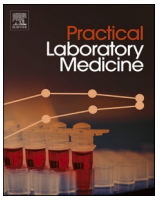
Practical Laboratory Medicine 33 (2023) e00305

Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm



Age and gender-specific reference intervals for a panel of lysophosphatidylcholines estimated by tandem mass spectrometry in dried blood spots

Archana Natarajan [a], Rita Christopher [a,*]

[a] Metabolic Laboratory, Department of Neurochemistry, National Institute of Mental Health and Neuro Sciences (NIMHANS), Bengaluru, India

ARTICLE INFO

Keywords:
Reference intervals
Lysophosphatidylcholines
Dried blood spots
Tandem mass spectrometry

ABSTRACT

Background and objectives: Very long-chain fatty acyl-lysophosphatidylcholines (VLCFA-LysoPCs) are measured in dried blood spots (DBS) for identifying X-linked adrenoleukodystrophy (X-ALD) and other inherited peroxisomal disorders. Our study aimed to establish age- and gender-specific reference intervals for a panel of LysoPCs measured by tandem mass spectrometry in DBS.

Methods: LysoPCs (26:0-, 24:0-, 22:0- and 20:0-LysoPCs) were estimated by flow injection analysis-tandem mass spectrometry (FIA-MS/MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods in 3.2 mm blood spots of 2689 anonymized, putative normal subjects (1375 males, and 1314 females) aged between 2 days and 65 years. Samples were divided into groups: Neonates (0–1month), Infants (>1m-1year), Children and Adolescents (>1–18years), and Adults (>18years). Reference intervals were determined using the percentile approach and represented as the median with the 1st and 99th percentile lower and upper limits.

Results: The percentage coefficient of variation (CV) for repeatability assays of internal and external quality control samples were within acceptable limits. Significant differences ($P < 0.0001$, $P < 0.01$) were observed in the concentrations of 26:0-, 24:0-, 22:0- and 20:0-LysoPCs and their ratios, 26:0/22:0-, 24:0/22:0-, 26:0/20:0-and 24:0/20:0-LysoPC in neonates and infants when compared to children, adolescents, and adults. Levels of 26:0-, 24:0- and 22:0-LysoPCs decreased, whereas 20:0-LysoPC increased with age. There were no significant gender-based differences in the concentration of LysoPCs.

Conclusion: We established age- and gender-specific reference intervals for a panel of LysoPCs in DBS. These reference values would be helpful when interpreting LysoPC values in DBS during screening for X-ALD and other peroxisomal disorders.

1. Introduction

Lysolecithins or lysophosphatidylcholines (LysoPCs/LPCs) are the most commonly occurring bioactive lysophospholipids. They are endogenously synthesised by the hydrolysis of phosphatidylcholines (PC) catalysed by enzymes like phospholipase (PLA_2), lecithin-cholesterol acyltransferase (LCAT), which cleaves PC present in lipoproteins (high density lipoprotein, HDL) at the C-2 or sn-2 position, thereby transferring a fatty acid to cholesterol and esterifying it. Endothelial or hepatic lipases also catalyse the formation of

* Corresponding author. Department of Neurochemistry National Institute of Mental Health and Neuro Sciences (NIMHANS), Bengaluru, 560029, India.

E-mail address: rita.nimhans@yahoo.com (R. Christopher).

https://doi.org/10.1016/j.plabm.2022.e00305
Received 19 May 2022; Received in revised form 3 November 2022; Accepted 16 December 2022
Available online 24 December 2022

Abbreviations

X-ALD	X-linked adrenoleukodystrophy
DBS	dried blood spot
PC	phosphatidylcholine
LysoPC	lysophosphatidylcholine
LCAT	lecithin-cholesterol acyltransferase
LPCAT	lysophosphatidylcholine acyl transferase
VLCFA-LysoPC	very long-chain fatty acyl-lysophosphatidylcholine
ZSD	Zellweger spectrum disorders
ACOX1	acyl CoA oxidase 1
AGS	Aicardi Goutieres Syndrome
RIs	reference intervals
FIA-MS/MS	flow injection analysis-tandem mass spectrometry
ESI	electrospray ionization
LC-MS/MS	liquid chromatography-tandem mass spectrometry
CDC	Centers for Disease Control and Prevention
NBS	newborn screening
NSQAP	Newborn Screening Quality Assurance Program

LysoPCs specifically by cleaving the PCs at the C-1 position as they have sn-1 phospholipase activity [1–4]. Very long-chain fatty acyl-lysophosphatidylcholines (VLCFA-LysoPCs) are LysoPCs with VLCFAs of variable lengths in the C-1/sn-1 position or at times in the C-2/sn-2 position. The R-group here varies in chain length (from C22:0) and saturation and this helps in distinguishing different VLCFA-LysoPCs species from each other [1]. Studies have shown that VLCFA-LysoPCs, specifically C26:0-LysoPC, is elevated in patients with X-linked adrenoleukodystrophy (X-ALD) and other peroxisomal disorders like Zellweger spectrum disorders (ZSD), acyl CoA oxidase 1 (ACOX1) deficiency, multifunctional protein (HSD17B4) defect and Aicardi Goutieres Syndrome (AGS) [5–10]. LysoPCs are generally measured by tandem mass spectrometry using the flow injection analysis (FIA-MS/MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques for identifying these disorders [6–21].

Recently, newborn screening (NBS) panels have included 26:0- and 24:0-LysoPC to screen for X-ALD along with the other inborn errors of metabolism [20,22,23]. To differentiate X-ALD cases from the healthy population, age and gender-specific reference intervals (RIs) and appropriate cut-offs specific to the population of that region are essential [24]. Although there are studies describing reference intervals for LysoPCs extracted from dried blood spots (DBS), there is no systematic study that has established age and gender-specific reference intervals in DBS for 26:0-, 24:0-, 22:0- and 20:0-LysoPCs and their ratios in the pediatric and adult population [5–9,11–15,21]. We therefore sought to establish reference intervals for a panel of LysoPCs (26:0-, 24:0-, 22:0- and 20:0-LysoPC) and their ratios (26:0/22:0-, 24:0/22:0-, 26:0/20:0- and 24:0/20:0-LysoPC) in DBS measured by FIA-MS/MS and LC-MS/MS methods in male and female neonates, infants, children, adolescents and adults, in India.

2. Materials and methods

2.1. Ethical approval

This study was carried out according to the national regulations, institutional policies and as per the tenets of the Helsinki Declaration (revised, 2013). The institutional ethics committee of the National Institute of Mental Health and Neuro Sciences (NIMHANS), Bengaluru, India, reviewed and approved the study protocol.

2.2. DBS samples

De-identified, left-over DBS of 2689 presumed normal subjects (1375 males, and 1314 females) aged between 2 days and 65 years, who were referred to the Metabolic Laboratory, Department of Neurochemistry, NIMHANS, Bengaluru, India, for routine testing, were used to establish the RIs by the FIA-MS/MS (n = 1074) and LC-MS/MS (n = 1615) methods. Subjects diagnosed with neurological disorders, inherited metabolic disease or with any abnormalities in brain or spine MRI were excluded from the study. Informed consent was obtained from the parents of children under 13 years and adult patients, while child assent was taken from children between 13 and 18 years before sample collection.

