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# Evaluation of C26:0-lysophosphatidylcholine and C26:0-carnitine as diagnostic markers for Zellweger spectrum disorders

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## Abstract

*Introduction* Zellweger spectrum disorders (ZSD) are a group of genetic metabolic disorders caused by a defect in peroxisome biogenesis. This results in multiple metabolic abnormalities, including elevated very long-chain fatty acid (VLCFA) levels. Elevated levels of C26:0-lysophosphatidylcholine (C26:0-lysoPC) have been shown in dried blood spots (DBS) from ZSD patients. However, little is known about the sensitivity and specificity of this marker and C26:0-carnitine, another VLCFA-marker, in ZSD. We investigated C26:0-lysoPC and C26:0-carnitine as diagnostic markers for ZSD in DBS and fibroblasts.

*Methods* C26:0-lysoPC levels in 91 DBS from 37 different ZSD patients were determined and compared to the levels in 209 control DBS. C26:0-carnitine levels were measured in 41 DBS from 29 ZSD patients and 97 control DBS. We measured C26:0-lysoPC levels in fibroblasts from 24 ZSD patients and 61 control individuals.

*Results* Elevated C26:0-lysoPC levels (>72 nmol/L) were found in 86/91 ZSD DBS (n=33/37 patients) corresponding to a sensitivity of 89.2%. Median level was 567 nmol/l (range 28–3133 nmol/l). Consistently elevated C26:0-carnitine levels (>0.077 µmol/L) in DBS were found in 16 out of 29 ZSD patients corresponding to a sensitivity of 55.2%. C26:0-lysoPC levels were elevated in 21/24 ZSD fibroblast lines.

*Discussion* C26:0-lysoPC in DBS is a sensitive and useful marker for VLCFA accumulation in patients with a ZSD. C26:0-carnitine in DBS is elevated in some ZSD patients, but is less useful as a diagnostic marker. Implementation of C26:0-lysoPC measurement in the diagnostic work-up when suspecting a ZSD is advised. This marker has the potential to be used for newborn screening for ZSD.

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**Abbreviations**

| | |
|---|---|
| ALD | Adrenoleukodystrophy |
| C26:0-lysoPC | C26:0-lysophosphatidylcholine |
| DBP | D-bifunctional protein deficiency |
| DBS | Dried blood spots |
| LC-MS/MS | Liquid chromatography-tandem mass spectrometry |
| SED | Single peroxisomal enzyme deficiency |
| UPLC-MS/MS | Ultra-performance liquid chromatography-tandem mass spectrometry |
| VLCFA | Very long-chain fatty acids |
| ZSD | Zellweger spectrum disorder |

## Introduction

Zellweger spectrum disorders (ZSD) are a group of genetic metabolic disorders caused by a defect in peroxisome biogenesis due to mutations in one of the *PEX* genes. Since peroxisomes are involved in a variety of metabolic pathways (e.g. β-oxidation of very long-chain fatty acids (VLCFA), α-oxidation of phytanic acid, bile acid synthesis, and plasmalogen biosynthesis) a defect in peroxisome biogenesis leads to multiple metabolic abnormalities in these patients (Wanders and Waterham 2006). Patients can present with a variety of symptoms and the severity ranges from death in infancy to adults with an isolated vision and hearing deficit (Klouwer et al 2015). With an estimated incidence of 1:50.000 (Gould et al 2001) ZSDs are considered rare, however, an increasing number of patients with a relatively mild phenotype have been identified in recent years (Régal et al 2010; Ebberink et al 2010; Sevin et al 2011; Mignarri et al 2012; Ebberink et al 2012; Ratbi et al 2015; Renaud et al 2016; Ventura et al 2016).

ZSD patients at the mild end of the spectrum can be especially challenging to diagnose since biochemical parameters typical for a peroxisomal disorder can be normal in plasma and urine. In addition, biochemical abnormalities tend to normalize in aging ZSD patients (Klouwer et al 2016; Berendse et al 2016). As a consequence, diagnosis is often delayed until additional symptoms occur or affected siblings are diagnosed. It is likely that an undiagnosed group of relatively mild ZSD patients exists as they lack the classical biochemical hallmarks. Hence, there is a need for new and more sensitive biomarkers for ZSD, preferably suitable for (neonatal) screening programs.

