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ORIGINAL ARTICLE

A COMPARATIVE STUDY OF PLASMA AND DRIED BLOOD SPOT ACYLCARNITINES IN FATTY ACID OXIDATION DISORDERS AND ORGANIC ACIDURIAS

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ABSTRACT

Analysis of blood acylcarnitines is used in the investigation of fatty acid oxidation disorders (FAOD) and organic acidurias. Dried blood spot (DBS) sample is regarded as the preferred sample matrix for newborn screening programmes due to its certain characteristics. However, there is limited data on the correlation of acylcarnitines in plasma and DBS. This study aims to compare individual acylcarnitines as well as the profile between plasma and DBS in FAOD and organic acidurias. We studied 55 paired plasma and DBS samples of known FAOD and organic acidurias. Individual acylcarnitines from both matrices were analysed as their butyl esters by electrospray ionisation tandem mass spectrometry (ESI-MS/MS) in multiple reaction monitoring (MRM) mode. The similarities and differences in acylcarnitines concentration and profile interpretation between plasma and DBS for individual diseases were described. Free carnitine elevations in patients with CPT1a deficiency were two times higher in DBS than that of plasma. Long-chain acylcarnitines elevations in patients with CACT and CPT2 deficiency were more strikingly demonstrated in plasma than in DBS. Long-chain hydroxylacylcarnitines elevations in MTP/LCHAD deficiency were equally detected on both matrices. Elevations of the main acylcarnitines were seen in both plasma and DBS samples of patients with propionic aciduria, glutaric aciduria type 1, and isovaleric aciduria. In methylmalonic aciduria, 3/19 (15.8%) patients had DBS C3-acylcarnitine within the reference range. DBS is a better sample for detecting CPT1a deficiency, while plasma is better at detecting CACT/CPT2 deficiency. Both plasma and DBS are equally useful in the investigation of suspected LCHAD/MTP deficiency and common organic acidurias.

Keywords: *acylcarnitine profile, dried blood spot, fatty acid oxidation disorders, organic acidurias, tandem mass spectrometry*

Abbreviations:
C0: Carnitine
C2: Acetylcarnitine
C3: Propionylcarnitine
C4: Butyryl-/isobutyrylcarnitine
C4OH: 3-Hydroxybutyrylcarnitine
C5: Isovalerylcarnitine/2-methylbutyrylcarnitine
C5DC: Glutarylcarnitine
C5OH: 3-Hydroxyisovaleryl-/2-methyl-3-hydroxybutyrylcarnitine
C5:1: Tiglyl-/3-methylcrotonylcarnitine
C8: Octanoylcarnitine
C16: Palmitoylcarnitine
C16OH: 3-Hydroxypalmitoylcarnitine
C18: Stearoylcarnitine
C18:1: Oleoylcarnitine
C18OH: 3-Hydroxystearoylcarnitine
C18:1OH: 3-Hydroxyoleoylcarnitine

INTRODUCTION

Acylcarnitines are formed through the esterification of carnitine and acyl-CoAs by the enzyme carnitine acyltransferase in the mitochondria. Defects in beta-oxidation of fatty acids and organic acids metabolism will lead to the accumulation of the corresponding acylcarnitines in body fluids (Reuter & Evans, 2012). Therefore, acylcarnitines serve as biomarkers in the detection and diagnosis of these fatty acid oxidation disorders (FAOD) and organic acidurias.

Fatty acid oxidation disorders (FAOD) and organic acidurias are a group of inborn errors of metabolism (IEM). Although individually rare, they are quite common collectively, with a reported incidence of 1:6565 (J. S. Lim et al., 2014). Diagnosing these disorders can be difficult due to the lack of specific symptoms and the variety of clinical manifestations. Early detection and management can prevent life-threatening metabolic events.