The samples were divided into the following four groups based on their age and gender; Group 1 – Neonates (0–1 month), Group 2 – Infants (above 1 month–1 year), Group 3 – Children (toddlers, pre-school and school-going children) and Adolescents (above 1 year - 18 years) and Group 4 – Adults (above 18 years). This age classification was done according to the National Institute of Child Health and Human Development (NICHD) guidelines [25,26] and Center for Drug Evaluation and Research, FDA [27] guidelines referenced by Job et al. in their study [28]. Neonates considered in the group were full-term with birth weight ≥2800g. All blood samples were collected on S&S 903 (*Schleicher & Schuell*) filter paper cards by heel-prick in neonates and by finger prick in infants, children,

adolescents, and adults.

2.3. Materials

Mass Spectrometry (MS)-grade solvents and additives like methanol, acetonitrile, isopropanol, formic acid, and ammonium acetate were sourced from Merck, Sigma-Aldrich Corp. (St. Louis, MO, USA). 26:0-, 24:0-, 22:0- and 20:0-LysoPC standards and an isotopically labelled internal standard (IS) of 26:0-LysoPC (26:0-d4-LysoPC) were procured from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Barnstead GenPure Pro water purification system (Thermo Fischer Scientific, Waltham, MA, USA) was used to generate deionized water. Microtitre 96-well plates, PEEK tubing, and columns were purchased from Waters Corporation (Milford, MA, USA). Newborn Screening Quality Assurance Program (NSQAP) for X-ALD conducted by Newborn Screening and Molecular Biology Branch, Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA, provided us enriched DBS cards for proficiency testing and external quality control.

2.4. Methods

2.4.1. Preparation, storage, and processing of DBS samples

The DBS samples were stored at −80 °C in sealed pouches with desiccants where humidity was maintained less than 30% until analysis. After the cards were thawed, 3.2 mm spots were punched from each card into a 96-well plate kept in an automated DBS puncher (PerkinElmer, Waltham, MA, USA). 150 μl of extraction solution containing internal standard (26:0-d4-LysoPC) of concentration 0.39 μmol/L reconstituted in absolute methanol was added to each well. The plate was covered with a polypropylene mat cap and kept in a thermomixer for 1 h at 26 °C and a mixing speed of 450 rpm.

2.4.2. LysoPC analysis

A panel of LysoPCs (26:0-, 24:0-, 22:0- and 20:0-LysoPC) were analysed by the FIA-MS/MS and the LC-MS/MS methods previously standardized in our laboratory [18,19]. Sample extract of 10 μl each, was injected into the Xevo TQD MS/MS (Waters Corporation, Milford, MA, USA) from an ACQUITY UPLC sample manager (Waters Corporation, Milford, MA, USA), directly for the FIA-MS/MS analysis and via a UPLC BEH Phenyl 1.7 μm*2.1*50 mm column maintained at 40 °C (ACQUITY) for the LC-MS/MS analysis. An isocratic flow of mobile phase mixture containing 80% (50% acetonitrile: 50% methanol) and 20% (2 mM ammonium acetate in water with 0.1% formic acid) was used to analyse each sample in 1.5 min by the FIA-MS/MS analysis. LC-MS/MS analysis required 6 min/sample with 0.5 mL/min gradient flow using mobile phases, 2 mM ammonium acetate in water, and a (45:45:10) mixture of acetonitrile: methanol: isopropyl alcohol (Supplementary Table 4). The MS/MS conditions for both the methods were set in the Electrospray ionization positive (ESI +) ion and multiple reaction monitoring (MRM) mode with 150 °C as source temperature and 600 °C as desolvation temperature. The mass transitions (m/z) for 26:0-d4-LysoPC and 26:0-, 24:0-, 22:0- and 20:0-LysoPCs were 640.65 > 104.09, 636.65 > 103.96, 608 > 104, 580 > 104.06 and 552.41 > 104.06, respectively. NeoLynx and TargetLynx applications of the MassLynx 4.1.1 software (Waters Corporation, Milford, MA, USA) were used to quantify the LysoPCs in micromoles/litre (μmol/L).

2.4.3. Quality control

Internal quality control (IQC) was monitored using spiked dried blood spots at concentrations 0.01,0.05,0.1,0.25 0.5 and 1 μg/mL (range: 0.079–0.362 μmol/L) containing 26:0-, 24:0-, 22:0- and 20:0-LysoPC mixture prepared in-house. For external quality control (EQC), enriched DBS specimens from CDC, Atlanta, containing 26:0- and 24:0-LysoPCs of an unknown concentration for proficiency testing and quality control samples containing C26:0-, C24:0-, C22:0- and C20:0-LysoPCs in the following concentrations: 0 μmol/L (un-enriched), 1 μmol/L and 5 μmol/L, were analysed. Accuracy and repeatability (inter-day intra-day) of both the MS/MS methods in measuring the LysoPCs were determined.

2.5. Statistical analysis

Graph Pad Prism 5.1(California, USA) and SPSS 17.0 (IBM Corporation, NY, USA) software were used for the statistical analyses. Since data distribution for the analytes was skewed and did not follow Gaussian distribution, a non-parametric percentile approach was applied to establish the reference intervals. Extreme outliers were removed using the Kolmogorov-Smirnov (KS) test, and RIs were represented as a box-whisker plot between the 1st and 99th percentile of the analytes measured in our population. RIs for LysoPCs and their ratios were established according to previously published studies [13,15,21]. The values below and above the 1st and the 99th percentile range that were flagged as outliers were removed, respectively. The median concentration of the LysoPCs and their ratios were compared between the four age groups in males and females using the Kruskal-Wallis Independent sample test (non-parametric one-way Analysis Of Variance- ANOVA) followed by *post-hoc* Dunn's multiple comparison test. Mann Whitney-U test (Non-parametric unpaired *t*-test) was used to compare the median concentration of the LysoPCs in males and females of each group. The significance value was set to $P < 0.05$. Accuracy (recovery) and coefficient of variation (CV) of repeatability assays were expressed as percentages. Bland-Altman analysis was performed to find out the agreement between the FIA-MS/MS and the LC-MS/MS methods.

3. Results

3.1. Quality control and method comparison

The accuracy and precision of the FIA-MS/MS and LC-MS/MS methods monitored as a part of IQC and EQC were within acceptable limits. The proficiency testing assessment for results reported by our method was 100% satisfactory. The details are represented in Supplementary Tables 1, 2, and 3.

The agreement between the two methods was represented by Bland-Altman Plots. The concentrations of 20:0-LysoPC, 22:0-LysoPC, 24:0-LysoPC and 26:0-LysoPC were found to be higher by 0.09. 0.22, 0.17, 0.17 μmol/L when measured by the FIA-MS/MS method in comparison to the LC-MS/MS method. This data has been represented in Supplementary Fig. 4.

3.2. Discussion RIs for LysoPCs measured by the FIA-MS/MS method

A total of 1074 putative normal samples (555 males, 519 females) were included in the study. The medians and reference intervals (1st and 99th percentiles) of LysoPCs and their ratios in the various age groups are represented in Table 1 and Fig. 1, Supplementary Fig. 1. Kruskal-Wallis Test with Dunn's *post-hoc* test was used to check for differences in the concentration of LysoPCs and their ratios in males and females according to age, and Mann Whitney-U test was used to check the gender-based variations in the LysoPCs and their ratios in each age group.

The concentrations of LysoPCs were not significantly different between males and females except for 20:0-LysoPC, which measured higher in males than females (0.38 μmol/L vs. 0.33 μmol/L, $P = 0.0001$) in the adult population. Similarly, no significant differences were observed in the LysoPC ratios except 24:0/22:0-LysoPC ratio, which was higher in females in comparison to male (1.93 vs. 1.78; $P = 0.0003$) children and adolescents considered in our study.

