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Age-Specific Reference Values for Free Carnitine and Short Chain Acylcarnitines Content in Dried Blood Spots in Newborns in Western Kazakhstan: A Tandem Mass Spectrometry Measurement

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Abstract: Measuring the level of acylcarnitines and free carnitine in the blood is one of the stages of early diagnosis of inborn errors of metabolism, including disorders of fatty acid oxidation, and ensures timely initiation of therapeutic measures. Currently, tandem mass spectrometry (MS/MS) is successfully used for these purposes. Dried blood spot acylcarnitine reference values developed for neonates are critical for interpreting test results and diagnosing fatty acid metabolic disorders. Objectives: To establish reference values for the concentrations of free carnitine and short-chain acylcarnitines in samples of dried blood spots of newborns in Western Kazakhstan using LC-MS/MS technology (liquid chromatography-tandem mass spectrometry). Methods: The cross-sectional study included 250 healthy newborns from Western Kazakhstan aged 1-3 days, born at term and breastfed, 49.2% boys and 50.2% girls. To establish age reference values for C0 and short-chain acylcarnitines, newborns were divided into three groups (1) 1-day, (2) 2-day and (3) 3-day. Guthrie blood samples were collected on days 1–3 of life and quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Nonparametric statistical approaches were used to obtain percentile distributions for newborns ranging from 2.5 to 97.5. Results: A statistically significant difference was found in the mean levels of acetylcarnitine (C2), butyrylcarnitine (C4) and tiglylcarnitine (C5:1) in men and women. The highest values were determined in the female group. Age-related differences were observed in the concentration levels of malonylcarnitine (C3DC), butyrylcarnitine (C4), isovalerylcarnitine (C5) and glutarylcarnitine (C5DC). No significant correlations were found between the content of C0 and 10 short-chain acetylcarnitines in dried blood spots and the body weight of newborns. Conclusion: The present study established concentrations of acylcarnitines and free carnitine that can be used as reference standards in a newborn screening program for inherited metabolic diseases in Kazakhstan.

Keywords: Newborn screening, Acylcarnitines, Free carnitine, Dried blood spots, Tandem mass spectrometry

Introduction

Inborn errors of metabolism (IED) belong to the group of so-called rare or orphan diseases. Their total frequency is low, which makes these diseases little studied and creates difficulties in diagnosis and treatment (Céspedes et al., 2017; Sarker et al., 2019). A major problem with these diseases is delay in diagnosis or

misdiagnosis due to lack of specialized laboratories, resulting in delayed or missing treatment. Early diagnosis and adequate treatment allow patients to lead almost normal lives, reducing the consequences or at least significantly reducing organ damage (Scolamieroet al., 2015).

Tandem mass spectrometry (MS/MS) is a technology that allows the simultaneous detection and identification of multiple analytes with high sensitivity, precision and accuracy, with high specificity (Mak et al., 2013). In plasma and whole blood, total carnitine is present in the form of free ester and acylcarnitine ester. total carnitine is present as free and acylcarnitine ester forms (Vieira -Neto et al., 2012). Acylcarnitine esters with short and medium chain length are formed in peroxisomes, then they are oxidized in mitochondria.

Acylcarnitine profiling is a powerful tool for diagnosis and neonatal screening of disorders of fatty acid oxidation and organic acid metabolism (Vieira- Neto et al., 2012). Age reference cutoff ranges for each analyte should be established for each population prior to screening/diagnosis of patients (Sarker et al., 2019). Threshold values for free carnitine and acylcarnitine esters in DBS have been published in the evaluation of newborn screening programs (Vieira Neto et al., 2012; He et al., 2021; Al-Riyami et al., 2022). However, these cutoffs are often very high percentiles (eg, 99.5th or 99.98th), which do not necessarily indicate reference intervals that can be used for diagnosis in neonates with suspected IEM. Some studies have established reference intervals of 5.0 to 95.0 for acylcarnitines in dried blood spots (Cavedon et al., 2005).