Acylcarnitines analysis are commonly performed on tandem mass spectrometry (Miller et al., 2021; Millington & Stevens, 2011). This technology enables multiple acylcarnitines detection in single sample analysis, allowing simultaneous screening of various FAODs and organic acidurias. Advancements in tandem mass spectrometry technology since the early 2000s have also led to increased implementation of expanded newborn screening for IEM in many countries (Belaramani et al., 2024; Chien & Hwu, 2023; Martín-Rivada et al., 2022). Dried blood spot (DBS) has been widely used in newborn screening programmes worldwide since it was first introduced in the early 1960s. DBS is an ideal sample for newborn screening due to its less invasive collection method, small blood volume requirement, and ease of transportation and storage (M. D. Lim, 2018).

However, there are a few concerns about the variability caused by the inherent imprecision in DBS samples (M. D. Lim, 2018). Generally, any presumptive positive result from newborn screening would require confirmatory testing with plasma or another alternative test method (Miller et al., 2021; Merritt et al., 2018). Interestingly, in recent years, the use of DBS has extended beyond newborn screening to potential applications in diagnostic testing for infectious diseases, molecular profiling, and toxicology (Malsagova et al., 2020). The data on the correlation between acylcarnitine concentrations in DBS and plasma samples remains limited. Understanding the biochemical variances between DBS and plasma is imperative for accurate diagnosis. This study aims to compare individual acylcarnitines concentrations and profiles between plasma and DBS in patients with FAOD and organic acidurias and evaluate whether the selection of sample matrix type would change the interpretation or diagnosis.

METHODS

Study Population and Sample Collection

Paired plasma and dried blood spot samples were obtained from fifty-five known FAOD and organic aciduria patients between January 2022 and August 2023. Plasma samples were collected in lithium heparin tubes, centrifuged, and separated. DBS samples were collected on Whatmann 903 filter paper and air-dried. All samples were sent to the Biochemistry Unit, Institute for Medical Research. Filter papers were then placed into seal bags with desiccant and stored at -30°C. Plasma was stored at -80°C until analysis. All plasma and urine data obtained were de-identified and anonymised. This study has been approved by the Medical Review and Ethics Committee (NMRR-20-2341-53827).

Sample Processing

Samples were processed according to published methods with some modifications (Habib et al., 2021; Millington & Stevens, 2011). Acylcarnitines extraction was performed on 3.2 mm punched filter paper disks and 10ul of plasma using methanol-containing internal standards. The internal standards used (NSK-B) were purchased from Cambridge Isotope Laboratories (USA), which contained the following stable-isotope acylcarnitines: d9-C0, d3-C2, d3-C3, d3-C4, d9-C5, d3-C8, d9-C14, and d3-C16. The extracted acylcarnitines were derivatized with butanolic-HCL and subsequently dried under nitrogen flow, followed by reconstitution with mobile phase (acetonitrile/water/0.1% formic acid) prior to analysis on the UPLC-MS/MS system.

Ultrahigh-performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS)

Sample analysis was performed on a Waters Aquity UPLC I-Class PLUS system connected to a Waters Xevo TQ-S micromass spectrometry and operated with electrospray in positive ionization mode. Multiple reaction monitoring (MRM) was used to scan for specific mass ion intensities. The individual acylcarnitine concentrations were determined by calculating the signal ratio from each acylcarnitine to the signal from the internal standard using Waters MassLynx software.

Statistical Analysis

The acylcarnitine levels and acylcarnitine ratio for specific FAOD and organic aciduria were compared to the reference cut-offs at 2.5th and 97.5th centiles. Agreement and bias between plasma and DBS were assessed via Bland-Altman plots. The difference between plasma and DBS of individual acylcarnitine in non-related disorder (as control) was assessed using paired T-test (parametric data) and Wilcoxon Signed Rank test (non-parametric data). Statistical analysis was conducted using SPSS version 26 and the findings were significant if the p-value was less than 0.05.