Median concentrations of 26:0-LysoPC and 26:0/22:0-LysoPC ratio was significantly higher in neonates ($P < 0.0001$) when compared with infants, children and adolescents while in adults, concentrations of 26:0-LysoPC and 26:0/22:0-LysoPC ratio were slightly higher ($P<0.01$) than that of neonates. 26:0/20:0-LysoPC was significantly higher in neonates ($P < 0.0001$) in comparison to infants, children & adolescents and adults.

Median concentrations of 24:0-LysoPC, 24:0/22:0-LysoPC, and 24:0/20:0-LysoPC were significantly higher in neonates ($P <$

Table 1
Distribution of LysoPCs and their ratios measured by the FIA-MS/MS method in different groups based on age and gender.

LysoPCs measured in DBS	[a]Median and reference interval (μmol/L) [b]Neonates		[a]Median and reference interval (μmol/L) [c]Infants		[a]Median and reference interval (μmol/L) [d]Children and Adolescents		[a]Median and reference interval (μmol/L) [e]Adults	
	M (n = 129)	F (n = 125)	M (n = 126)	F (n = 120)	M (n = 158)	F (n = 151)	M (n = 142)	F (n = 123)
26:0-LysoPC	0.21 (0.12–0.33)	0.21 (0.13–0.38)	0.16 (0.10–0.34)	0.18 (0.10–0.34)	0.14 (0.10–0.29)	0.15(0.10.-0.21)	0.25 (0.13–0.46)	0.24 (0.12–0.41)
24:0-LysoPC	0.38 (0.20–0.69)	0.39 (0.19–0.73)	0.29 (0.16–0.57)	0.28 (0.14–0.47)	0.26 (0.15–0.46)	0.28 (0.17–0.46)	0.32 (0.21–0.48)	0.32 (0.19–0.45)
22:0-LysoPC	0.15 (0.08–0.29)	0.16 (0.10–0.45)	0.15 (0.09–0.32)	0.15 (0.08–0.26)	0.14 (0.09–0.24)	0.14 (0.09–0.30)	0.19 (0.11–0.32)	0.17 (0.12–0.26)
20:0-LysoPC	0.25 (0.10–0.52)	0.27 (0.09–0.55)	0.31 (0.16–0.61)	0.28 (0.12–0.46)	0.28 (0.14–0.55)	0.29 (0.17–0.49)	0.38 (0.19–0.61) (***)	0.33 (0.19–0.54)
LysoPC ratios measured in DBS	[a]Median and reference interval		[a]Median and reference interval		[a]Median and reference interval		[a]Median and reference interval	
26:0/22:0-LysoPC	1.29 (0.73–2.00)	1.31 (0.56–2.56)	1.11 (0.58–1.82)	1.16 (0.65–2.00)	0.94 (0.50–1.45)	1.06 (0.48–1.75)	1.30 (0.79–1.85)	1.36 (0.74–2.24)
24:0/22:0-LysoPC	2.48 (1.67–3.24)	2.42 (1.05–3.53)	1.88 (1.27–2.56)	2.00 (1.31–2.43)	1.78 (1.17–2.36) (***)	1.93 (1.40–2.40)	1.81 (1.12–2.15)	1.81 (1.27–2.13)
26:0/20:0-LysoPC	0.83 (0.44–1.38)	0.80 (0.43–2.19)	0.52 (0.32–1.11)	0.59 (0.30–1.31)	0.50 (0.31–0.93)	0.50 (0.29–0.95)	0.63 (0.41–1.21)	0.67 (0.42–1.24)
24:0/20:0-LysoPC	1.60 (0.96–2.58)	1.52 (0.73–3.19)	0.94 (0.64–1.50)	1.00 (0.56–1.69)	0.93 (0.62–1.56)	0.94 (0.61–1.48)	0.87 (0.65–1.29)	0.90 (0.64–1.33)

M = Males; F= Females; n = No. of samples; FIA-MS/MS- Flow injection analysis tandem mass spectrometry; ***- $P < 0.0001$.
LysoPCs-lysophosphatidylcholines.
[a] Median (Reference intervals are expressed as the 1st and the 99th percentile).
[b] Neonates - 0–1month.
[c] Infants - >1 month-1 year.
[d] Children and Adolescents - >1 year-18 years.
[e] Adults - >18 years.

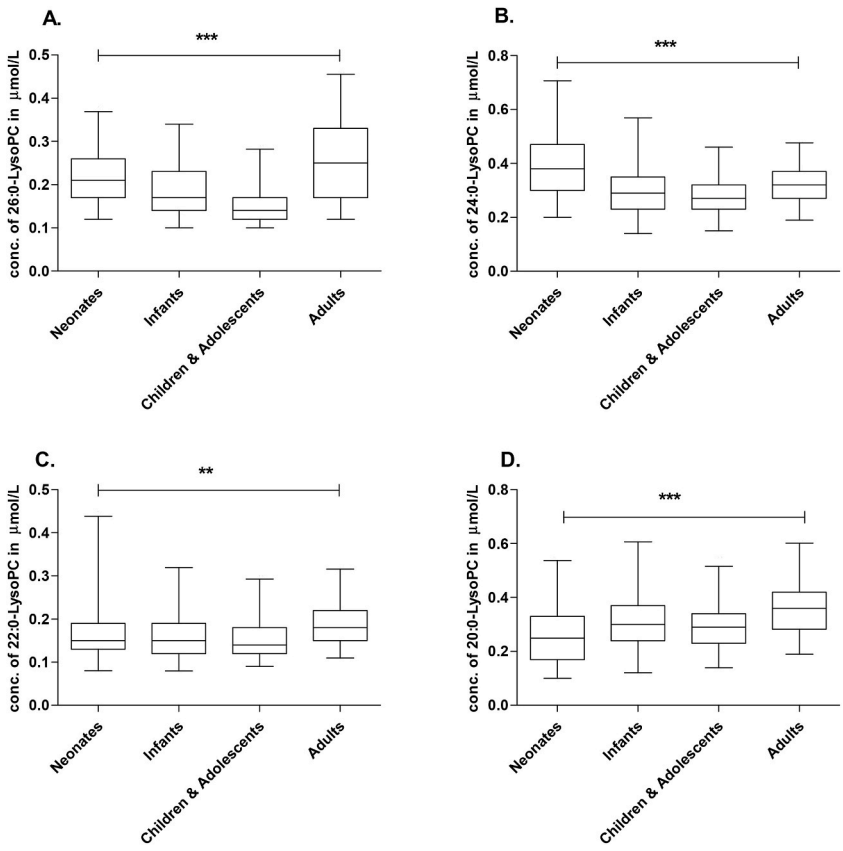


Fig. 1. Box and whisker plot for reference intervals of (A) 26:0-LysoPC, (B) 24:0-LysoPC, (C) 22:0-LysoPC and (D) 20:0-LysoPC measured in DBS by the FIA-MS/MS method in males and females divided across different age groups.
conc. – concentration; DBS- dried blood spots; FIA-MS/MS- Flow injection analysis tandem mass spectrometry; **- $P < 0.01$, ***- $P < 0.0001$ The lower and the upper end of the whiskers represent the 1st and the 99th percentile with the central median value (50th percentile) bound by 25th and 75th percentile values.

0.0001) in comparison to infants, children & adolescents, and adults.

The concentration of 22:0-LysoPC was significantly higher in neonates and infants ($P<0.01$) in comparison to children & adolescents but lower ($P < 0.01$) when compared with the adult group.

On the contrary, 20:0-LysoPC concentration was low in neonates ($P < 0.0001$) in comparison to infants, children & adolescents, and adults.

