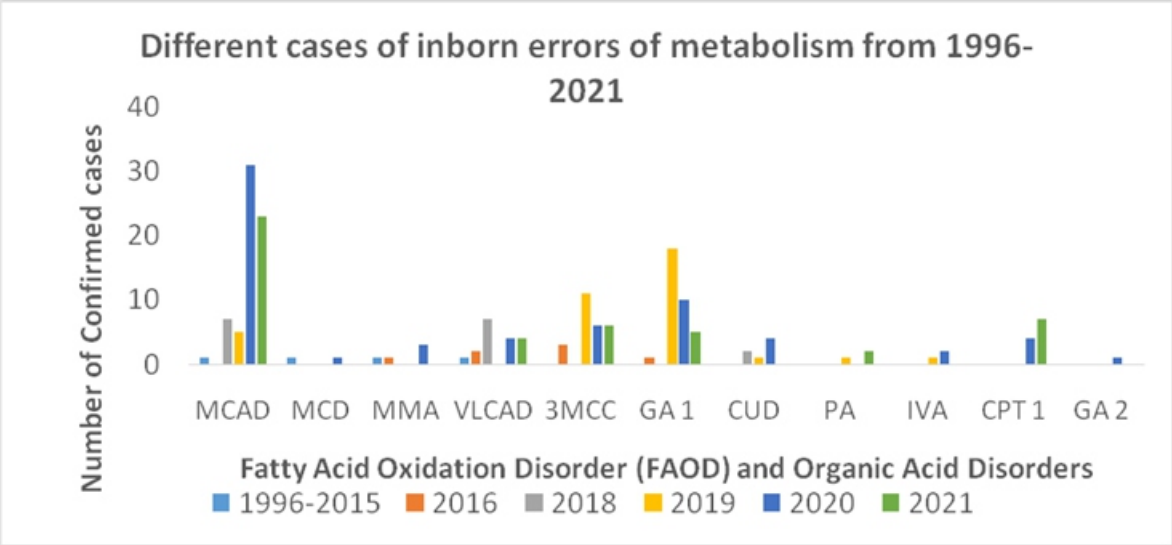

Figure 1. *Distribution of different cases of FAODs and organic aciduria from 1996-2021.*

from 68% in 2018 to 80% in 2021. Overall, satisfactory analytical performance has been accomplished by BGL for the quantification of free and acyl carnitines using LC-MS/MS as analytical technique from 2018 to 2021. As shown, BGL's performance in 2020 EQAS has improved significantly garnering satisfactory performance for 17 acylcarnitines out of 17 results which the laboratory has submitted while in 2021, BGL achieved satisfactory performance for 17 out of 18 carnitine results submitted. BGL's EQAS performance in 2017 was satisfactory for 8 out of 11 carnitines while from 2018 to 2019, satisfactory performances were achieved in 7 out of 10 acylcarnitine results submitted.

Biochemical confirmatory testing of FAOD and Organic acidurias in Filipino newborn babies.

During the six-year period, a total of 4203 patients have been referred to BGL for confirmatory testing; out of this, there were 4033 ENBS patients screened positive for different IEMs including amino acidopathies and were endorsed by NSCs to BGL for confirmatory testing while the rest (170 pay patients) were from endorsements from private/general practitioners, private and public hospitals and others. As described in Table 1, a total of 1642 patients were screened with disorders in which biomarkers may be detected and quantified by MS/MS and GC-MS techniques described above. Out of 1642 endorsed patients, 925 were screened positive for FAODs while 717 were screened positive for organic acidurias. The most common FAOD endorsed for confirmatory testing was MCAD with 335 patients screened positive. In the case of organic acidurias, there were 192 newborn babies flagged for methylmalonic aciduria (MMA).

As can be gleaned from Table 1, second tier testing comprised of MS/MS and GC-MS techniques has accounted the top three FAOD and organic aciduria with glutaric aciduria type 1 (GA 1) topping the list with 32.1% true positive cases followed by 3-methylcrotonyl glycine Co-A carboxylase deficiency (3MCC) with 28.3% diagnosis rate. MCAD deficiency came in as the third with highest % of true positive cases at 20%.