Identification of normal levels of acylcarnitine concentrations is necessary to use them as a reference for establishing a metabolic screening program for newborns (Céspedes et al., 2017). Some researchers use umbilical cord blood to determine reference intervals for acylcarnitines and create metabolic profiles (Walter et al., 2009; Vieira Neto et al., 2012), however, most define reference intervals in blood collected during the first three days of life from the heel of a newborn. Naturally, there will be significant differences between the acylcarnitine profiles of umbilical blood and those of blood collected from neonates after birth from the heel due to adaptation from a continuous supply of glucose in utero to a neonatal diet based on breast milk and therefore fat as a source of nutrition (Walter et al., 2009; Vieira -Neto et al., 2012).

Currently in Kazakhstan there are no developed reference intervals for the concentrations of acylcarnitines in dried blood spots for different age groups of the child population, including newborns. We initiated selective screening to obtain data on the frequency of IEM in children at risk in Western Kazakhstan. From October 2022 to December 2024, the incidence of 37 inborn errors of metabolism including amino acid disorders (AAD), organic acidemias (OA) and fatty acid oxidation disorders (FAOD) was assessed using LC-MS/MS technology in a group of high-risk children. The results of selective screening tests in different age groups of children examined should be interpreted by comparison with reference values and/or threshold levels established for these groups.

Therefore, one of the objectives of this study is to establish reference intervals for the concentration of acylcarnitines in dried blood spots in newborn children of Western Kazakhstan. Due to the urgent need for a highly sensitive diagnostic method and effective screening for IEM in Western Kazakhstan, this study focused on establishing the concentration values of free carnitine and short chain acylcarnitines in dried blood spot samples of Western Kazakhstan newborns using MS/MS technology.

Study Objectives

To establish reference values for free carnitine(C0) and short chain acylcarnitines (ACs) concentrations in samples of DBS from newborns in Western Kazakhstan using LC-MS/MS (liquid chromatography-tandem mass spectrometry) technology.

Tasks

1. To set reference ranges of C0 and short chain ACs concentrations in samples of DBS of 250 newborns of Western Kazakhstan aged 1-3 days using LC-MS/MS technology.
2. To evaluate factors that may affect C0 and short chain ACs levels.
3. To compare findings of the determined analytes in newborns of Western Kazakhstan DBS with the results of previously published studies in other populations.

Methods

Data Sources

The data of this study were obtained during the examination of 250 healthy newborns aged 1-3 days to establish reference values of C0 and short chain ACs (Figure 1).