RESULTS

Diseases included in this study are carnitine uptake defect (CUD) (n=2), Carnitine palmitoyltransferase type 1a (CPT1a) deficiency (n=2), Carnitine-acylcarnitine translocase (CACT)/Carnitine palmitoyltransferase type 2 (CPT2) deficiency (n=3), Long-chain 3-hydroxy acyl-CoA dehydrogenase (LCHAD)/Mitochondrial trifunctional protein (MTP) deficiency (n=2), Multiple acyl-CoA dehydrogenase deficiency (MADD) (n=2), Methylmalonic Aciduria/Cobalamin disorders (MMA/Cbl) (n=19), Propionic acidemia (PA) (n=6), Glutaric aciduria type 1 (GA1) (n=7), Isovaleric acidemia (IVA) (n=7), 3-Hydroxy methyl-glutaryl-CoA lyase (HMG) deficiency/3-Methylcrotonyl-CoA carboxylase (MCC) deficiency/3-Methylglutaconic aciduria type 1 (MGA) (n=4), and Beta-ketothiolase (BKT) deficiency (n=1).

Plasma and DBS acylcarnitines concentrations of patients with FAOD and organic aciduria are presented in Table 1 and Table 2, respectively. The commonly used acylcarnitine ratios were also calculated. Figure 1 shows the distribution of acylcarnitines differences between plasma and DBS in all samples.

The free carnitine (C0) concentration in CPT1a deficiency was more than two times higher in DBS than in plasma (Table 1, Figure 1a). One plasma sample of CPT1a deficiency had C0 and C0/(C16+C18) ratio values below the reference cut-off. Long-chain acylcarnitines (C16, C18, and C18:1) elevations in patients with CACT/CPT2 deficiency were more strikingly demonstrated in plasma than in DBS (Table 1, Figure 1g, Figure 1h). Two out of three patients with CACT/CPT2 deficiency had within-range DBS long-chain acylcarnitines, and one had a normal (C16+C18:1)/C2 ratio.

In MMA/Cbl, 3/19 (15.8%) patients had C3 levels below the reference cut-off in DBS samples. However, the C3/C2 and C3/C16 ratios in these patients were both raised and indicative of the disease. Propionic aciduria showed raised C3 values of at least 12 times above the reference cut-off in plasma compared to 2.5 times in DBS.

The elevation of the primary acylcarnitine in GA1, IVA, and BKT was seen higher in plasma than in DBS (Table 2, Figure 1c, Figure 1e), whereas the elevation of C5OH acylcarnitine in HMG/MCC/MGA was higher in DBS (Table 2, Figure 1d). Nevertheless, the changes of specific acylcarnitines in these disorders were significantly raised when compared to their matrix-match reference cut-offs.