On another note, mean ages at the time of diagnosis ranges from 1.2 to 3 months. The data depicted quite late diagnosis of these cases which may be due to delayed referral, poor sample collection, delays in recall of patients and others. Ideally diagnosis of IEMs should be on or before the 28th day of life of newborn babies.

The use of MS/MS has leveled up the capability of BGL to diagnose FAODs in Filipino newborns without the need for send-out of samples abroad. BGL uses tandem mass spectrometry (MS/MS) in the simultaneous quantification of acylcarnitines in plasma sample in a single analytical run. Accordingly, this has contributed in facilitating management and care for babies afflicted with IEM as well as in reducing

Table 1. *Number of Filipino newborn babies (ENBS) endorsed for various FAODs and organic acidurias (positive screen) from 2015 to 2021, and % true positive cases, with mean age at diagnosis.*

Category	Disorder	Number of newborn babies screened positive	% True Positive Cases	Mean age at diagnosis (months)
Fatty acid oxidation disorders (FAODs)	MCAD	335	20.0	2.1
	VCLAD	160	11.2	1.7
	CUD	100	7.0	3.0
	CPT 1	198	5.6	2.2
	CPT II	82	Not available	1.9
	GA II	50	2.0	1.6
Organic aciduria	Methyl malonic aciduria (MMA)	192	2.6	1.7
	Propionic acidemia (PA)	185	1,6	1.7
	Isovaleric aciduria (IVA)	142	2.1	1.2
	Glutaric aciduria type 1	106	32.1	2.8
	Beta-ketothiolase deficiency (BKT)/3-methylcrotonyl glycine Co-A carboxylase deficiency (MCC)	92	28.3	2.8