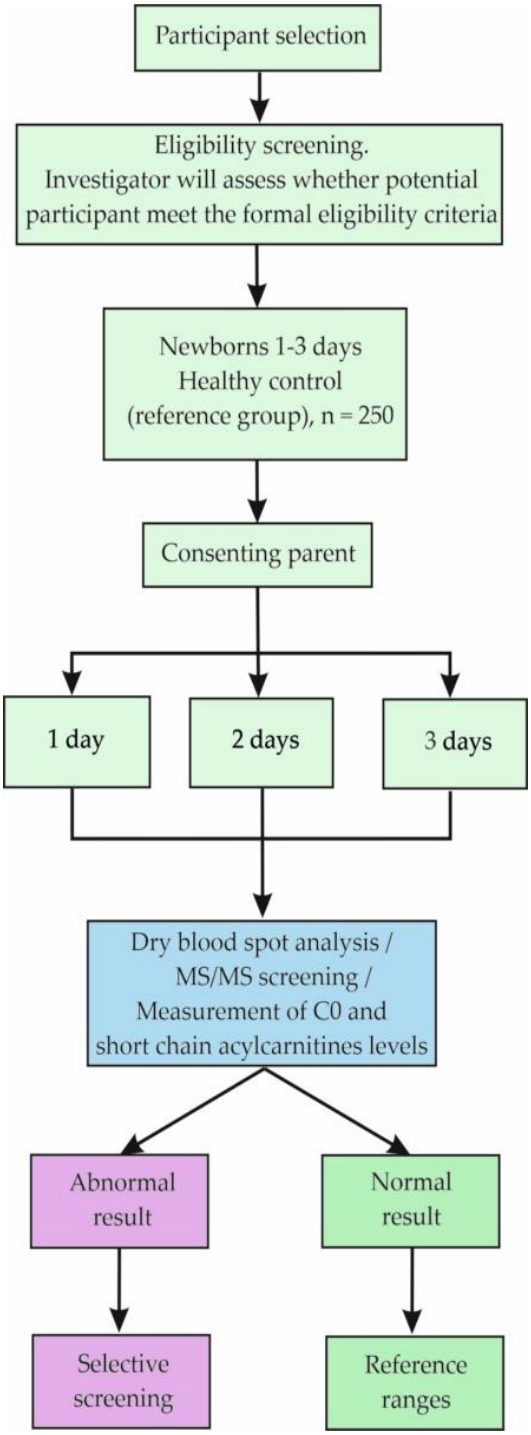

Figure 1. Study flowchart

The study was approved by the Bioethics Committee of the West Kazakhstan Marat Ospanov Medical University (Ref. No. 7, 09/09/2020.) Written informed consent (IS) was obtained from the parents and/or legal guardians of children after birth to collect a DBS sample. Demographic and anthropometric data of newborns are presented in Table 1.

Table 1. Demographic and anthropometric data of study participants

	Healthy newborns (n=250)			
	Group A 1 day (n=36)	Group B 2 days (n=116)	Group C 3 days (n=98)	The whole sample
Weight in grams, Median (IQR)	3434 (3180;3560)	3600 (3300;3833)	3615 (3470;3860)	3560 (3298;3830)
Gender				
Male, n, %	16 (44,4 %)	63 (54,3 %)	44 (44,9 %)	123 (49,2 %)
Female, n, %	20 (55,6 %)	53 (45,7 %)	54 (55,1 %)	127 (50,2 %)
Geographic distribution				
Urban population, n, %	22 (61,1 %)	68 (58,6 %)	54 (55,1 %)	144 (57,6 %)
Rural population, n, %	14 (38,9 %)	48 (41,4 %)	44 (44,9 %)	106 (42,4 %)

Criteria for Inclusion in the Study

Pediatricians examine all children in this study to ensure they do not suffer from any disorder or chronic disease. Healthy male and female newborns born after an uncomplicated pregnancy and vaginal delivery should have a body weight of 2500–4000 g, gestational age of 37–42 weeks, and an APGAR score greater than 7 in 10 minutes after birth. None should be diagnosed with birth asphyxia, defined as an Apgar score ≤6 at 5 min. All newborns must be breastfed, and their mothers must be healthy between 24 and 36. They must not have any food restrictions (vegetarian, vegan, etc.). Echograms of the placenta and fetus, as well as laboratory tests, should be normal throughout pregnancy.

Mass Spectrometry Analysis

Specimen Collection and Storage

Neonatal whole blood samples were collected from infants no earlier than 3 hours after feeding by heel prick using a heel stick. Five drops of whole blood (each ~75 µl) were applied to Guthrie cards, Ahlstrom 226 filter paper, and PerkinElmer 226 Five-Spot Card (PerkinElmer Health Sciences, Greenville, USA) to form dried blood spots (DBSs) for LC-MS/MS analysis. Samples were dried for 4 hours at room temperature and then stored at 4°C in labeled individual zip-lock plastic envelopes with desiccants until analyzed by LC-MS/MS. Samples were sent to the laboratory within five days. In the case of long-term storage of samples, it was carried out at a temperature of –20°C.