3.3. RIs for LysoPCs measured by the LC-MS/MS method

A total of 1615 putative normal samples (820 males, 795 females) were included for analysis. The medians and reference intervals (1^{st} and 99^{th} percentiles) of LysoPCs and their ratios in the various age groups are represented in Table 2 and Fig. 2, Supplementary Fig. 2. Kruskal-Wallis Test with Dunn's *post-hoc* test was used to check for differences in the concentration of LysoPCs and their ratios in males and females with respect to age, and Mann Whitney-U test was used to check the gender-based variations in the LysoPCs and their ratios in each age group.

The concentrations of LysoPCs and their ratios were not significantly different between males and females when measured by this method also. But, 26:0/22:0-LysoPC was significantly higher in males in comparison to females (1.00 vs. 0.80, $P < 0.0001$) in the infant group, while among children and adolescents, the same ratio was higher in females in comparison to males (1.0 vs. 0.80, $P < 0.0001$). 24:0/22:0-LysoPC was higher in males compared to females (2.00 vs. 1.67, $P = 0.0014$) in our population's infant group.

Median concentration of 26:0-LysoPC, 26:0/22:0-LysoPC, and 26:0/20:0-LysoPC were higher in neonates ($P < 0.01$) than infants, children & adolescents and adults.

A significantly higher concentration of 24:0-LysoPC was observed in neonates ($P < 0.01$) in comparison to infants, children & adolescents but almost the same in adults. 24:0/20:0-LysoPC median concentration was higher in neonates ($P < 0.01$) when compared with infants, children& adolescents and adults. 24:0/22:0-LysoPC concentration was significantly lower ($P < 0.0001$) in neonates and adults compared to infants, children & adolescents.

A significantly higher concentration of 22:0-LysoPC was observed in neonates ($P < 0.01$) in comparison to infants, children, and

Table 2
Distribution of LysoPCs and their ratios measured by the LC-MS/MS method in different groups based on age and gender.

LysoPCs measured in DBS	[a]Median and reference interval (μmol/L) [b]Neonates		[a]Median and reference interval (μmol/L) [c]Infants		[a]Median and reference interval (μmol/L) [d]Children and Adolescents		[a]Median and reference interval (μmol/L) [e]Adults	
	M (n = 125)	F (n = 128)	M (n = 207)	F (n = 201)	M (N = 299)	F (n = 296)	M (n = 189)	F (n = 170)
26:0-LysoPC	0.05 (0.03–0.09)	0.05 (0.03–0.10)	0.03 (0.02–0.09)	0.04 (0.02–0.11)	0.03(0 01–0.05)	0.03(0.02.-0.07)	0.03 (0.01–0.07)	0.04 (0.02–0.06)
24:0-LysoPC	0.09 (0.05–0.15)	0.10 (0.04–0.15)	0.07 (0.03–0.14)	0.07 (0.04–0.14)	0.07 (0.04–0.15)	0.07 (0.04–0.16)	0.10 (0.05–0.17)	0.09 (0.05–0.17)
22:0-LysoPC	0.05 (0.02–0.08)	0.05(0.03–0.09)	0.04 (0.02–0.12)	0.05 (0.02–0.10)	0.03 (0.02–0.08)	0.03 (0.02–0.10)	0.05 (0.02–0.11)	0.06 (0.02–0.11)
20:0-LysoPC	0.13 (0.05–0.26)	0.16 (0.05–0.33)	0.16 (0.04–0.31)	0.18 (0.06–0.42)	0.14 (0.05–0.27)	0.13 (0.05–0.32)	0.18 (0.09–0.35)	0.17 (0.08–0.33)
LysoPC ratios measured in DBS	[a]Median and reference interval		[a]Median and reference interval		[a]Median and reference interval		[a]Median and reference interval	
26:0/22:0-LysoPC	1.00 (0.67–2.00)	1.00 (0.62–1.78	1.00 (0.40–2.00) (***)	0.80 (0.33–1.71)	0.80 (0.33–2.00) (***)	1.00 (0.33–3.00)	0.67 (0.25–1.50	0.67 (0.32–1.60)
24:0/22:0-LysoPC	1.82 (1.33–3.20)	1.86 (1.00–3.20)	2.00 (1.00–4.00) (**)	1.67 (0.86–4.00)	2.25 (1.33–4.00)	2.44 (1.43–4.50)	1.78 (1.00–4.00)	1.67 (1.07–3.30)
26:0/20:0-LysoPC	0.42 (0.2–1.05)	0.35 (0.14–0.78)	0.24 (0.09–0.75)	0.22 (0.08–0.50)	0.21 (0.09–0.50)	0.21 (0.06–0.50)	0.20 (0.08–0.42)	0.20 (0.09–0.47)
24:0/20:0-LysoPC	0.72 (0.38–1.40)	0.62 (0.31–1.29)	0.47 (0.21–1.00)	0.44 (0.19–0.89)	0.56 (0.27–1.00)	0.57 (0.24–1.00)	0.56 (0.32–0.93)	0.55 (0.31–1.00)

M = Males; F= Females; n = No. of samples; LC-MS/MS- Liquid chromatography tandem mass spectrometry; **- $P < 0.01$, ***- $P < 0.0001$. LysoPCs-lysophosphatidylcholines.

[a] Median (Reference intervals are expressed as the 1st and the 99th percentile).
[b] Neonates - 0–1month.
[c] Infants - >1 month-1 year.
[d] Children and Adolescents - >1 year-18 years.
[e] Adults - >18 years.

adolescents but almost the same in adults.

The analyte, 20:0-LysoPC showed a slow increase ($P < 0.01$) in the concentration from neonates < infants < and adults.

4. Discussion

In X-ALD, very long-chain fatty acids (VLCFAs), ≥ C22, accumulate in the blood because of a deficiency of a peroxisomal ABC transport protein required for the transport of VLCFA, due to mutations in the *ABCD1* gene [22,29]. Affected persons may be asymptomatic or have varied clinical presentations across different ages (2.5 years to over 60 years) in males with the adrenocortical insufficiency presenting as early as 5 weeks after birth [22,29–33]. Affected females can also be symptomatic, typically above 30 years of age, the earliest reported symptomatic case being a 7-year old girl. It has been reported that over 80% of females with X-ALD will develop signs of neurological dysfunction like having a progressive spinal cord problem by the age of 60 years. Women with ALD have less than 1% chances of being affected by adrenal dysfunction or cerebral ALD [22,29–32].

Measurement of 26:0-LysoPC and 24:0-LysoPC in DBS by tandem mass spectrometry has been included [20,23] or is being considered for inclusion in newborn screening panels of many countries [16,17,21]. Consequently, appropriate age and gender-specific reference intervals need to be established. In our study, we estimated a panel of LysoPCs by the FIA-MS/MS and the LC-MS/MS methods, which are widely used as a part of the two-tier screening strategy for biochemically identifying X-ALD. The FIA-MS/MS method with an analysis time of 1.5 min/sample is a simple, fast and high-throughput method suitable for mass screening of X-ALD but less sensitive and specific in comparison to the LC-MS/MS method which takes 6 min/sample and utilizes a column to separate the analytes based on their retention time. False positives are reported by the FIA-MS/MS method, while the LC-MS/MS is 100% sensitive and specific in the identification of X-ALD.

We have established the reference intervals of LysoPCs and their ratios across all age groups: neonates, infants, children (toddlers, pre-school and school-going children) & adolescents and adults in males and females of our population. Since significant metabolic changes occur from the neonatal period to adulthood, which involves dynamic changes in their growth and feeding patterns, we divided our study population into different age groups [25–28,34]. Previous reports on the concentration of some of the LysoPCs in various populations are detailed in Table 3.

