Lipotoxicity in adrenoleukodystrophy

Size matters!

van de Beek, M.-C.

Citation for published version (APA):
van de Beek, M-C. (2018). Lipotoxicity in adrenoleukodystrophy: Size matters!.

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Comparison of C26:0-carnitine and C26:0-lysophosphatidylcholine as diagnostic markers in dried blood spots from newborns and patients with adrenoleukodystrophy

Chapter 4

Irene Huffnagel1*, Malu-Clair van de Beek1*, Amanda L. Showers3, Joseph J. Orsini3, Femke C.C. Klouwer1,2, Inge M.E. Dijkstra1, Peter C. Schielen4, Henk van Lenthe1, Ronald J.A. Wanders1, Mark A. Morrissey1, Marc Engelen2 & Stephan Kemp1,2

1Laboratory Genetic Metabolic Diseases, Departments of Pediatrics and Clinical Chemistry, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.
2Departments of Pediatrics and Pediatric Neurology, Emma Children’s Hospital, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.
3Newborn Screening Program, Wadsworth Center, New York State Department of Health, Albany, NY, USA.
4Center for Infectious Diseases Research, Diagnostics and Screening, National Institute for Public Health and the Environment, Bilthoven, The Netherlands.

*The authors contributed equally to this work.

Molecular Genetics and Metabolism (2017), http://dx.doi.org/10.1016/j.mggme.2017.10.012
Abstract

X-linked adrenoleukodystrophy (ALD) is the most common leukodystrophy with a birth incidence of 1:14,700 live births. The disease is caused by mutations in ABCD1 and characterized by very long-chain fatty acids (VLCFA) accumulation. In childhood, male patients are at high-risk to develop adrenal insufficiency and/or cerebral demyelination. Timely diagnosis is essential. Untreated adrenal insufficiency can be life-threatening and hematopoietic stem cell transplantation is curative for cerebral ALD provided the procedure is performed in an early stage of the disease. For this reason, ALD is being added to an increasing number of newborn screening programs. ALD newborn screening involves the quantification of C26:0-lysoPC in dried blood spots that requires a dedicated method. Recently, C26:0-carnitine was identified as a potential new biomarker for ALD. It can be added as one more analyte to the routine analysis of amino acids and acylcarnitines already in use. The first objective of this study was a comparison of the sensitivity of C26:0-carnitine and C26:0-lysoPC in dried blood spots from control and ALD newborns. C26:0-carnitine was measured in 270,000 newborn dried blood spots in the New York State screening program. While C26:0-lysoPC was elevated in all ALD newborns, C26:0-carnitine was elevated only in 83%. Therefore, C26:0-carnitine is not a suitable biomarker to use in ALD newborn screen. In women with ALD, plasma VLCFA analysis results in a false negative result in approximately 15-20% of cases. The second objective of this study was to compare plasma VLCFA analysis with C26:0-carnitine and C26:0-lysoPC in dried blood spots of women with ALD. Our results show that C26:0-lysoPC was elevated in dried blood spots from all women with ALD, including from those with normal plasma C26:0 levels. This shows that C26:0-lysoPC is a better and more accurate biomarker for ALD than plasma VLCFA levels. We recommend that C26:0-lysoPC be added to the routine biochemical tests for peroxisomal beta-oxidation disorders.
1. Introduction

X-linked adrenoleukodystrophy (ALD) (MIM #300100) is a progressive neurodegenerative disorder with an estimated incidence of 1 in 14,700 live births. All ALD patients have a mutation in the ABCD1 gene (www.x-ald.nl) that codes for the ABCD1 protein. The ABCD1 protein transports very long-chain fatty acids (VLCFA, ≥C22:0) as CoA-esters across the peroxisomal membrane. Mutations in ABCD1 result in deficient peroxisomal beta-oxidation of VLCFA, and consequently VLCFA accumulation in plasma and tissues, including the white matter of the brain, spinal cord, and adrenal cortex.