Table 1: Acylcarnitine profiles in FAOD

Disorder	Acylcarnitine	No. of sample	Age	Patient Range in Plasma (umol/L)	2.5th Cut-off in Plasma	Flag	Patient Range DBS (umol/L)	2.5th Cut-off in DBS	Flag
CUD	C0	2	1m-1y	0.6-17.6	21.2	↓↓-↓	0.6-11.5	13.2	↓↓-↓
	C2	2	1m-1y	4.7-12.6	5.8	↓-n	2.5-7.8	7.0	↓↓-↓
	C3	2	1m-1y	0.01-0.39	0.19	↓↓-n	0.04-0.39	0.42	↓↓-↓
Disorder	**Acylcarnitine**	**No. of sample**	**Age**	**Patient Range in Plasma (umol/L)**	**97.5th Cut-off in Plasma**	**Flag**	**Patient Range DBS (umol/L)**	**97.5th Cut-Off in DBS**	**Flag**
CPT1a	C0	2	1m-1y	48-92	59	n-↑	187-197	57	↑↑
	C0/(C16+C18) ratio	2	1m-1y	472-1487	789	n-↑	145-2069	50	↑↑
CACT/CPT2	C16	1	1m-1y	1.13	0.33	↑↑	1.9	2.78	n
		2	>1y	1.48-7.03	0.31	↑↑	2.0-6.3	2.51	n-↑
	C18	1	1m-1y	0.22	0.09	↑↑	0.51	1.11	n
		2	>1y	0.3-1.92	0.07	↑↑	0.71-2.40	1.02	n-↑
	C18:1	1	1m-1y	0.79	0.33	↑	0.70	1.93	n
		2	>1y	0.46-6.78	0.36	↑-↑↑	0.47-4.50	1.60	n-↑↑
	C18:2	1	1m-1y	0.38	0.21	↑	0.25	0.51	n
		2	>1y	0.24-1.83	0.18	↑-↑↑	0.19-0.99	0.37	n-↑
	(C16+C18:1)/C2 ratio	1	1m-1y	0.12	0.07	↑	0.36	0.30	↑
		2	>1y	0.14-2.18	0.07	↑-↑↑	0.31-3.34	0.37	n-↑↑
LCHAD/MTP	C16OH	1	<1m	1.42	0.05	↑↑	1.88	0.04	↑↑
		1	1m-1y	1.32	0.02	↑↑	0.76	0.02	↑↑
	C18:1OH	1	<1m	1.41	0.03	↑↑	1.09	0.02	↑↑
		1	1m-1y	1.47	0.01	↑↑	0.48	0.02	↑↑
	C18OH	1	<1m	0.39	0.02	↑↑	0.71	0.02	↑↑
		1	1m-1y	0.42	0.01	↑↑	0.26	0.01	↑↑
	C16OH/C16 ratio	1	<1m	0.49	0.18	↑	0.24	0.01	↑↑
		1	1m-1y	0.38	0.19	↑	0.16	0.01	↑↑
MADD	C8	1	1m-1y	0.03	0.25	n	0.02	0.13	n
		1	>1y	0.49	0.23	↑	0.41	0.13	↑↑
	C10	1	1m-1y	0.05	0.48	n	0.04	0.25	n
		1	>1y	0.90	0.46	↑	0.73	0.22	↑↑

Note: ↓=1-2 times below lower limit, ↓↓= 3 times below lower limit, n=within reference range, ↑=1-2 times above upper limit, ↑↑=3 times above upper limit