In our study we did not see any significant difference in the concentration of LysoPCs between males and females, except for 20:0-LysoPC and 26:0/22:0-LysoPC, 24:0/22:0-LysoPC ratios, which were higher in males. Although statistically significant, the magnitude of variation was minimal. So the data generated for males and females were taken together for representation in Figs. 1 and 2 and Supplementary Figs. 1 and 2.

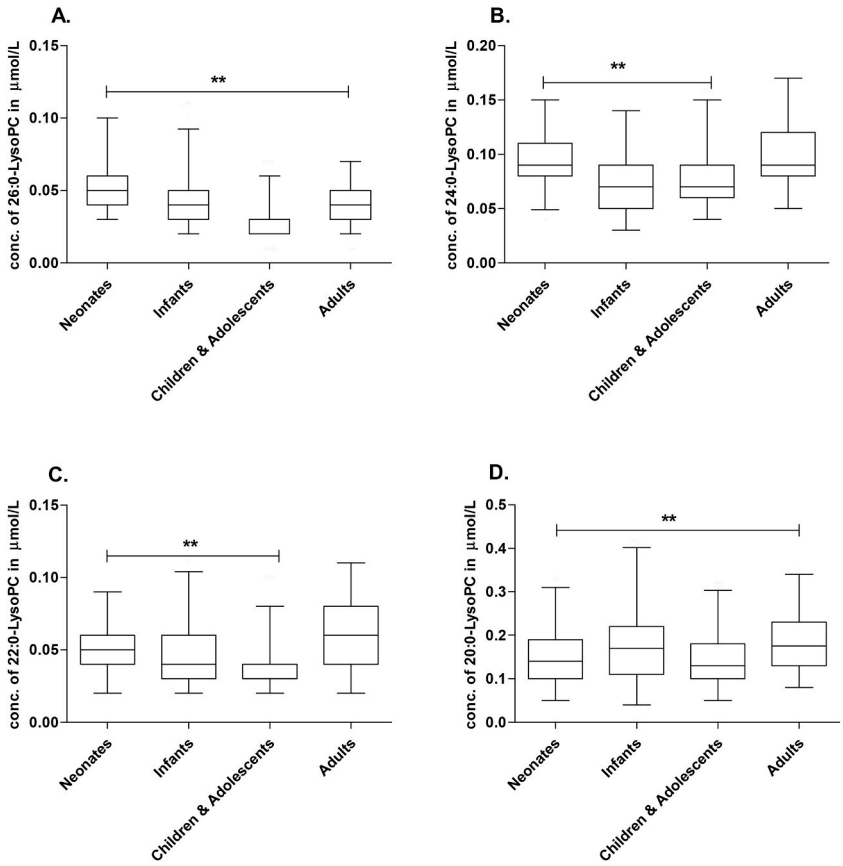


Fig. 2. Box and whisker plot for reference intervals of (A) 26:0-LysoPC, (B) 24:0-LysoPC, (C) 22:0-LysoPC and (D) 20:0-LysoPC measured in DBS by the LC-MS/MS method in males and females divided across different age groups.
conc. – concentration; DBS- dried blood spots; LC-MS/MS- Liquid chromatography tandem mass spectrometry; **- $P < 0.01$ The lower and the upper end of the whiskers represent the 1st and the 99th percentile with the central median value (50th percentile) bound by 25th and 75th percentile values.

The variations seen in the concentration of LysoPCs and their ratios measured by both the methods have been represented in Supplementary Fig. 3 by plotting trend lines. In our study we found that 26:0-LysoPC, 24:0-LysoPC, 22:0-LysoPC and 26:0/22:0-LysoPC, 26:0/20:0-LysoPC, 24:0/22:0-LysoPC and 24:0/20:0-LysoPC ratios (FIA-MS/MS and LC-MS/MS) were significantly higher in neonates in comparison to infants, children & adolescents and adults, while the concentration of 20:0-LysoPC increased after birth. Even though, the median concentration of 22:0-LysoPC (FIA-MS/MS and LC-MS/MS) was higher in neonates in comparison to infants, children &adolescents, its upper 99th percentile limit was almost the same in adults. These observations are in line with the study results published by various study groups whose observations are described further.

Tian and co-workers reported that the concentration of 26:0-LysoPC and 26:0/22:0-LysoPC, 24:0/22:0-LysoPC ratios (FIA-MS/MS) were significantly higher in Chinese newborns in comparison to children. The Newborn Screening Program conducted by the Washington State Department of Health, USA, has shown that 26:0-LysoPC concentration was significantly high in newborns aged 0–7days than newborns ≥7 days old (mean [±SD], 0.088 µmol/L [±0.024] vs. 0.051 µmol/L [±0.018]) [35]. We observed a similar pattern in the newborns included in our study, with a minor statistical difference in the median concentration of 26:0-LysoPC (LC-MS/MS) in neonates aged between 2 and 7 days when compared with ≥7–30 days old neonates (0.06 µmol/L vs. 0.05 µmol/L). However, the upper cut-off limit of the two sub-groups and the medians were not significantly different so we clubbed the data into one group (from birth to 1 month). Studies by Barendsen et al. [20] and Huffnagel et al. [8] in the Dutch population estimated higher levels of 26:0-LysoPC (LC-MS/MS) in the DBS of adults than newborns. Additionally, Huffnagel et al. [8] also concluded that even though the median concentration of 26:0-LysoPC in newborns was higher than that of the adults (62 nmol/L vs. 43 nmol/L), there wasn't any clear separation seen in its level between both the groups. Further, Wu et al. [17] also found the concentrations of 26:0-LysoPC and 24:0-LysoPC (LC-MS/MS) to be significantly high in Japanese newborns in comparison to adults. Here, the researchers indicated that either the low sample size or difference in the dietary pattern between the newborns of western Japan and the adults of eastern Japan to be possible reasons for this variation in the concentration of the 26:0-LysoPC and 24:0-LysoPC in the two groups.

The increased concentration of 26:0-LysoPC, 24:0-LysoPC, and 22:0-LysoPC in neonates in comparison to infants, children &

Table 3
Comparison of reference values determined for the LysoPCs and their ratios measured by tandem mass spectrometry methods in various study population.