ALD is characterized by a striking and unpredictable clinical spectrum including primary adrenal insufficiency, progressive myelopathy and cerebral inflammatory disease (cerebral ALD). Patients with ALD are free of symptoms at birth. In 80% of male patients, the first manifestation of the disease is adrenal insufficiency. In adulthood, virtually all male patients and >80% of women with ALD develop a chronic progressive myelopathy. Additionally, male patients are at risk of developing cerebral ALD. Boys between 3 and 18 years of age are estimated to have a 35-40% risk and although the risk seems to decrease during adulthood, male patients have a lifetime risk of about 60% to develop cerebral ALD. In the absence of a genotype–phenotype correlation, predicting the disease course of an individual patient is impossible, even within individual families.

Hematopoietic stem cell transplantation (HSCT) can stop or even reverse the progression of cerebral ALD provided the procedure is performed in an early stage of the disease before extensive MRI white matter abnormalities are present. Unfortunately, cerebral ALD can be relentlessly progressive and therefore the therapeutic window is narrow. If the index patient presents with cerebral ALD this window is often missed.

Newborn screening is ideally suited to achieve early identification and intervention thereby giving ALD patients a significantly improved prognosis. This is one of the reasons why ALD is being added to an increasing number of newborn screening programs, and that ALD has been added to the US Recommended Uniform Screening Panel for newborn screening.

ALD newborn screening involves the quantification of 1-hexacosanoyl-2-lyso-sn-3-glycero-phosphorylcholine (C26:0-lysoPC) by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in dried blood spots (DBS). The procedure has been modified to combine the existing method for acylcarnitine analysis and C26:0-lysoPC, which enables simultaneous extraction and screening for peroxisomal and mitochondrial fatty acid oxidation disorders and certain organic acidurias.

Recently, C26:0-carnitine was identified as a potential new biomarker in DBS derived from ALD mice and patients. Other studies have reported increased levels of C26:0-carnitine in plasma, urine and DBS from patients with a peroxisomal biogenesis disorder. The first objective of this study was to compare the sensitivity of C26:0-lysoPC and C26:0-carnitine in DBS from control and ALD newborns. At the New York State Newborn Screening program, C26:0-carnitine was tested as an alternative to the C26:0-lysoPC marker, since the C26:0-
carnitine could be added as one more analyte to the routine analysis of amino acids and acylcarnitines already in use. The use of the C26:0-carnitine as a marker for ALD could potentially save time and effort in sample handling and data processing. In males, diagnosis of ALD can be achieved with >99.9% sensitivity by analysis of VLCFA levels in plasma. However, 2 cases have been reported with false-negative plasma VLCFA results\textsuperscript{25, 26}. Importantly, additional biochemical studies in cultured skin fibroblasts gave clearly abnormal values in both cases\textsuperscript{26}. In women, false negative results are found in approximately 15 - 20% of cases\textsuperscript{6, 12}. Therefore, \textit{ABCD1} mutation analysis is the most reliable diagnostic method, provided that the mutation in the family has been defined in an affected male or female\textsuperscript{27}. It is our experience, however, that an increasing number of symptomatic heterozygous women with a myelopathy are identified as the index patient in a family. Recently, we were confronted with two unrelated women with symptoms of a spinal cord disorder and a variant of unknown significance (VUS) in the \textit{ABCD1} gene, but with normal C26:0 levels in plasma and fibroblasts. We developed an elegant, but time-consuming strategy that was based on the generation of clonal cell lines that express only one of the two alleles followed by biochemical studies to demonstrate that the variants were not pathogenic\textsuperscript{28}. The second objective of our study was to investigate whether the analysis of C26:0-lysoPC and/or C26:0-carnitine in DBS may lower the number of false negative results in women with ALD.