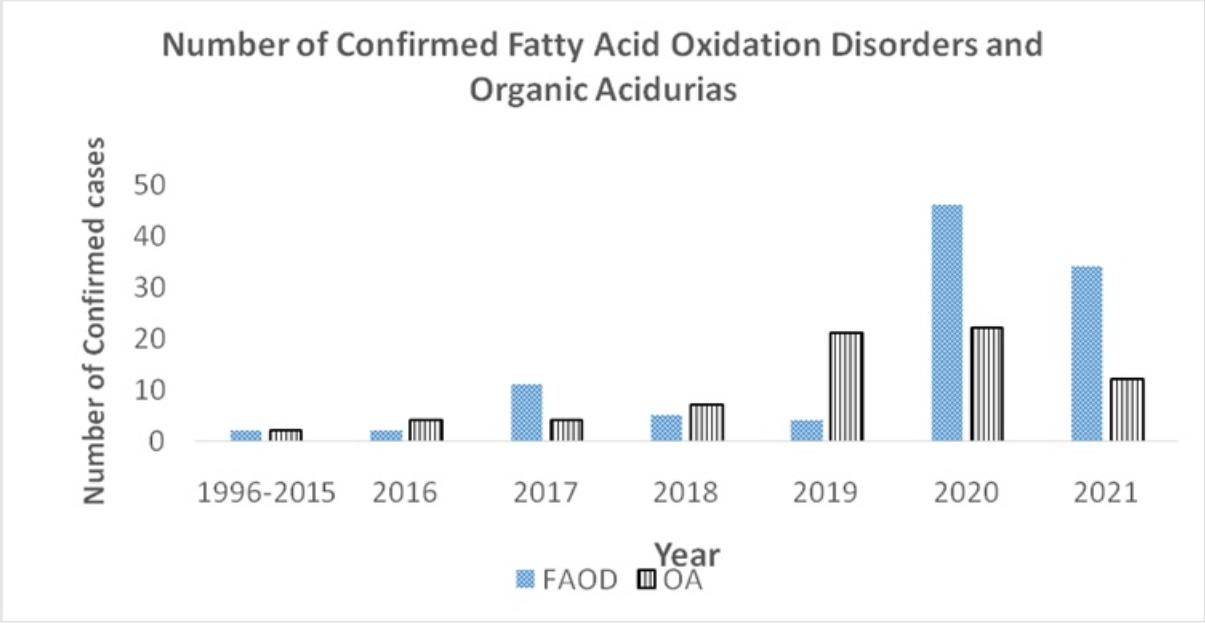

Figure 2. *Number of confirmed FAODs and organic acidurias from 1996 to 2021.*

the number of possible mortality and morbidity rates among babies with IEM. As shown in Figure 2, confirmed cases for FAODs and organic acidurias rose beginning 2020.

Detection rate of true positive cases for FAOD was 11.2% in which 104 confirmed cases out of 925 patients screened positive while detection rate of true positive organic acidurias was at 10.0% with 72 confirmed cases out of 717 patients flagged in newborn screening.

True positive cases as determined via MS/MS

In this retrospective study, one hundred seventy-six (176) patients with different IEMs have been confirmed using a combination of MS/MS (with plasma as test specimen) and GC-MS (with urine a test sample) analytical techniques, out of 1642 patients screened as high risk via ENBS screening (using dried blood spot), as shown in Table 2. Overall detection rate is 10.7% (176 confirmed cases of both FAOD and organic aciduria out of 1642 patients with positive NBS) with fatty acid oxidation defects presented in 6.3% patients (n= 104) with positive ENBS screens (n = 1642) while organic acidurias are presented in Filipino newborn babies with 5.3% detection rate (72 confirmed cases out of 1642 endorsed for second tier testing). Frequently, patients with fatty acid oxidation defects present features of acute metabolic decompensation arising out of the inability to respond to the need to utilize fatty acids as an energy source during late fasting. The clinical symptoms may be related to toxicity of the suddenly released fatty acids, to the accumulation of toxic intermediates of the inhibited fatty acid oxidation pathway.

While organic acidurias generally present with hyperammonemia and high anion gap metabolic acidosis, the major clinical features are developmental delay/mental retardation, seizures, lethargy, coma, hypotonia, vomiting, failure to thrive, hepatomegaly, respiratory distress, and cardiac dysfunction. The symptoms worsen in the absence of supporting care and can proceed to coma/death [12]. The above mentioned clinical conditions therefore highlight the need for both NBS screening using dried blood spot as test specimen and second tier biochemical testing using MS/MS with plasma as test specimen as well as GC-MS with urine as test specimen, to obtain true positive cases of FAODs or organic acidurias. And it is in this regard, that a highly reliable biochemical test such as plasma acylcarnitine profiling using MS/MS adequately addresses such demand, to accompany the clinical assessment that later on will be conducted by genetic metabolic specialists, after confirmation of the disorder.

As shown in Table 2, the FAOD that yielded the highest number of cases among Filipino newborn babies screened is MCAD with 67 confirmed cases. MCAD deficiency is the most common disorder of fatty-acid oxidation in other population [13-14]. Numbers of MCAD confirmed cases rose in 2020 and 2021 which could be due to, among others, improvements in the quantitative performance of the laboratory as manifested by its analytical performance in the ERNDIM External Quality Assurance Scheme (EQAS). Results of MS/MS analysis shows elevated concentrations of octanoylcarnitine (C8) and other medium-chain acylcarnitines such as C6, C10:1 and C10. Mean

Table 2. *Number of true positive cases of FAOD and Organic acidurias from 2015 to 2021 as determined via MS/MS and GC-MS analytical techniques.*