Authors	Population	Methodology, (Unit), (Min-Max) range	[a]Sample Size	Analytes measured in DBS	26:0-LysoPC	24:0-LysoPC	22:0-LysoPC	20:0-LysoPC	26:0/22:0-LysoPC	24:0/22:0-LysoPC	26:0/20:0-LysoPC	24:0/20:0-LysoPC
FIA-MS/MS method												
Tian et al. [21]	Chinese	FIA-MS/MS (Noebase2 kit, Perkin Elmer) (μmol/L) (1–99)percentile	n = 3078 (24hrs–7 days)	**NB**	0.10–0.34	0.20–0.67	0.06–0.35	0.08–0.40	0.49–3.00	1.24–5.15	NA	NA
			n = 396 (2–11 years)	**Children**	0.05–0.30	0.13–0.70	0.07–0.42	0.09–0.54	0.32–2.12	0.93–4.04	NA	NA
Turgeon et al. [13]	American	FIA-MS/MS (Lab developed) ([a]μmol/L) (1–99)percentile	n = 130	**NB**	0.18–0.36	0.23–0.42	0.10–0.23	0.10–0.60	NA	NA	NA	NA
			n = 20	**Adults**	0.20–0.67	0.14–0.27	0.12–0.23	0.13–0.42	NA	NA	NA	NA
Armangue et al. [9]	Multicenter study	FIA-MS/MS (μmol/L) (95% CI)	n = 670524	**NB**	0.21–0.21	NA	NA	NA	NA	NA	NA	NA
Present study	Indian	FIA-MS/MS (Lab developed) (μmol/L) (1–99)percentile	n = 254	**NB (M+F)**	0.12–0.36	0.20–0.70	0.08–0.43	0.10–0.53	0.58–2.50	1.11–3.50	0.43–2.08	0.74–3.06
			n = 309	**Children (M+F)**	0.10–0.28	0.15–0.46	0.09–0.29	0.14–0.51	0.48–1.67	1.17–2.38	0.30–0.94	0.61–1.55
			n = 275	**Adults (M+F)**	0.12–0.45	0.19–0.47	0.11–0.31	0.19–0.60	0.74–2.22	1.12–2.15	0.41–1.23	0.64–1.33
LC-MS/MS method												
Hubbard et al. [5, 6]	American	HPLC-MS/MS ([b]μmol/L) Range	n = 663	**NB**	0.07–0.28	NA	NA	NA	NA	NA	NA	NA
Sandlers et al. [11]	American	HPLC-MS/MS ([b]μmol/L) Range	n = 89	**NB**	0.016–0.21	NA	NA	NA	NA	NA	NA	NA
Theda et al. [12]	American	HPLC-MS/MS ([b]μmol/L)	n = 4689	**NB**	0.02–0.39	NA	NA	NA	NA	NA	0.03–0.71	NA
Wu et al. [17]	Japanese	HPLC-MS/MS ([c]μmol/L)	n = 604	**NB**	0.01–0.57	0.003–0.432	NA	0.009–2.406	NA	NA	NA	NA
			n = 50	**Adults**	0.003–0.168	0.003–0.198	NA	0.234–1.296	NA	NA	NA	NA
Barendsen et al. [20]	Dutch	HPLC-MS/MS (μmol/L)	n = 250	**NB**	0.029–0.165	NA	NA	NA	NA	NA	NA	NA
			n = 126	**Adults**	0.019–0.078	NA	NA	NA	NA	NA	NA	NA
Armangue et al. [9]	Multicenter study	HPLC-MS/MS (μmol/L) (95% CI)	n = 11353	**NB**	0.08–0.08	NA	NA	NA	NA	NA	NA	NA
Huffnagel et al. [8]	Dutch	UPLC-MS/MS ([d]μmol/L) (Range)	n = 200	**NB**	0.036–0.138	NA	NA	NA	NA	NA	NA	NA
			n = 126	**Adults**	0.019–0.078							
			n = 20	**Females**	0.021–0.077							
Klouwer et al. [10]	Dutch	UPLC-MS/MS ([d]μmol/L)	n = 209	**Males (0–81 years)**	0.028–0.060 Later Upper cut-off was increased to 0.100	NA	NA	NA	NA	NA	NA	NA
Present study	Indian	UPLC-MS/MS (Lab developed) (μmol/L) (1–99)percentile	n = 253	**NB (M+F)**	0.03–0.10	0.04–0.15	0.02–0.09	0.05–0.31	0.62–2.0	1.00–3.20	0.14–1.00	0.32–1.33
			n = 595	**Children&Adol (M+F)**	0.02–0.06	0.04–0.15	0.02–0.08	0.05–0.30	0.33–2.70	1.35–4.00	0.07–0.50	0.24–1.00
			n = 359	**Adults (M+F)**	0.02–0.07	0.05–0.17	0.02–0.11	0.08–0.34	0.26–1.50	1.07–3.85	0.08–0.44	0.32–0.98

M = Males; F= Females; n = No. of samples; NB= Newborn/Neonates; CI- Confidence Interval; UPLC/HPLC/LC-MS/MS- Ultra/High performance-liquid chromatography tandem mass spectrometry; LysoPCs-lysophosphatidylcholines; Adol- Adolescents; NA- Not available.

[a] Converted the unit μg/mL to μmol/L.

[b] Converted the unit pmol/1/8″ mm blood spot to μmol/L (1/8″ mm blood spot = 3 mm = 3 μL of blood).

[c] Converted the unit pmol/punch of blood spot to μmol/L (3 mm blood spot = 3.3 μL of blood).

[d] Converted the unit nmol/L of blood spot to μmol/L.

adolescents and adults could be associated with the amount of feeding, the feeding method (breastfeeding/formula feeding), stage of lactation (early/late) for the newborn and the mother's long term diet and genetic characteristics or lifestyle-related differences in the population [1,2,21]. LysoPC is present in human breast milk, and it could add to the initially high concentration seen in newborns during early lactation [2]. Another potential reason could be the presence of an increased number of red blood cells in newborns, which decline with age, thus decreasing LysoPC concentration in children or adults. This could be because LysoPCs in the blood are predominantly present in the cell membrane of the RBCs, and LysoPCs which we extracted from DBS are mainly derived from these RBCs [20,36,37].

In our study, the increased concentration of 26:0-LysoPC measured by FIA-MS/MS in the adult group in comparison to neonates could be due to the interfering artefact. This observation is similar to the findings reported by Turgeon et al. [13]where, 26:0-LysoPC was measured by the FIA-MS/MS method in the American population.

An increase in the concentration of 20:0-LysoPC with age could be due to changes in the metabolism from birth. In a couple of studies on LysoPCs, Takatera, and co-workers [1,38] reported that the concentration of LysoPCs increased with age. They proposed that it could be due to the difference in immunity between neonates and adults.

5. Conclusion

We established age, and gender-specific reference intervals for a panel of lysophosphatidylcholines and their ratios in dried blood spot extracts estimated by the two tandem mass spectrometry techniques (FIA-MS/MS and LC-MS/MS) which are used for screening and identification of X-ALD. We infer that the age-related variations in 26:0-, 24:0-, 22:0- and 20:0-LysoPCs and the ratios are small and unlikely to have any significant impact on identifying X-ALD, but such studies could be useful to understand the age-related variations where other biochemical abnormalities are subtle. Our data may help in the accurate identification of X-ALD and other peroxisomal disorders in the Indian population.

Author contributions

AN: Performed the experiments, analysed data, and wrote the paper, RC: Conceived and designed the study and approved the manuscript.

Research funding

This work was supported by Department of Biotechnology, Ministry of Science and Technology, New Delhi, Government of India [Grant No.BT/PR16043/MED/97/275/2016].

Declaration of competing interest

None declared.

Data availability

Data will be made available on request.

Acknowledgment

This work was supported by the Department of Biotechnology (DBT), Ministry of Science and Technology, New Delhi, Government of India. We thank Centers for Disease Control and Prevention, Atlanta, GA, USA, for including us in the Newborn Screening and Quality Assurance Programme and for providing us with quality control and proficiency testing samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2022.e00305.

References

[1] A. Takatera, A. Takeuchi, K. Saiki, I. Morioka, N. Yokoyama, M. Matsuo, Blood lysophosphatidylcholine (LPC) levels and characteristic molecular species in neonates: prolonged low blood LPC levels in very low birth weight infants, Pediatr. Res. 62 (2007) 477–482, https://doi.org/10.1203/PDR.0b013e31814625ca.

[2] C. Hellmuth, O. Uhl, H. Demmelmair, M. Grunewald, R. Auricchio, G. Castillejo, I.R. Korponay-Szabo, I. Polanco, M. Roca, S.L. Vriezinga, K.J. Werkstetter, B. Koletzko, M.L. Mearin, F.F. Kirchberg, The impact of human breast milk components on the infant metabolism, PLoS One 13 (2018), e0197713, https://doi.org/10.1371/journal.pone.0197713.