2. Materials and Methods

2.1 Controls and patients (The Netherlands)

Newborn DBS cards from 200 control newborns were obtained through a collaboration with the National Institute for Public Health and the Environment (RIVM), which is the governmental agency in the Netherlands that stores all DBS cards from the Netherlands newborn screening program for 5 years. Because only a single Dutch boy diagnosed with ALD currently under the age of 5 years is known to us, solely 1 Dutch ALD DBS card could be retrieved from the RIVM repository. To increase the sample size, we obtained 5 additional ALD newborn DBS cards through a collaboration with the New York State ALD newborn screening program. In addition, we received 5 “repeat samples” from New York State. These are ALD DBS cards that were generated within 8 weeks after birth to confirm the initial findings. DBS cards from adult ALD patients (47 males and 49 females) were obtained from ALD patients currently participating in a prospective natural history study (referred to as “The Dutch ALD cohort”; IRB: METC 2014_347 and 2015_079). Written informed consent was received from each patient. For an adult control group, we collected all routine C26:0-lysoPC and C26:0-carnitine measurements performed at the Laboratory Genetic Metabolic Diseases
in the Academic Medical Center between January 2012 and April 2017. Measurements of C26:0-lysoPC and C26:0-carnitine in DBS of patients with a peroxisomal fatty acid oxidation disorder such as the Zellweger spectrum disorders, peroxisomal acyl-CoA oxidase 1 (ACOX1) and d-bifunctional protein deficiency (HSD1B4) were excluded. The remainder of the C26:0-lysoPC and C26:0-carnitine measurements were combined and labeled as the control group (108 samples, males and females). Approval of the Research Ethics Committee for the analysis of C26:0-lysoPC and C26:0-carnitine in DBS cards was not required, since all measurements were performed as part of diagnostic procedures or standard patient care and data were anonymized for further analysis.

2.1.1 Analysis of C26:0-lysoPC and C26:0-carnitine in DBS cards (The Netherlands)

To better compare the correlation between C26:0-lysoPC and C26:0-carnitine, both metabolites were analyzed in the same DBS punch using a combined analysis. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was performed as described before, with minor modifications. A single punch of a DBS (¼ inch in diameter) was extracted with 10 μL of an internal standard solution containing 1 μM d4-C26:0-lysoPC and 10 μL of 0.1 μM d4-C26:0-carnitine in 0.5 mL of methanol by ultrasonication in a sonicator bath (Branson 3510) for 5 minutes at room temperature. The extract was transferred to a 4 mL glass tube and the methanol was evaporated under a constant stream of nitrogen at 40°C. The residue was dissolved in 50 μL of methanol, transferred to a sample vial, and capped. Analysis was done using an ACQUITY UPLC system (Waters Corp., Milford, MA, USA) consisting of a binary solvent manager, a vacuum degasser, a column heater and a sample manager. Ten μL of extract was injected onto a Kinetex C8 column at 50°C (50 × 2.1 mm, 2.6 μm particle diameter from Phenomenex, Torrance, CA, USA). Metabolites were separated by a linear gradient between solution A (0.1% formic acid in H2O) and solution B (0.1% formic acid in methanol). The UPLC run was 12 minutes at a flow rate of 0.4 ml/min. All gradient steps were linear and as follows: at T = 0 min: 64% B, towards T = 6 min: 100% B; T = 6 -11 min 100% B isocratic, and T = 11 - 12 min back to 64% B. For the mass spectrometric detection, a Quattro Premier XE (Waters, Milford, MA, USA) was used in the positive electrospray ionization mode. The spray voltage used was 3.5 kV, nitrogen was used as desolvation gas (900 L/h) and cone gas (50 L/h). Desolvation temperature was 350°C and the source temperature was 130°C. Argon was used as collision gas (2.5 x 10e-3 mbar). For acylcarnitines, the following multiple reaction monitoring (MRMs) were used: C26:0-carnitine (540.50 > 85.00), D4-C26:0-carnitine (544.50 > 85.00) both using a dwell time of 0.030 s, a cone voltage of 54 V and a collision energy of 32 eV. For C26:0-lysoPC, the following MRMs were used: C26:0-lysoPC (636.50 > 104.10) and D4-C26:0-lysoPC (640.50 > 104.10) both using a dwell time of 0.030 s, a cone voltage 53 V and collision energy of 31 eV. C26:0-carnitine
2.2 Newborn samples (New York State)