Fatty Acid Oxidation Disorders	Number of True Positive Cases
Carnitine uptake defect (CUD)	7
Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency	67
Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency	18
Multiple acyl-CoA dehydrogenation deficiency (MADD)	1
Carnitine Palmitoyl Transferase type 1 deficiency (CPT 1)	11
TOTAL	104
Organic Acidurias	**Number of True PositiveCases**
3-Methylcrotonyl-CoA carboxylase (3-MCC) deficiency	26
Glutaric aciduria type I (GA 1)	34
Isovaleric aciduria (IVA)	3
Methylmalonic aciduria (MMA)	5
Propionic acidemia (PA)	3
Multiple carboxylase deficiency (MCD)	1
TOTAL	72

level of C8 typically measured was 1.04 µM and ranging from 0.40 to 5.82 µM, while concentration of C6 typically measured using FIA-MS/MS were 0.41 µM with concentration range from 0.03 to 1.90 µM. Average concentration of C10:1 in plasma of confirmed patients was 0.58 µM which may vary between 0.23 and 1.38 µM. In the case of C10, mean level was 0.34 µM but may vary from 0.10 to 0.82 µM. Recently, BGL's algorithm also includes the determination of ratios of octanoylcarnitine (C8) to the following acylcarnitines: acetylcarnitine (C2), decanoylcarnitine (C10) and dodecanoylcarnitine (C12) to increase the specificity for MCAD deficiency. C8/C2 > 0.020, C8/C10 > 1.60 and C8/C12 > 1.60 ratios indicate highly suggestive for MCAD.

Another prominent IEM established via FIA-MS/MS and GC-MS analyses was glutaric aciduria type 1 (GA 1), with 34 confirmed cases. Patients with this condition have deficiency or absence of glutaryl-CoA dehydrogenase (GCDH) enzyme that is involved in the lysine metabolism. Plasma acylcarnitine results obtained via FIA-MS/MS would show extremely high concentrations of glutarylcarnitine (C5DCA) with average concentration of 4.80 µMin plasma of confirmed cases and levels may vary from 3.40 to 19.0 µM.

Meanwhile, 3-Methylcrotonyl-CoA carboxylase (3-MCC) deficiency has the most number of organic aciduria cases among Filipino patients with 26 confirmed cases. Newborn babies with this disorder have a limited or missing enzyme, 3-Methylcrotonyl-CoA carboxylase that helps break down proteins containing the amino acid, leucine. Results MS/MS analysis shows elevation in the concentration of 3-hydroxyisovalerylcarnitine (C5OH), a primary marker, with average value of 3.46 µM. BGL was able to establish concentration range for C5OH of 0.30 to 15.10 µM. Also, crucial to the confirmation of 3MCC is the evaluation of C5OH in maternal samples. Algorithm for the diagnosis of 3MCC includes the measurement of C5OH in the plasma of the mothers. BGL, apart from testing of baby's samples, also receives maternal plasma samples for testing using MS/MS.

Another FAOD confirmed among Filipino newborn babies was very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, which results from defects in peroxisome biogenesis or deficiency of a single peroxisomal enzyme [15], and accounts to 18 confirmed cases in the Philippines during the period 2015-2021. Plasma acylcarnitine profile of VLCAD shows high concentrations of the markers: 1) dodecanoylcarnitine (C12) with average concentration of 0.60 µM with 1.25 µM as the highest concentration measured; 2) cis-5-tetradecenoyl carnitine (C14:1) yielded mean level of 2.07 µM but may go as high as 8.90 µM and 3) palmitoyl (C16) acylcarnitine which gave a mean level of 0.74 µM but may be as high as 9.3 µM, when measured using MS/MS.

Table 3. *Organic acids excreted in urine of patients and Acylcarnitine markers in plasma of newborn babies who are at high risk for FAOD or organic aciduria.*