[3] S.-H. Law, M.-L. Chan, G.K. Marathe, F. Parveen, C.-H. Chen, L.-Y. Ke, An updated review of lysophosphatidylcholine metabolism in human diseases, Int. J. Mol. Sci. 20 (2019), https://doi.org/10.3390/ijms20051149.

[4] P. Liu, W. Zhu, C. Chen, B. Yan, L. Zhu, X. Chen, C. Peng, The mechanisms of lysophosphatidylcholine in the development of diseases, Life Sci. 247 (2020), 117443, https://doi.org/10.1016/j.lfs.2020.117443.

[5] W.C. Hubbard, A.B. Moser, S. Tortorelli, A. Liu, D. Jones, H. Moser, Combined liquid chromatography-tandem mass spectrometry as an analytical method for high throughput screening for X-linked adrenoleukodystrophy and other peroxisomal disorders: preliminary findings, Mol. Genet. Metabol. 89 (2006) 185–187, https://doi.org/10.1016/j.ymgme.2006.05.001.

[6] W.C. Hubbard, A.B. Moser, A.C. Liu, R.O. Jones, S.J. Steinberg, F. Lorey, S.R. Panny, R.F. Vogt, D. Macaya, C.T. Turgeon, S. Tortorelli, G.V. Raymond, Newborn screening for X-linked adrenoleukodystrophy (X-ALD): validation of a combined liquid chromatography-tandem mass spectrometric (LC-MS/MS) method, Mol. Genet. Metabol. 97 (2009) 212–220, https://doi.org/10.1016/j.ymgme.2009.03.010.

[7] C.A. Haynes, V.R. De Jesús, Improved analysis of C26:0-lysophosphatidylcholine in dried-blood spots via negative ion mode HPLC-ESI-MS/MS for X-linked adrenoleukodystrophy newborn screening, Clin. Chim. Acta Int. J. Clin. Chem. 413 (2012) 1217–1221, https://doi.org/10.1016/j.cca.2012.03.026.

[8] I.C. Huffnagel, M.-C. van de Beek, A.L. Showers, J.J. Orsini, F.C.C. Klouwer, I.M.E. Dijkstra, P.C. Schielen, H. van Lenthe, R.J.A. Wanders, F.M. Vaz, M. A. Morrissey, M. Engelen, S. Kemp, Comparison of C26:0-carnitine and C26:0-lysophosphatidylcholine as diagnostic markers in dried blood spots from newborns and patients with adrenoleukodystrophy, Mol. Genet. Metabol. 122 (2017) 209–215, https://doi.org/10.1016/j.ymgme.2017.10.012.

[9] T. Armangue, J.J. Orsini, A. Takanohashi, F. Gavazzi, A. Conant, N. Ulrick, M.A. Morrissey, N. Nahhas, G. Helman, H. Gordish-Dressman, S. Orcesi, D. Tonduti, C. Stutterd, K. van Haren, C. Toro, A.D. Iglesias, M.S. van der Knaap, R.G. Mansky, A.B. Moser, R.O. Jones, A. Vanderver, Neonatal detection of Aicardi Goutières Syndrome by increased C26:0 lysophosphatidylcholine and interferon signature on newborn screening blood spots, Mol. Genet. Metabol. 122 (2017) 134–139, https://doi.org/10.1016/j.ymgme.2017.07.006.

[10] F.C.C. Klouwer, S. Ferdinandusse, H. van Lenthe, W. Kulik, R.J.A. Wanders, B.T. Poll-The, H.R. Waterham, F.M. Vaz, Evaluation of C26:0-lysophosphatidylcholine and C26:0-carnitine as diagnostic markers for Zellweger spectrum disorders, J. Inherit. Metab. Dis. 40 (2017) 875–881, https://doi.org/10.1007/s10545-017-0064-0.

[11] Y. Sandlers, A.B. Moser, W.C. Hubbard, L.E. Kratz, R.O. Jones, G.V. Raymond, Combined extraction of acyl carnitines and 26:0 lysophosphatidylcholine from dried blood spots: prospective newborn screening for X-linked adrenoleukodystrophy, Mol. Genet. Metabol. 105 (2012) 416–420, https://doi.org/10.1016/j.ymgme.2011.11.195.

[12] C. Theda, K. Gibbons, T.E. Defor, P.K. Donohue, W.C. Golden, A.D. Kline, F. Gulamali-Majid, S.R. Panny, W.C. Hubbard, R.O. Jones, A.K. Liu, A.B. Moser, G. V. Raymond, Newborn screening for X-linked adrenoleukodystrophy: further evidence high throughput screening is feasible, Mol. Genet. Metabol. 111 (2014) 55–57, https://doi.org/10.1016/j.ymgme.2013.10.019.

[13] C.T. Turgeon, A.B. Moser, L. Mørkrid, M.J. Magera, D.K. Gavrilov, D. Oglesbee, K. Raymond, P. Rinaldo, D. Matern, S. Tortorelli, Streamlined determination of lysophosphatidylcholines in dried blood spots for newborn screening of X-linked adrenoleukodystrophy, Mol. Genet. Metabol. 114 (2015) 46–50, https://doi.org/10.1016/j.ymgme.2014.11.013.

[14] C.A. Haynes, V.R. De Jesús, Simultaneous quantitation of hexacosanoyl lysophosphatidylcholine, amino acids, acylcarnitines, and succinylacetone during FIA-ESI-MS/MS analysis of dried blood spot extracts for newborn screening, Clin. Biochem. 49 (2016) 161–165, https://doi.org/10.1016/j.clinbiochem.2015.09.011.

[15] S. Tortorelli, C.T. Turgeon, D.K. Gavrilov, D. Oglesbee, K.M. Raymond, P. Rinaldo, D. Matern, Simultaneous testing for 6 lysosomal storage disorders and X-adrenoleukodystrophy in dried blood spots by tandem mass spectrometry, Clin. Chem. 62 (2016) 1248–1254, https://doi.org/10.1373/clinchem.2016.256255.

[16] R. Mashima, M. Tanaka, E. Sakai, H. Nakajima, T. Kumagai, M. Kosuga, T. Okuyama, A selective detection of lysophosphatidylcholine in dried blood spots for diagnosis of adrenoleukodystrophy by LC-MS/MS, Mol. Genet. Metab. Rep. 7 (2016) 16–19, https://doi.org/10.1016/j.ymgmr.2016.02.007.

[17] C. Wu, T. Iwamoto, J. Igarashi, T. Miyajima, M.A. Hossain, H. Yanagisawa, K. Akiyama, H. Shintaku, Y. Eto, Application of a diagnostic methodology by quantification of 26:0 lysophosphatidylcholine in dried blood spots for Japanese newborn screening of X-linked adrenoleukodystrophy, Mol. Genet. Metab. Rep. 12 (2017) 115–118, https://doi.org/10.1016/j.ymgmr.2017.06.004.

[18] A. Natarajan, R. Christopher, M. Netravathi, M. Bhat, S.R. Chandra, Liquid chromatography-tandem mass spectrometry method for estimation of a panel of lysophosphatidylcholines in dried blood spots for screening of X-linked adrenoleukodystrophy, Clin. Chim. Acta Int. J. Clin. Chem. 485 (2018) 305–310, https://doi.org/10.1016/j.cca.2018.07.007.