Newborn DBS specimens were obtained by heel prick and spotted on Schleicher and Schuell 903 filter paper, or equivalent. New York State guidelines recommend that all infants be screened when they are at least 24 hours of age29. All samples are sent to the Newborn Screening Program by courier or the US mail. As part of mandatory screening all samples are tested for C26:0-lysoPC, the primary marker for ALD. Quantification of the C20:0-, C22:0-, C24:0- and C26:0-carnitine markers was conducted on newborn screening samples to determine the effectiveness of these markers in screening for ALD. Approximately 270,000 samples were screened for the very long-chain acylcarnitine analytes in parallel with the routine screen for C26:0-lysoPC. Since no new specimens were taken from any of the samples for the C26:0-carnitine testing and since ALD screening is mandated by New York State law, approval of the Institutional Review Board (IRB) was not required. In addition, 64 samples from confirmed cases of ALD (detected through routine screening or confirmed clinically were retrieved from the program archive) and screen positive samples identified by the program during routine testing for C26:0-lysoPC were tested for the very long chain acylcarnitines analytes. These positive samples all had a higher than normal concentration of C26:0-lysoPC when tested by HPLC-MS/MS21.

2.2.1 Analysis of C26:0-carnitine in DBS cards (New York State)

The D3-labeled C26:0-carnitine internal standard was purchased from Cambridge Isotope Laboratories (Andover, MA). A stock solution was prepared at a concentration of 1 mg/mL in methanol. From this, a working internal standard with a concentration of 1.8 ng/mL D3-C26:0-carnitine was prepared. This corresponded to a concentration of 0.20 μmole of C26:0-carnitine per liter of blood in a 3.2 mm dried blood spot specimen (assuming 3.25 μL of blood in the 3.2 mm specimen). Unlabeled C26:0-carnitine standard for preparation of fortified quality control samples was obtained as a generous gift of Dr. Ann Moser of Kennedy Krieger Institute (purchased from Dr. Herman ten Brink). The stock solution of the unlabeled standard used for formulating quality control samples was prepared at a concentration of 1.4 mg/mL in methanol. High- and low-concentration quality control DBS samples were prepared in-house according to standard operating procedures. High concentration quality control samples were fortified with 1.0 μmole/L of C26:0-carnitine. All other chemicals and solvents were of the highest purity available and were used without further purification. C26:0-carnitine was quantified using a modification of our routine amino acid/acylcarnitine assay which is based on previously published methods of analysis30,31. Briefly, a 1/8-inch
Comparison of C26:0-carnitine and C26:0-lysoPC as diagnostic markers in dried blood spots

A (3.2mm) punch from each DBS specimen is placed in a well of a polypropylene, 96-well, micro-titer plate. A 200 μL aliquot of methanol based internal standard containing stable isotope analogs of amino acids and acylcarnitines (including D₃-C26:0-carnitine) is added to each well, the plates are covered, and the samples extracted at room temperature for 20 minutes with gentle shaking. The extract is transferred to a clean plate and evaporated. The residue is derivatized with 3N butanolic HCL. After derivatization the butanol is evaporated and the samples are reconstituted in ACN:H₂O (80:20) containing 0.1% formic acid.

Sample extracts were analyzed using a Waters ACQUITY UPLC system coupled to a Xevo TQ MS triple-quadrupole MS/MS system (Waters Corp., Milford MA), operated in positive ion mode. A 10 μL aliquot of each sample extract was injected into a mobile phase (ACN:H₂O, 80:20) for analysis by flow injection mass spectrometry (FIA-MS/MS). Data was acquired in the MRM mode for the butyl esters of the very long-chain acylcarnitines for ALD screening. C20:0- (512.5 > 85.1), C22:0- (540.5 > 85.1), C24:0- (568.5 > 85.1), and C26:0-carnitine (596.5 > 85.1) butyl esters were quantified relative to the butyl ester of D₃-C26:0-carnitine (599.5 > 85.1). The cone and collision voltages of the mass spectrometer were 50V and 32 eV, respectively, for these analytes.