Fatty Acid Oxidation Disorders	Primary markers in Urine	Primary markers in Plasma
Carnitine uptake defect (CUD)	absence of dicarboxylic acids	Total Carnitine (-) Free Carnitine (-) Acetylcarnitine (-)
Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency	suberylglycine; hexanoylglycine	Hexanoylcarnitine (+) Octanoylcarnitine (+) Decanoylcarnitine (+) Decenoylcarnitine (+)
Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency	C6–C14 dicarboxylic acids	Tetradecenoylcarnitine (+) Tetradecanoylcarnitine (+)
Organic Acidurias	**Primary markers in Urine**	**Primary markers in Plasma**
3-Methylcrotonyl-CoA carboxylase (3-MCC) deficiency	3-OH-isovaleric acid; 3-methylcrotonylglycine	3-Hydroxyisovalerylcarnitine (+)
Glutaric aciduria type I (GA1)	Glutaric acid; glutaconate; 3-hydroxy glutarate; 3- hydroxy butyrate; 2- hydroxy butyrate; 2-keto glutarate	Glutarylcarnitine (+)
Isovaleric aciduria (IVA)	Isovaleryl glycine; 3-hydroxy isovalerate	Isovalerylcarnitine (+)
Methylmalonic aciduria (MMA)	Methylmalonic acid; 3-OH-propionic acid and methylcitric acid	Propionylcarnitine (+)
Propionic acidemia (PA)	3-OH-propionic acid; propionylglycine and methylcitric acid	Propionylcarnitine (+)

(+) Increased and (-) Decreased level of Acylcarnitines in Plasma Sample

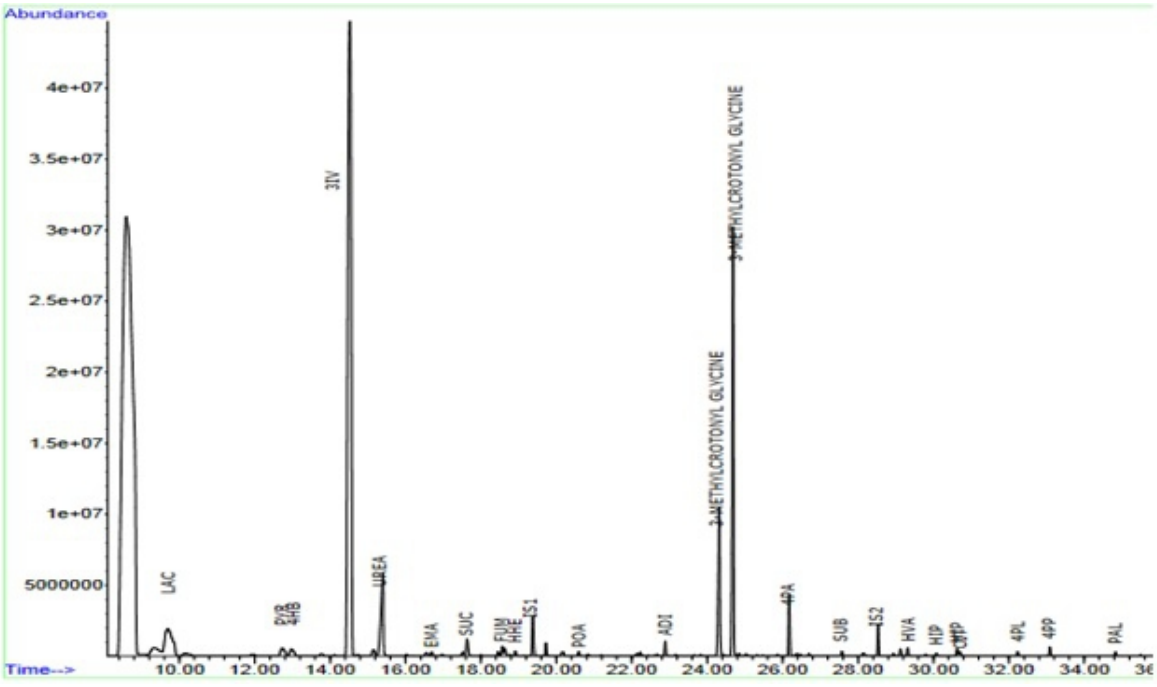

Figure 3. *GC-MS total ion chromatogram of a patient with 3 MCC deficiency on a HP-5MS column.*