C26:0-lysophosphatidylcholine (C26:0-lysoPC) is known to be elevated in dried blood spots (DBS) of patients with adrenoleukodystrophy (ALD), a single peroxisomal enzyme deficiency leading to the accumulation of VLCFAs (Hubbard et al 2006). A high-throughput liquid chromatography-tandem mass spectrometric (LC-MS/MS) method has been established to measure C26:0-lysoPC in DBS (Hubbard et al 2009) and this method has already been implemented in newborn screening programs for ALD in some countries (Vogel et al 2015). In studies on C26:0-lysoPC levels in ALD patients a limited number of ZSD patients with elevated C26:0-lysoPC levels have also been identified (Hubbard et al 2006; Haynes and De Jesús 2016), but no specific studies on C26:0-lysoPC accumulation in ZSD patients have been performed. Also, little is known about the sensitivity and specificity of this biomarker for ZSD.

In addition, recent studies have suggested C26:0-carnitine as a potential new biomarker for ALD (van de Beek et al 2016). Since acylcarnitine analysis is already implemented in most newborn screening panels, it is hypothesized that a minor modification of this existing method would allow high-throughput screening for ALD without additionally measuring C26:0-lysoPC (van de Beek et al 2016). Elevated levels of C26:0-carnitine has been demonstrated in urine of ZSD patients (Duranti et al 2008), but it has not been studied yet whether C26:0-carnitine in DBS could be a good marker for ZSDs.

In this article we present the results of the measurement of C26:0-lysoPC and C26:0-carnitine measurements in DBS in a well characterized, large cohort of phenotypically and genetically heterogeneous ZSD patients and in a large cohort of healthy individuals. In addition, we studied C26:0-lysoPC levels as marker in fibroblasts of ZSD patients.

## Methods

### Patients

All routine measurements of C26:0-lysoPC and C26:0-carnitine performed at the Laboratory Genetic Metabolic Diseases in the AMC between January 2012 and April 2016 were collected and used for this study. C26:0-lysoPC and C26:0-carnitine measurements of patients with a known ZSD or D-bifunctional protein (DBP) deficiency, a peroxisomal single enzyme deficiency (SED) affecting peroxisomal VLCFA oxidation, were analyzed separately. Patients known to have ALD were excluded. The remainder of the C26:0-lysoPC and C26:0-carnitine results were combined and labeled as the control group. Approval of the Research Ethics Committee was not required, since all measurements were performed as part of diagnostic procedures or standard patient care and data were anonymized for further analysis.

### Biochemical measurements

#### Measurement of C26:0-lysoPC and C26:0-carnitine in DBS

Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was performed as described before (van de Beek et al 2016) with minor modifications as summarized below. A punch (quarter-inch (6.4 mm) diameter) of a DBS was transferred to a 2.0 ml tube, to which 10 μl of 1 μM $d_4$-C26:0-lysoPC and 10 μl of 0.1 μM $d_4$-C26:0-carnitine was added, followed by 500 μL methanol. This was incubated for 5 min at room temperature in a sonicator bath (Branson 3510). The extraction fluid was transferred to a 4-mL glass tube and the sample was taken to dryness under nitrogen flow at 40 °C. The residue was reconstituted in 50 μl of methanol, transferred to a sample vial, and capped. Ten μL of extract was injected into the UPLC-MS system. LC was performed on an Acquity UPLC system (Waters, Milford, MA) using a Kinetex C8 column (50*2.1 mm, 2.6 μm particle size (Phenomenex, Utrecht, The Netherlands) at 50 °C. Metabolites were