2.3 Plasma very long-chain fatty acid analysis
Plasma VLCFA levels were analyzed as described previously³².

3. Results

3.1 Comparison of the sensitivity of C26:0-lysoPC and C26:0-carnitine in DBS from control and ALD newborns
To investigate whether C26:0-carnitine can be used as an alternative biomarker for ALD newborn screening, we measured C26:0-lysoPC and C26:0-carnitine levels in 200 DBS from newborn controls and 11 DBS from 6 different ALD newborns. For C26:0-lysoPC, the average level in control newborns was 65 ± 16 nmol/L (range 36 – 138 nmol/L) and in ALD newborns it was 532 ± 340 nmol/L (range 211 – 1140 nmol/L). All ALD newborns had elevated C26:0-lysoPC levels, and there was a clear separation between controls and ALD newborns (Figure 1A). This corresponds to a sensitivity of 100%. For C26:0-carnitine, the average level in newborn controls was 15 ± 5 nmol/L (range 5 – 31 nmol/L) and in ALD newborns it was 57 ± 23 nmol/L (range 28 – 90 nmol/L). In our sample set, 2 independent samples derived from 1 ALD newborn had a C26:0-carnitine level that fell into the control range (Figure 1B). This corresponds to a sensitivity of 82%.

We investigated the correlation between the C26:0-lysoPC levels and the C26:0-carnitine levels measured in the same ALD DBS. This showed a non-significant correlation between C26:0-lysoPC levels and C26:0-carnitine levels in newborn DBS (Figure 1C).
Figure 1.
Scatterplots of C26:0-lysoPC levels (A) and C26:0-carnitine (B) in DBS from control newborns (green circles) and ALD newborns (blue squares). The upper control values for C26:0-lysoPC (138 nmol/L) and C26:0-carnitine (31 nmol/L) are indicated by the dashed red lines. (C) Correlation plot showing the correlation between C26:0-lysoPC and C26:0-carnitine levels measured in the same ALD DBS (n = 11). Statistical analysis performed using Spearman’s correlation test showed a non-significant correlation. The upper control value of C26:0-carnitine (31 nmol/L) is indicated by the horizontal dotted red line,
and the upper control value of C26:0-lysoPC (138 nmol/L) is indicated by the vertical dotted red line. (D) Scatterplot of C26:0-carnitine levels vs. the ratio of C26:0/C20:0-carnitine in DBS from newborn screening samples (black diamonds) (n = 270,000) and samples positive for the C26:0-lysoPC marker (green triangles) (n = 64). The proposed cut-off values for the C26:0-carnitine marker and the C26:0/C20:0 ratio are indicated by the dashed red lines (60 nmol/L and 1.00, respectively).

The New York State Newborn Screening Program analyzed approximately 270,000 DBS samples for C26:0-carnitine along with other very long-chain acylcarnitines. The average concentration of C26:0-carnitine in the samples was 30 ± 10 nmol/L (range <10 – 450 nmol/L). In addition, 64 DBS from C26:0-lysoPC positive newborns were tested, the average concentration was 130 ± 107 nmol/L (range 30 – 640 nmol/L). While on average samples positive for C26:0-lysoPC had a higher concentration of the C26:0-carnitine than normal newborn screening samples, there was overlap between the two groups. A C26:0-carnitine cut-off of greater than or equal to 60 nmol/L would result in 3,380 positive results from the population of approximately 270,000 samples (1.25%). A slightly more conservative cut-off of 50 nmol/L would result in 1,529 positive results from the population of approximately 270,000 samples (4.27%). For a cut-off of 60 nmol/L, 11 of the 64 positive cases would be at or below the cut-off and risk being missed. At the more conservative cut-off of 50 nmol/L, 8 of the 64 positive cases would still be at or below the proposed cut-off. The use of the ratio of the C26:0- to C20:0-carnitine was tested to improve the separation of the normal and positive cases. However, results of the ratio for the positive samples showed considerable overlap with the normal samples. Indeed, there are cases where a positive C26:0-carnitine result (>60 nmol/L) has a C26:0/C20:0 ratio that is less than 1.10. This could result in evaluation as a false negative. A representation of the results for C26:0-carnitine concentrations and C26:0-/C20:0-carnitine ratios for normal and positive samples is shown in Figure 1D.

3.2 Comparison of C26:0-lysoPC and C26:0-carnitine in DBS for diagnosing ALD in women.

Typically biochemical diagnosis of ALD involves the analysis of total VLCFA (C26:0 and the C26:0/C22:0 ratio). In women, false negative results are found in approximately 15 - 20% of cases. To investigate whether C26:0-lysoPC and/or C26:0-carnitine can be used as an alternative biomarker for the diagnosis of ALD in adults, we measured C26:0-lysoPC and C26:0-carnitine levels in DBS from 108 adult controls (males and females), 47 males with ALD and 49 women with ALD.