Carnitine uptake defect (CUD) results in systemic and intracellular carnitine deficiencies [16] and has an incidence of around 1 in 67,000 (95% CI: one in 31,600–512,000), after 2007 [17]. There were 7 Filipino newborn babies confirmed to be afflicted with CUD, where plasma acyl carnitine results show below normal ranges of total carnitine and free carnitine (C0). Typically, total carnitine and free carnitine levels are below 8 and 4 µM, respectively, for newborn babies who are less than 28 days old. As part of BGL's differential diagnosis, CUD cases were usually subjected to repeat sample collection and testing after 2 months. In addition, similar to 3MCC cases, maternal CUD testing were also undertaken by BGL to confirm suspicion of CUD in newborn babies.

On the other hand, propionic acidemia, methylmalonic aciduria, and isovaleric aciduria constitute the other organic acidurias established in this biochemical assessment, with 3, 5, and 3 confirmed cases in the Philippines from 2015 to 2021, respectively. Propionic acidemia (PA) is a disorder characterized by elevated propionylcarnitine (C3) obtained via FIA-MS/MS as well as the presence of propionylglycine, 3-hydroxypropionate, and methylcitrate in the urine as established via GC-MS analysis [18]. While methylmalonic aciduria (MMA) is also characterized with elevated concentration of propionylcarnitine (C3) and presence of 3-hydroxypropionate, and methylcitrate in urinary organic acids, it can be differentiated from PA via observed elevations in the level of methylmalonic acid in the GC-MS chromatogram. Further, BGL performs urine metabolic screening via the use of high voltage electrophoresis (HVE) which is able to detect elevations in methylmalonic acid in urine.

Isovaleric aciduria (IVA), on the other hand, is a rare autosomal recessive disorder in leucine metabolism characterized by episodes of acute metabolic crisis and psychomotor development retardation [19]. IVA was the first organic acidemia discovered in 1966 [20]. Incidence of IVA in Germany is around 1:62, 500 [21] while 1: 365,000 in Taiwan [22]. The use of MS/MS for the detection of isovalerylcarnitine (C5) may not be enough to confirm isovaleric aciduria. This is because BGL is yet to resolve the 3 different physiological isomers of C5 which are: 2-methylbutyrylcarnitine, isovalerylcarnitine and valerylcarnitine as well as the non-physiological pivaloylcarnitine which may be due to intake of pivalate-containing antibiotics [23]. All of these isomers may contribute to the total amount of C5 that can be measured using MS/MS. Therefore, BGL confirms IVA via GC-MS in which chromatogram is marked by the presence of isovaleric acid, 3-hydroxyisovaleric acid, and isovalerylglycine (IVG) in the urine of patients.

GC-MS for differential diagnosis of FAOD and Organic Aciduria

As earlier discussed, analysis of organic acids in urine is of paramount importance to the diagnosis of organic acidurias and fatty acid oxidation disorder. There may be cases that plasma acylcarnitine profiles do not necessarily follow a pattern suggestive of a specific IEM but nonetheless yielded either a decrease or an increase in the acylcarnitine concentrations when compared against the reference range. As already highlighted, GC-MS technique may complement the results generated from MS/MS since there are key markers for IEM that are also present in urine. Table 3 gives the organic acids and other important metabolites that could be detected in urine and can be correlated with corresponding levels of acylcarnitine markers, in case a patient is found to be afflicted with FAOD or organic acidurias.

To further explain the information given in Table 3, those cases which gave patterns suggestive of MCAD as indicated by their plasma acylcarnitine profile, were subjected to further scrutiny using their urinary organic acid profiles. Typically, observed in the GC-MS chromatogram of MCAD patients are the acylglycines namely, suberylglycine and hexanoylglycine (dicarboxylic acid esters of glycine) in the urine of the patients. Such consistency in the results of the biochemical tests (GC-MS with MS/MS) would then lead to an interpretation of a highly suggestive MCAD case and shall be referred to a clinical geneticist or metabolic specialist for correlation with clinical presentations of MCAD. Likewise, those PLAC results highly suggestive of organic acidurias were correlated with their urine organic acid profiles.