Table 2: Acylcarnitine profiles in Organic Aciduria

Disorder	Acylcarnitine	No. of sample	Age	Patient Range in Plasma (umol/L)	97.5th Cut-off in Plasma	Flag	Patient Range in DBS (umol/L)	97.5th Cut-off in DBS	Flag
MMA/Cbl	C3	3	<1m	9.5-25.3	0.96	↑↑	5.3-10.5	3.50	↑-↑↑
		4	1m-1y	56.1-85.9	1.12	↑↑	17.5-30.5	3.03	↑↑
		12	>1y	1.3-64.5	0.91	↑↑	1.5-22.4	2.34	n-↑↑
	C3/C2 ratio	3	<1m	0.91-1.3	0.08	↑↑	0.32-0.62	0.14	↑-↑↑
		4	1m-1y	0.56-2.8	0.11	↑↑	0.56-1.2	0.15	↑↑
		12	>1y	0.12-1.5	0.14	↑-↑↑	0.29-2.6	0.19	↑-↑↑
	C3/C16 ratio	3	<1m	40.8-76.1	17.4	↑-↑↑	4.6-9.2	1.4	↑↑
		4	1m-1y	397.2-553.0	18.9	↑↑	29.1-35.9	4.5	↑↑
		12	>1y	6.8-309.7	15.2	n-↑↑	1.2-23.4	2.4	↑-↑↑
PA	C3	2	<1m	11.5-37.5	0.96	↑↑	8.7-12.4	3.50	↑-↑↑
		4	>1y	43.5-78.8	0.91	↑↑	14.2-35.6	2.34	↑↑
	C3/C2 ratio	2	<1m	0.9-3.4	0.08	↑↑	0.8-3.0	0.14	↑↑
		4	>1y	1.7-6.4	0.14	↑↑	1.5-5.3	0.19	↑↑
	C3/C16 ratio	2	<1m	69.4-304.1	17.4	↑↑	8.1-22.1	1.4	↑↑
		4	>1y	363.1-508.6	15.2	↑↑	12.9-43.8	2.4	↑↑
IVA	C5	2	<1m	4.6-16.8	0.34	↑↑	4.7-6.5	0.16	↑↑
		5	>1y	11.2-14.4	0.22	↑↑	9.5-13.3	0.15	↑↑
	C5/C2 ratio	2	<1m	1.7-2.8	0.03	↑↑	1.9-2.3	0.01	↑↑
		5	>1y	1.2-1.8	0.04	↑↑	0.57-3.3	0.02	↑↑
GA1	C5DC	1	1m-1y	3.28	0.06	↑↑	0.83	0.02	↑↑
		6	>1y	2.46-8.05	0.08	↑↑	0.64-1.86	0.03	↑↑
	C5DC/C4 ratio	1	1m-1y	6.9	0.79	↑↑	2.6	0.20	↑↑
		6	>1y	10.6-22.9	0.87	↑↑	3.9-13.8	0.18	↑↑
HMG/MCC/MGA	C5OH	2	1m-1y	0.20-1.36	0.06	↑↑	0.72-5.23	0.19	↑↑
		2	>1y	0.26-0.32	0.06	↑↑	1.12-1.69	0.27	↑↑
	C5OH/C8 ratio	2	1m-1y	3.4-53.1	3.3	↑-↑↑	25.6-1257.0	8.5	↑↑
		2	>1y	0.9-1.0	6.5	n	11.9-13.1	14.5	n
	C5OH/C0 ratio	2	1m-1y	0.005-0.36	0.001	↑↑	0.02-0.74	0.01	↑-↑↑
		2	>1y	0.005	0.001	↑↑	1.1-1.7	0.01	↑↑
BKT	C5:1	1	<1m	0.66	0.02	↑↑	0.21	0.02	↑↑
	C5OH	1	<1m	0.45	0.02	↑↑	0.63	0.02	↑↑
	C4OH	1	<1m	0.91	0.15	↑↑	1.7	0.23	↑↑