For C26:0-lysoPC, the average level in controls was 46 ± 12 nmol/L (range 21 – 78 nmol/L), in ALD males it was 453 ± 207 nmol/L (range 177 – 1187 nmol/L), and in ALD females it was 261 ± 111 nmol/L (range 80 – 510 nmol/L). All ALD males had elevated C26:0-lysoPC levels, and there was a clear separation between controls and ALD males. Interestingly, all ALD females
had elevated C26:0-lysoPC levels (Figure 2A). This corresponds with a sensitivity of 100% for both males and females.
For C26:0-carnitine, the average level in controls was 35 ± 18 nmol/L (range 17 – 100 nmol/L), in ALD males it was 151 ± 43 nmol/L (range 61 – 259 nmol/L), and in ALD females it was 90 ± 26 nmol/L (range 44 – 154 nmol/L). In contrast to C26:0-lysoPC, there was no complete separation between controls and ALD patients: 7/47 ALD males and 32/49 females had a C26:0-carnitine in the control range (Figure 2B). This corresponds with a sensitivity of 85% for ALD males and 35% for ALD females, respectively.
Analysis of the correlation between the C26:0-lysoPC levels and the C26:0-carnitine levels measured in the same ALD DBS showed a significant positive correlation between C26:0-lysoPC levels and C26:0-carnitine levels in DBS from control and ALD patients (Figure 2C).

**Figure 2.**
Scatterplots of C26:0-lysoPC levels (A) and C26:0-carnitine (B) in DBS from adult controls (green circles), ALD males (blue squares) and ALD females (purple triangles). The upper control values for C26:0-lysoPC (78 nmol/L) and C26:0-carnitine (100 nmol/L) are indicated by the dashed red lines. (C) Correlation plot showing the correlation between C26:0-lysoPC and C26:0-carnitine levels measured in the same ALD DBS (n = 96). Statistical analysis was performed using Spearman’s correlation test, the correlation coefficient is indicated by r. The upper control value of C26:0-carnitine (100 nmol/L) is indicated by the horizontal dotted red line, and the upper control value of C26:0-lysoPC (78 nmol/L) is indicated by the vertical dashed red line.
3.2.1 Comparison of C26:0 and C26:0/C22:0 in plasma to C26:0-lysoPC and C26:0-carnitine in DBS for diagnosing ALD in women.

To investigate whether the analysis of C26:0-lysoPC and/or C26:0-carnitine allows a more accurate diagnosis of women with ALD by biochemical testing alone, we compared the sensitivity of C26:0 levels and the C26:0/C22:0 ratio in plasma with the sensitivity of C26:0-lysoPC and C26:0-carnitine measured in DBS. To this end, VLCFA levels were analyzed in plasma from the same blood sample that was used to generate the DBS for the C26:0-lysoPC and C26:0-carnitine measurements. A complete data set was available from 20 female controls and 46 women diagnosed with ALD (Figure 3). The average C26:0 level in female controls was 1.40 ± 0.40 (range 0.72 – 2.20) and the average C26:0/C22:0 ratio in female controls was 0.023 ± 0.005 (range 0.015 – 0.033). Six out of 46 women with ALD had a normal C26:0 level and C26:0/C22:0 ratio in plasma (Figure 3A and B). This corresponds to an 87% sensitivity for plasma VLCFA analysis in this cohort. The average C26:0-lysoPC level in female controls was 49 ± 16 nmol/L (range 21 – 77 nmol/L). None of the women with ALD had a C26:0-lysoPC level within the normal range (Figure 3C). The lowest measured C26:0-lysoPC level was 80 nmol/L. In our cohort the sensitivity of C26:0-lysoPC was 100%. The average C26:0-carnitine level in female controls was 56 ± 22 nmol/L (range 23 – 100 nmol/L). Twenty-nine of the 46 women with ALD had a C26:0-carnitine level in the normal range (Figure 3D), which corresponds to a sensitivity of 37%. Analysis of the correlation between the plasma C26:0/C22:0 ratio and the C26:0-lysoPC level measured in the same blood sample showed a significant positive correlation between C26:0/C22:0 ratios and C26:0-lysoPC levels (Figure 3E). Importantly, the 6 samples from ALD females with a normal VLCFA profile in plasma had elevated C26:0-lysoPC levels in DBS.
Figure 3.
Scatterplots of C26:0 levels (A) and C26:0/C22:0 ratio (B) in plasma, and C26:0-lysoPC levels (C) and C26:0-carnitine levels (D) in DBS from female controls (green circles) and ALD females (purple triangles). The upper control values for C26:0 (2.20 μmol/L), C26:0/C22:0 (0.033), C26:0-lysoPC (77 nmol/L) and C26:0-carnitine (100 nmol/L) are indicated by the dashed red lines. (E) Correlation plot showing the
correlation between the C26:0/C22:0 ratio in plasma and the C26:0-lysoPC level measured in a DBS collected from the same blood sample (n = 46). Statistical analysis was performed using Spearman's correlation test, the correlation coefficient is indicated by $r$. The upper control value of the plasma C26:0/C22:0 ratio (0.033) is indicated by the vertical dashed red line, and the upper control value of C26:0-lysoPC (77 nmol/L) is indicated by the horizontal dashed red line.