Specimen Preparation and LC-MS/MS Analysis

The Neobase2 TM Non-derivatized MSMS kit (PerkinElmer, Wallac Oy, Turku, Finland) will be used to quantify free carnitine, 10 short chain acylcarnitines in dried blood spots according to the manufacturer's instructions. Vial with lyophilized isotope-labeled internal standards (IS) containing 2H9-free carnitine (C0 IS), 2H3-Acetylcarnitine (C2 IS), 2H3-Propionylcarnitine (C3 IS), 2H3-C4-Malonylcarnitine+3-Hydroxybutyrylcarnitine (C3DC/C4OH IS), 2H3-Butyrylcarnitine (C4 IS), 2H9-C5-Methylmalonylcarnitine+3-Hydroxyisovalerylcarnitine (C4DC/C5OH IS), 2H9-Isovalerylcarnitine (C5 IS), 2H9-C5-Tiglylcarnitine (C5:1 IS), 2H3-Glutarylcarnitine (C5DC IS), was being recovered by adding 1.4 ml of the extraction solution that is included in the Neobase 2 kit. The Extraction Working Solution (EWS) IS was prepared by diluting the recovered internal standards with the extraction solution of 1:100 (v/v).

DBS were analyzed using a Shimadzu LCMS-8050 Triple Quadrupole Mass Spectrometer (Shimadzu Corporation, Kyoto, Japan). Level I and Level II (low standard and high standard) dried blood drops were included with each assay lot of the Neobase2 TM Non-derivatized MSMS kit to monitor system accuracy and precision.

To analyze free carnitine and short chain acylcarnitines, stored DBS card samples are brought to room temperature (+18 to +25°C) before extraction. A 3.2 mm disc (equivalent to ~3.1 µl of whole blood) is punched out of one dried blood spot with a diameter of 3.2 mm using a Wallac DBS Puncher (PerkinElmer, Wallac Oy, Mustionkatu 6, FI-20750 Turku, Finland) into the well of the 96-well polystyrene U-bottom microplate supplied

with the Neobase2 TM Non-derivatized MSMS kit. After adding 125 μL of working extraction solution to each well of the microplate, the plate is covered with an adhesive aluminum film and incubated for 30 minutes at room temperature on a microplate shaker with a shaking speed of 650 rpm. After incubation, 100 μL of the supernatant is transferred to a new 96-well U-bottom microplate, covered with aluminum foil to reduce evaporation, and incubated for 1 hour. The plate is then placed into the Shimadzu LCMS-8050 Triple Quadrupole Mass Spectrometer autosampler, and 5 μL of supernatant is injected into the LCMS for analysis.

Metabolites to Measure

Free carnitine (C0), Acetylcarnitine (C2), Propionylcarnitine (C3), Malonylcarnitine+3-Hydroxybutyrylcarnitine (C3DC/C4OH), Butyrylcarnitine (C4), 2H9-C5-Methylmalonylcarnitine+3-Hydroxyisovalerylcarnitine (C4DC/C5OH), Isovalerylcarnitine (C5), Tiglylcarnitine (C5:1), Glutarylcarnitine (C5DC).

Statistical Analysis

Shapiro-Wilk and Kolmogorov-Smirnov tests were used to check the normality of the distribution. The data obtained in the study demonstrated that the distribution of free carnitine and short chain acylcarnitines in DBS differs from normal. Me (median) and quartiles (IQR interquartile range) were used for descriptive statistics of the samples. Nonparametric tests (Mann-Whitney U test, Kruskal-Wallis H test) were used to test differences in C0 and short chain ACs concentrations depending on various factors (gender, age, place of residence). Reference ranges in the group of healthy newborns aged 1-3 days were determined non-parametrically and corresponded to the 2.5-97.5th percentile of the experimental distribution. Considering the skewed distribution, correlations between body weight, age, and the concentration of C0 and short chain ACs in dry blood spots were performed using Spearman's test. Two-sided levels <0.05 are assumed to be statistically significant. Statistical analysis was done using the software IBM SPSS v. 23.0 (IBM, Armonk, NY, USA) and Statistica (StatSoft, Inc., Tulsa, OK, USA, v. 10).