separated by a linear gradient between solution A (0.1% (v/v) formic acid in water) and solution B (0.1% (v/v) in methanol). The UPLC run was 12 min at a flow rate of 0.4 ml/min. All gradient steps were linear and the gradient was as follows: 0–6 min, 64%–100% B; 6–11 min, 100% B isocratic; 11–12 min, equilibration at 64% B. For the mass spectrometric detection, a Micromass Quattro Premier XE Tandem Mass Spectrometer (Waters, Milford, MA) was used in the multiple reaction monitoring mode (MRM) in the positive electrospray ionization mode. The spray voltage used was 3.5 kV, source temperature was 130 °C, desolvation temperature was 350 °C, cone gas flow was 50 l/h, desolvation gas flow was 900 l/h and collision gas pressure 2.5–3.0 $10^{-3}$ mbar. For C26:0-carnitine the following MRMs were used: C26:0-carnitine (540.5 > 85.0), $d_4$-C26:0-carnitine (544.5 > 85.0) 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 (636.5 > 104.1) and $d_4$-C26:0-lysoPC (640.5 > 104.1) both using a dwell time of 0.030 s, a cone voltage 53 V and collision energy of 31 eV. C26:0-carnitine and C26:0-lysoPC levels were calculated using Masslynx 4.0 software using the Quanlynx module.

### Measurement of C26:0-lysoPC in fibroblast homogenates

Fibroblasts pellets were homogenized in 300 µl of PBS and the suspension was sonicated for 20 s at 8 W output with a sonicator probe (Vibra-Cell, Sonics & materials, Newtown, CT). The measurement of C26:0-lysoPC was performed as described for DBS except that 0.1 mg of fibroblast protein homogenate was used as starting material.

### Other peroxisomal parameters

For some patients, additional data on peroxisomal parameters in blood was available. Plasma VLCFA, phytanic acid, pristanic acid (Vreken et al 1998), plasma bile acids (Bootsma et al 1999), pipecolic acid (Rashed et al 2001), and plasmalogens in erythrocytes (Dacremont and Vincent 1995) were measured in the Laboratory Genetic Metabolic Diseases in the AMC as part of standard diagnostic procedures or standard patient care.

## Results

### Patient characteristics

C26:0-lysoPC levels were measured in 91 DBS from 37 different ZSD patients. The average age of the patients was 15.6 years (range 0–49 years). The average age of the control group consisting of 209 individuals was 16.6 years (range 0–81.7 years). Specific biallelic *PEX* mutations were known for

all ZSD patients, except one. Twenty-nine patients had mutations in *PEX1* of whom 18 were homozygous for the common *PEX1* p.G843D mutation (50% of the patients). Three patients had mutations in *PEX6*, two in *PEX10*, and two in *PEX26* (Table 1).

C26:0-carnitine levels were measured in 41 DBS from 29 different ZSD patients. The average age of the patients was 15.2 years (range 0–49 years). The average age of the control group for C26:0-carnitine measurements consisting of 97 individuals was 16.1 years (range 0–81.7 years). Specific biallelic *PEX* mutations were known for 28 ZSD patients (Table 1).

C26:0-lysoPC levels were measured in skin fibroblasts of 24 ZSD patients with mutations in nine different *PEX* genes (average age of patients 3.1 years, range 0–22.1 years) and in control skin fibroblasts of 61 individuals (average age of 14.8 years, range 0–68.3 years). Specific biallelic *PEX* mutations were known for 23 ZSD patients (Table 2)

### C26:0-lysoPC

The median level of all 91 C26:0-lysoPC measurements in DBS of ZSD patients was 567 nmol/L (range 28–3133 nmol/L) (Fig. 1a). The current control reference range of C26:0-lysoPC in DBS in our laboratory is 29–72 nmol/L. Four out of 37 ZSD patients had levels of C26:0-lysoPC within this control reference range corresponding to a sensitivity of 89.2% in this cohort (median 40 nmol/L, range 28–60 nmol/L). For one of these patients, two different DBS sampled at

**Table 1** Patient characteristics for C26:0-lysoPC and C26:0-carnitine measurements in DBS

| C26:0-lysoPC in DBS | ZSD (n = 37) | Control (n = 209) |
|---|---|---|
| Gender (male) | 16 (43%) | 135 (65%) |
| Average age (years) | 15.6 (range 0–49) | 16.6 (range 0–81.7) |
| Mutations | | |
| *PEX1* | n = 29 | |
| *PEX6* | n = 3 | |
| *PEX10* | n = 2 | |
| *PEX26* | n = 2 | |
| Unknown | n = 1 | |
| C26:0-carnitine in DBS | ZSD (n = 29) | Control (n = 97) |
| Gender (male) | 13 (45%) | 58 (60%) |
| Average age (years) | 15.2 (range 0–49) | 16.1 (range 0–81.7) |
| Mutations | | |
| *PEX1* | n = 23 | |
| PEX6 | n = 2 | |
| PEX10 | n = 2 | |
| PEX26 | n = 1 | |
| Unknown | n = 1 | |