4. Discussion

The first objective of this study was to compare the sensitivity of C26:0-lysoPC and C26:0-carnitine in DBS from control and ALD newborns. To this end, we performed an experiment with 200 controls and 11 ALD newborn DBS in our laboratory. The analysis of newborn samples showed that C26:0-lysoPC levels were increased in all ALD newborns. In contrast, 2 independent samples derived from 1 ALD newborn had a C26:0-carnitine level that fell into the control range. Whereas the sensitivity of C26:0-lysoPC was 100%, the sensitivity of C26:0-carnitine was 82%. In parallel, C26:0-carnitine was measured in 270,000 newborn DBS in the New York State screening program. The possibility of using C26:0-carnitine as a marker instead of C26:0-lysoPC was appealing as this would allow for it to be included with other MS/MS testing using similar markers and would eliminate the need for a separate extraction method and test for ALD. In the evaluation of C26:0-carnitine as a potential marker, New York State retested samples from screen positive newborns that were identified using C26:0-lysoPC and determined a significant overlap between the concentrations of routine newborn screening samples and samples determined to be screen positive by C26:0-lysoPC HPLC-MS/MS analysis. In the first year of ALD newborn screening 20 referrals for ALD were reported by the New York State program based on the C26:0-lysoPC. Of these, 15 had a mutation in the $ABCD1$ gene, 4 were diagnosed as another disorder, and one baby passed away before a diagnosis was obtained. However, when 64 screen positive samples identified by the New York State program were tested for the C26:0-carnitine marker, 11 cases (17%) were at or below a cut-off set at 60 nmol/L. These cases are at risk of being missed. From these data we conclude that C26:0-lysoPC is superior in comparison with C26:0-carnitine for the diagnosis and screening of ALD in newborns.

Why did C26:0-carnitine fail as a sensitive biomarker for ALD? Our data show that there is no significant difference between the C26:0-lysoPC levels in newborn controls (65 ± 16 nmol/L (range 36 – 138 nmol/L)) and the C26:0-lysoPC levels in adult controls (46 ± 12 nmol/L (range 21 – 78 nmol/L)). In contrast, the levels of C26:0-carnitine in newborn controls (mean 15 ± 5 nmol/L (range 5 – 31 nmol/L)) were strikingly lower than those in adult controls (35 ± 18 nmol/L (range 17 – 100 nmol/L)). This difference between newborns and adults may be related to a dietary effect. An earlier study in a cohort of 26 disabled patients showed that plasma levels of free carnitine were significantly higher in the patients with solely food intake in comparison with formula fed patients. We hypothesize that a similar dietary effect
causes our observed variation in C26:0-carnitine levels between newborns and adults. In the newborn control group about 10% of newborns seem to have a slightly higher level of C26:0-carnitine than the gross majority. This variation could possibly be attributed to a difference in nutrition (e.g. breast feeding versus formula). In 2015, 80% (95% CI 78-82) of Dutch newborns were breastfed directly after birth. In a cohort of 20 formula-fed and 18 breast-fed preterm babies an increase in free carnitine levels in plasma was observed in the formula, but not in the breast-fed group.