Results and Discussion

Descriptive statistics and reference intervals for the concentrations of C0 and short chain ACs in whole blood of healthy newborns divided into subgroups according to age are presented in Table 2. For each analyte, the upper cut-off limit is set above the 97.5th percentile, while the lower limit is set below 2.5th percentile. Differences in the distribution of free carnitine and short chain acylcarnitines levels in DBS between groups of newborns aged 1, 2 and 3 days, determined using the Kruskal-Wallis test, are noted in Table 2. Statistically significant differences between age groups are noted in concentration malonylcarnitine (C3DC), butyrylcarnitine (C4), isovalerylcarnitine (C5) and glutarylcarnitine (C5DC) (Table 2). In addition, significant weak negative correlations with age were established for the concentrations in DBS of malonylcarnitine (C3DC), butyrylcarnitine (C4), isovalerylcarnitine (C5) and glutarylcarnitine (C5DC) (Table 3).

Significant differences between the groups of female and male newborns were established by the concentration of acetylcarnitine (C2), butyrylcarnitine (C4), and tiglylcarnitine (C5:1) in DBS (Table 3). In a study by Ruoppolo et al. (2015), who examined the metabolome of newborns, including blood acylcarnitine profiles, statistically significant differences were also found between male and female newborns in the level of free carnitine and short chain acylcarnitines in DBS.

Assessing the effect of newborn body weight on the level of free carnitine and short chain acylcarnitines, no significant correlations were found between the concentration of free carnitine and short chain acylcarnitines in dry blood spots and newborn body weight. Reference values for free carnitine and short chain acylcarnitines, like other blood metabolites, are highly dependent on various factors. such as genetic background, geographical location of the population, diet and age (Cavedon et al., 2005; Ruoppolo et al., 2015; Dogan et al., 2017; Sarker et al., 2019). We compared the results of measuring free carnitine and short chain acylcarnitines levels in DBS in newborns of western Kazakhstan with the results of previously published studies in other populations. A significant number of researchers confirm the relationship between gender and the level of certain acylcarnitines in DBS (Ruoppolo et al., 2015; He et al., 2021; Al-Riyami et al., 2022), but denies Céspedes et al. (2017).

The relationship between free carnitine and short chain acylcarnitines levels and birth weight was confirmed by Ruoppolo et al. (2015), Manta-Vogli et al. (2020), He et al. (2021), but is not confirmed by Céspedes et al. (2017).

Table 2. C0 and short chain ACs levels in dried blood spots of 250 healthy newborns aged 1-3 days in Western Kazakhstan