Note: n=within reference range, ↑=1-2 times above upper limit, ↑↑=3 times above upper limit

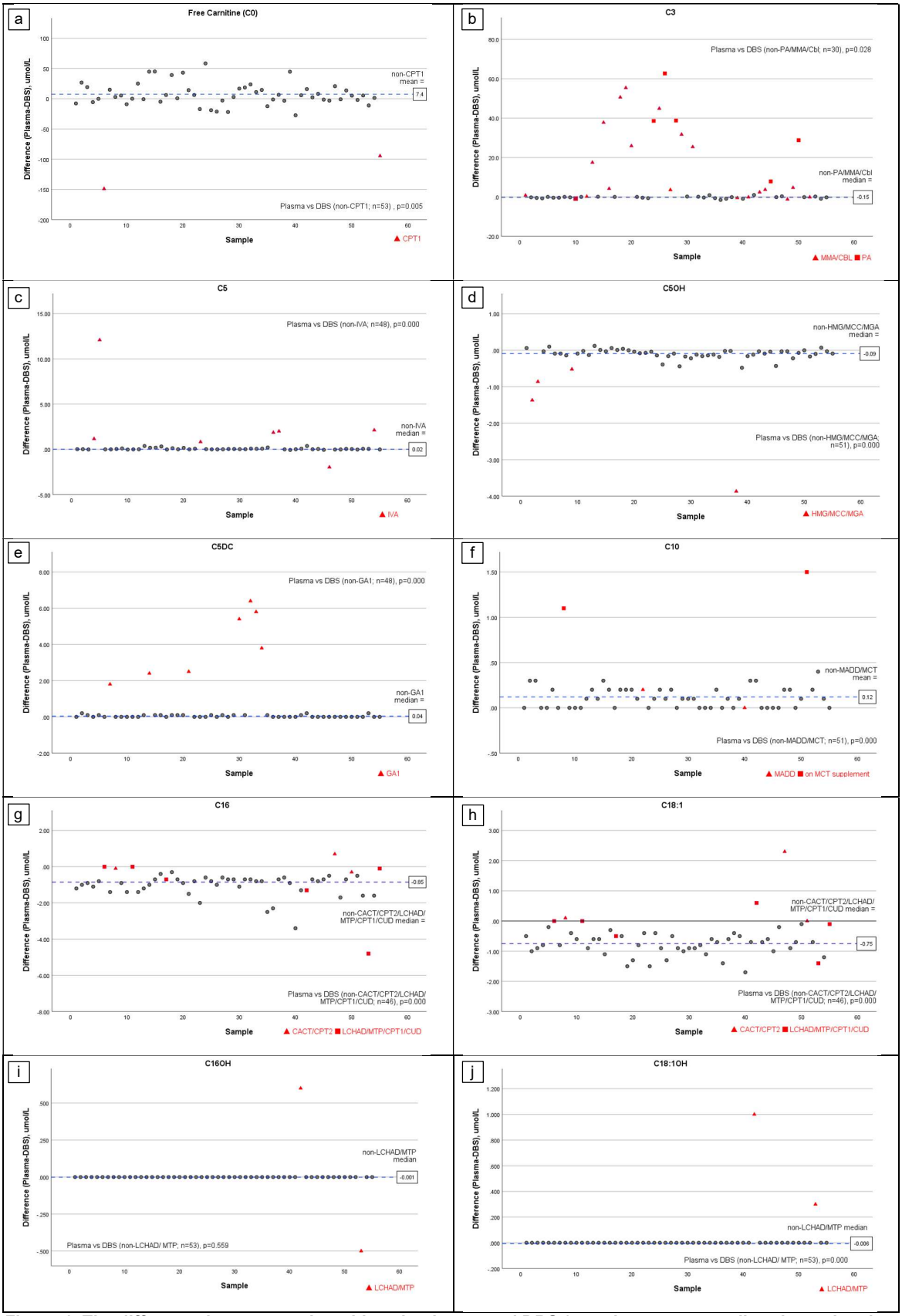

Figure 1. The difference between acylcarnitines in plasma and DBS (▲and ■ represents disorders related to specific acylcarnitines. Blue line indicates mean/median differences in non-related disorders).

DISCUSSION

Our main aim was to compare the acylcarnitine profile in plasma and DBS of FAOD and organic aciduria samples and evaluate whether the selection of sample type would change the interpretation or diagnosis. Based on this aim, we analysed paired samples from eleven confirmed FAOD patients and forty-four confirmed organic acidurias. Our results showed that the concentrations of most acylcarnitines in plasma and DBS varied. Thus, interpreting the acylcarnitine profile based on matrix-matched reference cut-off is fundamental for accurate diagnosis.

From our findings, CPT1a deficiency can be confidently identified in DBS by the significantly elevated free carnitine levels and C0/(C16+C18) ratio in DBS. This suggests that DBS is more sensitive than plasma in diagnosing CPT1a deficiency, and this condition could be missed in plasma. Our finding aligns with two previous case reports where the initial DBS screening suggested CPT1a deficiency, but the confirmatory testing in plasma showed a normal acylcarnitine profile (Borch et al., 2012; Dowsett et al., 2017). Even though a raised C0/(C16+C18) ratio is typically considered the most reliable indicator of CPT1a deficiency, there have been reported cases of patients with normal C0/(C16+C18) ratio (Fingerhut et al., 2001; Zhou et al., 2022). It is noteworthy that presymptomatic or milder form CPT1a deficiency could present with normal free carnitine levels and C0/(C16+C18) ratios or subtle changes (Borch et al., 2012;Dowsett et al., 2017).

Conversely, in CACT/CPT2 deficiency, plasma is superior to DBS, as evidenced by the markedly raised long-chain acylcarnitines and the ratio of C16+C18:1)/C2 in all plasma samples of CACT/CPT2 patients. DBS naturally has a higher composition of long-chain acylcarnitines, which could potentially obscure the elevation of long-chain acylcarnitines caused by fatty acid oxidation defects (Reuter & Evans, 2012). Our findings further support the preference of plasma samples for investigating CACT/CPT2 deficiency (Al-Thihli et al., 2014;de Sain-van der Velden et al., 2013). Nevertheless, elevated C16+C18:1)/C2 ratio is still a sensitive marker in DBS, as demonstrated by Habib et al. (2021), where all patients were successfully identified in DBS, in contrast to the normal ratio seen in a few patients of our patients and other studies (Al-Thihli et al., 2014; de Sain-van der Velden et al., 2013). The differences in DBS findings could be attributed to the varying metabolic statuses of the study populations. Habib et al. (2021) worked with the samples in acute metabolic crises, whereas our samples were relatively in metabolically stable state.