For those cases of 3 MCC deficiency with elevated C5OH in their plasma acylcarnitine profile, these were correlated clinically and with urinary organic acids which have shown presence of 3-methylcrotonylglycine and increased 3-hydroxyisovaleric acid as shown in Figure 3.

In the case of patients with GA1, characteristic metabolites of GA1 were also found present in urine samples as obtained via GC-MS and these include the following: 3-hydroxyglutaric acid, glutaconic acid and glutaric acid. The GC-MS chromatogram of a GA 1 patient is shown in Figure 4.

Challenges of using MS/MS and GC-MS as confirmatory tools for the diagnosis of IEMs

As demonstrated, diagnosis of FAODs such as VLCAD and CPT II and organic aciduria entails the establishment of

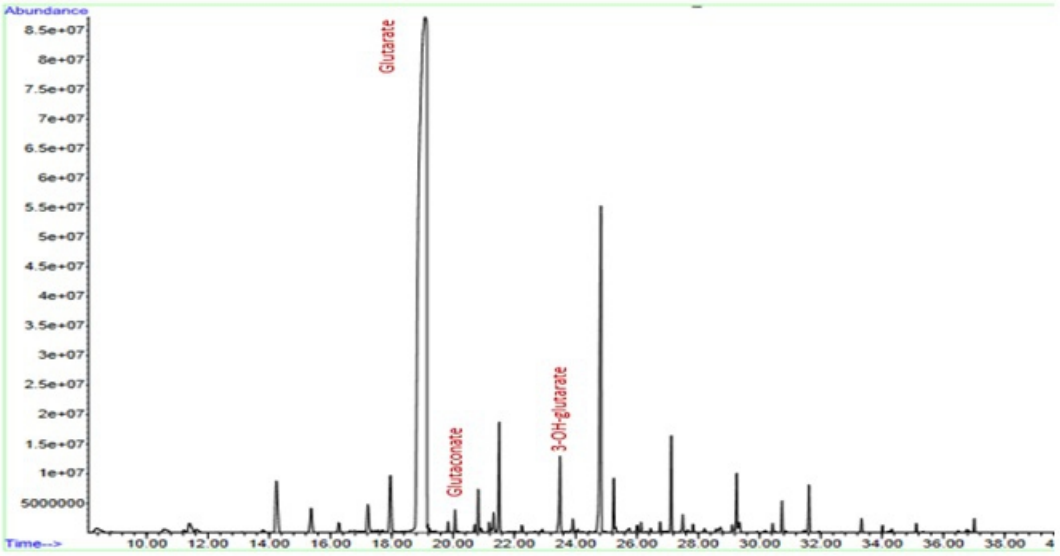

Figure 4. *GC-MS chromatogram of a patient with glutaric aciduria type 1 on a HP-5MS column.*

metabolic fingerprint in other matrices such as urine and plasma in addition to dried blood spot (DBS). Though for CPT I, DBS is still the most suitable sample matrix however, DBS especially if obtained from 1 to 2 day old babies, is not a good matrix as some markers for IEMs cannot be detected in DBS. Hence, plasma and urine samples are the better alternative samples for MS/MS and GC-MS analyses.

There have been cases that plasma acylcarnitine profiles may be outside the reference ranges and the ratios or profiles are not also adequate to rule out an IEM. In 2021, the Newborn Screening Reference Center (NSRC) in coordination with the panel of metabolic experts, has introduced gene testing as another second-tier method to confirm the diagnosis of FAOD cases. Recent experience has shown that gene test results have complemented the biochemical test results. As of this writing, there have been at least 3 cases in which gene testing supported the biochemical laboratory findings, thus confirming that these were indeed FAOD case based on the mutations observed.