The second objective of our study was to investigate if the measurement of C26:0-lysoPC and/or C26:0-carnitine in DBS may lower the number of false negative results in women with ALD. The analysis of adult ALD patients revealed that 7/47 ALD males (15%) and 32/49 females (65%) had a C26:0-carnitine level that fell into the control range. In contrast to C26:0-carnitine, C26:0-lysoPC levels were increased in all adult ALD males and also in all ALD females. This is of great importance for the diagnosis of women suspected of ALD. It is known that only 85% of women with ALD have abnormal VLCFA levels in plasma and fibroblasts. Also in our cohort 6/46 (13%) women with ALD had a normal plasma VLCFA profile. Therefore, it is recommended to perform ABCD1 mutation analysis in women suspected of ALD. However, if DNA analysis yields a sequence variant of unknown significance (VUS) in combination with normal levels of VLCFA in plasma and/or fibroblasts a diagnostic dilemma arises. Recently, a diagnostic test was described that is based on generating clonal cell lines that express only one of the two alleles. The test allows accurate analysis of the effect of a variant allele within a physiological intact system. However, the disadvantage is that the clonal method is both costly and time-consuming. Therefore, the availability of a fast and accurate test that is based on routine analysis of C26:0-lysoPC in DBS is a major step forward. Importantly, C26:0-lysoPC was elevated in DBS from the 6 ALD females with a normal VLCFA levels in plasma. This observation is in line with findings in the ALD knockout mouse. We and others have previously demonstrated that ALD knockout mice have normal plasma C26:0 levels and a normal C26:0/C22:0 ratio, but C26:0-lysoPC in DBS was highly elevated. This indicates that, at least in mice, the plasma C26:0/C22:0 ratio does not reflect the elevated C26:0 levels in spinal cord or brain. In contrast, C26:0-lysoPC levels in mouse DBS do correlate with the VLCFA levels in CNS tissues. The availability of a more sensitive biomarker that better reflects the biochemical deficiency in ALD is warranted. Using plasma VLCFA levels, false-negative results are seen in 15 – 20% of women with ALD, but they have also been reported in 2 male ALD cases. False-positive results in individuals without a defect in peroxisomal VLCFA beta-oxidation have also been reported. For example, hemolysis of the blood sample, diabetic ketoacidosis, a ketogenic diet and non-fasted blood samples obtained from individuals with a high peanut (butter) consumption can cause increased VLCFA levels. In plasma obtained from patients with an impaired liver function, the C26:0/C22:0 ratio may become increased, but this is due to a decrease in C22:0 levels and not an elevation in
C26:0 levels. It remains to be determined whether C26:0-lysoPC levels remain unaffected in the above described conditions. Interestingly, the newborn screening program in New York State unintentionally identified a newborn with elevated C26:0-lysoPC, but without a variant in the \textit{ABCD1} gene. The infant was diagnosed with Aicardi Goutières Syndrome (AGS). Subsequent analysis of 18 retrospectively collected newborn screening cards from children with confirmed AGS revealed that 13 out of 19 had elevated C26:0-lysoPC in DBS.

\textbf{5. Concluding remarks}

From these data, we conclude that C26:0-lysoPC is superior in comparison with C26:0-carnitine for the diagnosis of ALD in newborns. Furthermore, we conclude that C26:0-lysoPC is a better and more accurate biomarker for ALD than plasma VLCFA levels. We recommend that C26:0-lysoPC be added to the routine biochemical tests for peroxisomal beta-oxidation disorders.

\textbf{Acknowledgements}

This work was supported by a grant from the European Leukodystrophy Association (ELA International grant (ELA 2015-015I to SK) and a research grant from Bluebird Bio.
References

Comparison of C26:0-carnitine and C26:0-lysoPC as diagnostic markers in dried blood spots