[19] A. Natarajan, R. Christopher, M. Netravathi, M.D. Bhat, S.R. Chandra, Flow injection ionization-tandem mass spectrometry-based estimation of a panel of lysophosphatidylcholines in dried blood spots for screening of X-linked adrenoleukodystrophy, Clin. Chim. Acta Int. J. Clin. Chem. 495 (2019) 167–173, https://doi.org/10.1016/j.cca.2019.04.059.

[20] R.W. Barendsen, I.M.E. Dijkstra, W.F. Visser, M. Alders, J. Bliek, A. Boelen, M.J. Bouva, S.N. van der Crabben, E. Elsinghorst, A.G.M. van Gorp, A.C. Heijboer, M. Jansen, Y.R.J. Jaspers, H. van Lenthe, I. Metgod, C.F. Mooij, E.H.C. van der Sluijs, A.S.P. van Trotsenburg, R.K. Verschoof-Puite, F.M. Vaz, H.R. Waterham, F. A. Wijburg, M. Engelen, E. Dekkers, S. Kemp, Adrenoleukodystrophy newborn screening in The Netherlands (SCAN study): the X-factor, Front. Cell Dev. Biol. 8 (2020) 499, https://doi.org/10.3389/fcell.2020.00499.

[21] G.-L. Tian, F. Xu, K. Jiang, Y.-M. Wang, W. Ji, Y.-P. Zhuang, Evaluation of a panel of very long-chain lysophosphatidylcholines and acylcarnitines for screening of X-linked adrenoleukodystrophy in China, Clin. Chim. Acta 503 (2020) 157–162, https://doi.org/10.1016/j.cca.2020.01.016.

[22] B.R. Turk, C. Theda, A. Fatemi, A.B. Moser, X-linked adrenoleukodystrophy: pathology, pathophysiology, diagnostic testing, newborn screening and therapies, Int. J. Dev. Neurosci. (2020) 1–21, https://doi.org/10.1002/jdn.100032.

[23] A.B. Moser, E. Seeger, G.V. Raymond, Newborn screening for X-linked adrenoleukodystrophy: past, present, and future, Int. J. Neonatal Screen. 8 (2022) 16, https://doi.org/10.3390/ijns8010016.

[24] S.A.V. Shah, K. Ichihara, A.J. Dherai, T.F. Ashavaid, Reference intervals for 33 biochemical analytes in healthy Indian population: C-RIDL IFCC initiative, Clin. Chem. Lab. Med. 56 (2018) 2093–2103, https://doi.org/10.1515/cclm-2018-0152.

[25] K. Williams, D. Thomson, I. Seto, D.G. Contopoulos-Ioannidis, J.P.A. Ioannidis, S. Curtis, E. Constantin, G. Batmanabane, L. Hartling, T. Klassen, StaR Child Health Group, Standard 6: age groups for pediatric trials, Pediatrics 129 (Suppl 3) (2012) S153–S160, https://doi.org/10.1542/peds.2012-0055I.

[26] S. Abdel-Rahman, G.L. Amidon, A. Kaul, V. Lukacova, A.A. Vinks, G. Knipp, Summary of the NICHD-BPCA pediatric formulation initiatives workshop-pediatric biopharmaceutics classification system (PBCS) working group, Clin. Therapeut. 34 (2012), https://doi.org/10.1016/j.clinthera.2012.09.014. S11–S24.

[27] Center for Drug Evaluation and Research, FDA, General clinical pharmacology considerations for pediatric Studies for Drugs and Biological Products, guidance for industry, Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/general-clinical-pharmacology-considerations-pediatric-studies-drugs-and-biological-products. (Accessed 13 March 2022). Published December 2014. Accessed:.

[28] K.M. Job, M. Gamalo, R.M. Ward, Pediatric age groups and approach to studies, Ther. Innov. Regul. Sci. 53 (2019) 584–589, https://doi.org/10.1177/2168479019856572.

[29] M. Engelen, S. Kemp, M. de Visser, B.M. van Geel, R.J. Wanders, P. Aubourg, B.T. Poll-The, X-linked adrenoleukodystrophy (X-ALD): clinical presentation and guidelines for diagnosis, follow-up and management, Orphanet J. Rare Dis. 7 (2012) 51, https://doi.org/10.1186/1750-1172-7-51.

[30] M. Foschi, V. Vacchiano, P. Avoni, A. Incensi, S. Battaglia, V. Donadio, E. Panzeri, M.T. Bassi, R. Liguori, G. Rizzo, Broadening the spectrum of adulthood X-linked adrenoleukodystrophy: a report of two atypical cases, Front. Neurol. 10 (2019), https://doi.org/10.3389/fneur.2019.00070.

[31] M. Engelen, M. Barbier, I.M.E. Dijkstra, R. Schür, R.M.A. de Bie, C. Verhamme, M.G.W. Dijkgraaf, P.A. Aubourg, R.J.A. Wanders, B.M. van Geel, M. de Visser, B. T. Poll-The, S. Kemp, X-linked adrenoleukodystrophy in women: a cross-sectional cohort study, Brain J. Neurol. 137 (2014) 693–706, https://doi.org/10.1093/brain/awt361.

[32] I.C. Huffnagel, F.K. Laheji, R. Aziz-Bose, N.A. Tritos, R. Marino, G.E. Linthorst, S. Kemp, M. Engelen, F. Eichler, The natural history of adrenal insufficiency in X-linked adrenoleukodystrophy: an international collaboration, J. Clin. Endocrinol. Metab. 104 (2019) 118–126, https://doi.org/10.1210/jc.2018-01307.

[33] J. Matteson, S. Sciortino, L. Feuchtbaum, T. Bishop, R.S. Olney, H. Tang, Adrenoleukodystrophy newborn screening in California since 2016: programmatic outcomes and follow-up, Int. J. Neonatal Screen. 7 (2021) 22, https://doi.org/10.3390/ijns7020022.

[34] B. Goldstein, B. Giroir, A. Randolph, International Consensus Conference on Pediatric Sepsis, International pediatric sepsis consensus conference: definitions for sepsis and organ dysfunction in pediatrics, Pediatr. Crit. Care Med. J. Soc. Crit. Care Med. World Fed. Pediatr. Intensive Crit. Care Soc. 6 (2005) 2–8, https://doi.org/10.1097/01.PCC.0000149131.72248.E6.
[35] Association of Public Health Laboratories, APHL Conference presentations-Documents 2019, Available from: https://www.aphl.org/conferences/proceedings/Documents/2019/. (Accessed 13 March 2022).
[36] H. Nishio, S. Kodama, S. Yokoyama, T. Matsuo, T. Mio, K. Sumino, A simple method to diagnose adrenoleukodystrophy using a dried blood spot on filter paper, Clin. Chim. Acta 159 (1986) 77–82, https://doi.org/10.1016/0009-8981(86)90169-5.
[37] K. Tanaka, M. Shimada, T. Naruto, H. Yamamoto, K. Nishizawa, Y. Saeki, Very long-chain fatty acids in erythrocyte membrane phospholipids in adrenoleukodystrophy, Acta Paediatr. Jpn. Overseas Ed. 31 (1989) 136–143, https://doi.org/10.1111/j.1442-200x.1989.tb01279.x.
[38] A. Takatera, A. Takeuchi, K. Saiki, T. Morisawa, N. Yokoyama, M. Matsuo, Quantification of lysophosphatidylcholines and phosphatidylcholines using liquid chromatography–tandem mass spectrometry in neonatal serum, J. Chromatogr. B 838 (2006) 31–36, https://doi.org/10.1016/j.jchromb.2006.03.006.