In the case of GC-MS analysis, the laboratory has sometimes experienced poor recovery due to interferences posed by medications and drugs administered to patients. Chromatograms often yield metabolic patterns that are quite ambiguous. In this regard that BGL requires the submission of clinical abstracts, which are generally not available in the case of ENBS cases. Further, as our approach is semi-quantitative, where we do pattern recognition in interpreting the GC-MS chromatograms, cut off values for each of the metabolite is also not available.

Importantly, interpretation of GC-MS chromatograms demand skills and expertise to be able to interpret the data correctly. Hence, one solution is to continually update our own database for chromatogram analysis. Since 2003, BGL has already analyzed 3678 urine samples using GC-MS. This has assisted us in figuring out the relevance of each peak in the overall pattern for a more accurate diagnosis.

In view of the foregoing discussion highlighting the relevance of MS/MS and GC-MS analytical techniques as second-tier methods for the diagnosis of FAODs and organic acidurias, BGL recognizes the need to: 1) tackle extraction efficiency including efficiency of derivatization for detection in order to minimize false elevation in metabolites; 2) periodic review of the diagnostic algorithm to include deriving ratios of specific biomarkers instead of just flagging markers that are outside the normal range; 3) adopt computational tools to simplify metabolic pattern recognition and facilitate the interpretation process. The above future direction is expected to increase the efficiency of the diagnosis, that is, minimizing the occurrence of false positives as well as false negatives from among those cases endorsed to BGL for confirmatory testing.

Conclusions

This retrospective study accounts for FAOD and organic aciduria cases among Filipino newborn babies over a course 6 years since the start of the full implementation of expanded newborn screening (ENBS) from 2015-2021 and the first time that MS/MS technique was introduced as another second-tier

method for the diagnosis of IEMs. After screening using dried blood spots, plasma and urine samples from these babies were sent to BGL for 2nd tier testing using MS/MS and GC-MS analytical techniques. The Biochemical Genetics Laboratory has received a total of 4033 positive ENBS screened patients out of which 1642 were suspected cases of FAOD and organic acidurias. 176 newborn babies were confirmed positive for FAODs and Organic Acidurias from 2015 to 2021. MCAD deficiency is the most prevalent among FAOD cases in the Philippines while glutaric aciduria type 1 is most common organic aciduria in the Philippines.The numbers presented in the paper indicate that there are FAOD cases and organic acidurias that have been documented in Filipino population.

Upon confirmation, each case is then forwarded to the concerned Newborn Screening Centers (NSCs) and/or to Clinical Genetics Research and Services unit of the Institute of Human Genetics, for appropriate patient's recall and management. To date, some of these cases are still managed by the country's metabolic specialists.

The determination of acylcarnitine profiles in plasma samples of Filipino newborn babies using FIA-MS/MS, as a reliable analytical tool to detect the occurrence of fatty acid oxidation disorders and organic acidurias has been presented in this paper. The MS/MS and GC-MS analytical techniques employed by BGL are crucial in the diagnosis of these inborn errors of metabolism. Plasma acylcarnitine profile when correlated with urinary organic acids profile obtained via GC-MS could offer reliable means to rapidly identify specific FAOD or organic aciduria among newborn babies. Further, the use of GC-MS provides an ample data which will complement the determination of true positives among FAOD and organic aciduria cases endorsed to BGL. Accordingly, a highly reliable diagnosis becomes available which shall become a scientifically sound basis for subsequent administration of intervention. The Biochemical Genetics Laboratory has demonstrated its capability to contribute to the diagnosis and management of Filipino newborn babies with genetic disorders as extensively as possible beginning 2015 to present.

Further, we have shown that when absolute concentrations of acylcarnitines are necessary for diagnosis, the choice of plasma as test specimen has been proven to be relevant and useful as majority of fatty acid oxidation defects can be detected in plasma using the MS/MS method. Nonetheless, a comprehensive assessment to include gene testing may be necessary to further sort true positives from carriers who may also yield elevations and/or attenuations in the levels of key biomarkers being considered for diagnosis.

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Statement of Authorship

All authors have approved the submitted final version.

Author Disclosure

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