Amino acid, μmol/l		All children 1-3 days (n = 250)	Group A 1 day (n = 36)	Group B 2 days (n = 116)	Group C 3 days (n = 98)	Kruskal – Wallis *H* test	p-values
C0	Median	29.35	28.90	31.62	27.82	4,66	0.097
	Range	24.92;37.13	23.53;35.18	25.35;38.39	25.39;34.30		
	2.5th-97.5th	15.79-55.32	21.81-51.32	17.89-64.62	15.25-45.52		
C2	Median	33.54	31.99	33.89	33.54	0.917	0.623
	Range	26.44;40.38	23.90;38.78	26.38;42.34	27.41;39.49		
	2.5th-97.5th	13.20-61.58	19.68-49.75	10.53-68.94	15.87-56.46		
C3	Median	1.78	1.67	1.89	1.62	4.18	0.124
	Range	1.37;2.34	1.28;2.42	1.45;2.50	1.34;2.11		
	2.5th-97.5th	0.926-3.72	0.928-4.14	0.994-4.13	0.796-2.90		
C3DC	Median	0.157	0.184	0.158	0.148	8.31	0.016
	Range	0.130;0.185	0.138;0.206	0.131;0.187	0.124;0.167		
	2.5th-97.5th	0.080-0.255	0.082-0.271	0.071-0.246	0.084-0.255		
C4	Median	0.290	0.378	0.290	0.278	18.86	0.0001
	Range	0.244;0.364	0.285;0.454	0.240;0.369	0.231;0.330		
	2.5th-97.5th	0.160-0.607	0.191-0.690	0.146-0.771	0.160-0.467		
C4DC	Median	0.504	0.497	0.498	0.513	2.11	0.348
	Range	0.419;0.592	0.404;0.573	0.410;0.589	0.445;0.645		
	2.5th-97.5th	0.285-0.872	0.311-0.872	0.271-0.922	0.285-0.819		
C4OH	Median	0.147	0.154	0.142	0.149	1.89	0.388
	Range	0.115;0.175	0.122;0.178	0.106;0.171	0.115;0.177		
	2.5th-97.5th	0.066-0.283	0.075-0.327	0.047-0.286	0.066-0.253		
C5	Median	0.144	0.166	0.142	0.139	8.47	0.015
	Range	0.121;0.174	0.141;0.212	0.118;0.181	0.115;0.167		
	2.5th-97.5th	0.076-0.289	0.096-0.370	0.072-0.281	0.076-0.288		
C5:1	Median	0.060	0.059	0.063	0.059	5.80	0.055
	Range	0.046;0.069	0.044;0.073	0.048;0.076	0.043;0.067		
	2.5th-97.5th	0.021-0.093	0.005-0.080	0.024-0.131	0.026-0.082		
C5DC	Median	0.135	0.153	0.139	0.126	7.05	0.029
	Range	0.112;0.157	0.115;0.183	0.116;0.159	0.109;0.148		
	2.5th-97.5th	0.075-0.233	0.071-0.273	0.065-0.233	0.078-0.225		
C5OH	Median	0.240	0.249	0.273	0.249	4.50	0.105
	Range	0.220;0.278	0.228;0.278	0.214;0.274	0.223;0.282		
	2.5th-97.5th	0.170-0.349	0.164-0.336	0.163-0.333	0.197-0.352		

Table 3. Statistical analysis according to age (Spearman's correlation) and gender (Mann Whitney U test)

Analyte	Spearman correlation		Male N=123		Female N=127		p-values
	ρ	p-values	Median (μmol/L)	Range	Median (μmol/L)	Range	
C0	-0.096	0.065	30.35	17.36;59.01	28.09	15.58;50.68	0.061
C2	0.013	0.419	31.32	15.87;55.16	35.14	13.20;68.93	0.017
C3	-0.070	0.135	1.76	0.956;3.58	1.78	0.755;3.72	0.370
C3DC	-0.174	0.006	0.156	0.081;0.231	0.159	0.080;0.271	0.125
C4	-0.240	0.000	0.273	0.164;0.558	0.308	0.151;0.690	0.020
C4DC	0.089	0.163	0.502	0.285;0.883	0.512	0.290;0.872	0.751
C4OH	-0.006	0.927	0.142	0.065;0.249	0.151	0.071;0.283	0.460
C5	-0.157	0.013	0.135	0.083;0.274	0.148	0.073;0.289	0.395
C5:1	0.071	0.262	0.057	0.015;0.088	0.064	0.031;0.105	0.003
C5DC	-0.169	0.007	0.137	0.075;0.216	0.133	0.071;0.237	0.786
C5OH	0.072	0.260	0.243	0.170;0.352	0.293	0.180;0.336	0.829

Conclusion

The present study established age- and sex-specific concentrations of free carnitine and short-chain acylcarnitines that can be used as reference standards in a newborn screening program for inherited metabolic diseases in Kazakhstan.

Conflicts of Interest

The authors declare no conflict of interest.

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Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS Journal belongs to the authors.

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