Acylcarnitine profiles in both plasma and DBS of LCHAD/MTP deficiency were equally informative, even though plasma was recommended by Van Hove et al. (2000) due to more numbers of elevated hydroxyl acylcarnitines observed. The remarkable elevation of C16OH and C18:1OH in our samples was considerably diagnostic and compatible with the diagnosis of LCHAD/MTP deficiency. Similar findings were reported by (Lotz-Havla et al., 2018), and the hydroxylacylcarnitines abnormalities were consistently demonstrated in following DBS or plasma samples.

Meanwhile, all organic acidurias in our study were successfully identified either in plasma or DBS samples, with the exception of a few cases of MMA/Cbl. It appears that the selection of blood samples may not significantly enhance the diagnostic value for organic aciduria compared to FAOD. Organic acidurias that shared a similar elevation of acylcarnitines pattern would warrant further confirmatory testing by urine organic acid analysis (Miller et al., 2021).

In Malaysia, presymptomatic newborn screening for FAOD and organic aciduria is not part of the national newborn screening programmes but is available as an optional test offered by certain private healthcare. Besides that, patients who presented with signs and symptoms suggestive of IEM would undergo high-risk screening, often in combination with other routine or specialised testing. As such, in the setting of high-risk screening, these findings can guide clinicians in selecting the appropriate sample types for specific targeted diseases, particularly when blood samples are limited.

While our study provides valuable insights into the differences in acylcarnitine profiles between plasma and DBS, the findings of this study have to be seen in light of some limitations. Due to their rarity, only a small number of cases of FAOD and organic aciduria were being explored. Therefore, the findings may not be representative of the entire spectrum of these disorders. However, the disorders included in our study were considerably more prevalent IEM disorders in our Malaysian population. Future studies with larger sample sizes and more comprehensive inclusion of other FAOD and organic aciduria types are recommended to further strengthen the findings of our study.

CONCLUSION

In conclusion, acylcarnitines in plasma and DBS are significantly different. It also highlights the need for a reference range for a specific sample matrix and the importance of using a similar sample matrix for treatment monitoring. DBS is better at detecting CPT1a deficiency, while plasma is better at detecting CACT/CPT2 deficiency. Both plasma and DBS are equally useful in investigating suspected LCHAD/MTP deficiency and common organic acidurias.

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AUTHORS' CONTRIBUTION

Azzah Hana A.Y. and Anasufiza H. formulated and designed the study. Muhd Irfan Bukhari A.N., Nurfarah Nabila M.A., and Marleena M. executed the experiments and prepared the data. Muhd Irfan Bukhari A.N. and Salina A.R. were responsible for methodology and validation. Chew H.B. and Moey L.H. were responsible for recruitment and sample collection. Azzah Hana A.Y., Saraswathy A., Anasufiza H., Chew H.B., and Moey L.H. played a key role in interpreting the results. Azzah Hana A.Y. spearheaded the writing of the manuscript. All authors actively contributed to providing critical feedback, shaping the research, guiding the analysis, and refining the manuscript.

CONFLICT OF INTEREST DECLARATION

We affirm that there is no Conflict of Interest among the author(s) concerning the subject matter or materials discussed in this manuscript. We further certify that the article represents the original work of the Authors and Co-Authors. The manuscript has not been previously published and is not currently under consideration for publication elsewhere. This research/manuscript has neither been submitted for publication nor published in whole or in part elsewhere. We attest that all authors have made significant contributions to the work, ensuring the validity and legitimacy of the data and its interpretation, thereby warranting its submission to the Malaysian Journal of Clinical Biochemistry.

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