*Abbreviations:* C26:0-lysoPC: C26:0-lysophosphatidylcholine, DBS: dried blood spot, ZSD: Zellweger spectrum disorder

**Table 2** Patient characteristics for C26:0-lysoPC measurements in cultured skin fibroblasts

| C26:0-lysoPC in fibroblasts | ZSD ($n = 24$) | Control ($n = 63$) |
|---|---|---|
| Gender (male) | 16 (67%) | 35 (56%) |
| Average age (years) | 3.1 (range 0–22.1) | 15.7 (range 0–68.3) |
| Mutations | | |
| *PEX1* | $n = 8$ | |
| *PEX2* | $n = 3$ | |
| *PEX3* | $n = 3$ | |
| *PEX6* | $n = 3$ | |
| *PEX10* | $n = 2$ | |
| *PEX12* | $n = 1$ | |
| *PEX13* | $n = 1$ | |
| *PEX16* | $n = 1$ | |
| *PEX19* | $n = 1$ | |
| Unknown | $n = 1$ | |

*Abbreviations:* C26:0-lysoPC: C26:0-lysophosphatidylcholine, ZSD: Zellweger spectrum disorder

different time points were analyzed, which showed comparable results. Two out of these four patients (10 and 49 years old respectively) were compound heterozygous for *PEX10* mutations. Both these patients have a relatively mild phenotype with predominantly ataxia and clearly elevated bile acid intermediates in plasma. One patient (34 years old) was reported earlier as having Heimler syndrome, a very mild ZSD variant (Ratbi et al 2015). The fourth patient was 2 years old; the results of genetic testing and VLCFA levels in plasma were not available for this patient. The three other patients had normal C26:0 levels in the plasma sample from which the DBS was generated. However, both *PEX10* patients did have subtle abnormalities of the C26:0/C22:0 ratio in plasma.

Interestingly, five ZSD patients showed clearly elevated C26:0-lysoPC levels in DBS (median 239 nmol/L, range 124–410 nmol/L), but normal C26:0 levels in the plasma

collected from the same blood sample. In one patient, an elevated C26:0-lysoPC level was the only detectable biochemical abnormality in blood clearly pointing toward a peroxisomal disorder (Table 3 patient #1). Other patients did have subtle abnormalities of additional peroxisomal parameters, but only the C26:0-lysoPC level was clearly elevated (for example Table 3, patient #2).

In general, more severe ZSD patients tended to have higher C26:0-lysoPC levels in DBS. This leads to a negative correlation between C26:0-lysoPC levels in DBS and age at the time of the analysis (Fig. 2a), since a natural selection of relatively mild patients occurred in this cohort over time.

To compare the sensitivity of C26:0-lysoPC in DBS with the sensitivity of VLCFA in plasma, we analyzed the levels of C26:0 and the C26:0/C22:0 and C24:0/C22:0 ratios in plasma taken from the same blood sample that was used to generate the DBS for the C26:0-lysoPC measurements. VLCFA data were available for 90 out of 91 measurements in ZSD patients. Eight out of 36 patients had normal levels of C26:0 in plasma, corresponding to a sensitivity of 77.1% in this cohort. C24:0/C22:0 ratio was normal in four out of 36 patients (sensitivity: 88.9%) and three out of 36 patients had a normal C26:0/C22:0 ratio (sensitivity: 91.7%) (Suppl. Fig. 1).

The median level of C26:0-lysoPC in the control group was 39 nmol/L (range 0–147 nmol/L) (Fig. 1a). Fourteen out of 209 individuals in the control group had levels of C26:0-lysoPC outside the control reference range (>72 nmol/L) (median 86.5 nmol/L, range 74–174 nmol/L). We re-analyzed nine of these 14 specific DBS, which showed similar results. Two out of the 14 individuals had a normal C26:0-lysoPC level in DBS from a different time point.

### C26:0-carnitine

The median level of all 41 C26:0-carnitine measurements in DBS of ZSD patients was 0.106 μmol/L (range 0.030–
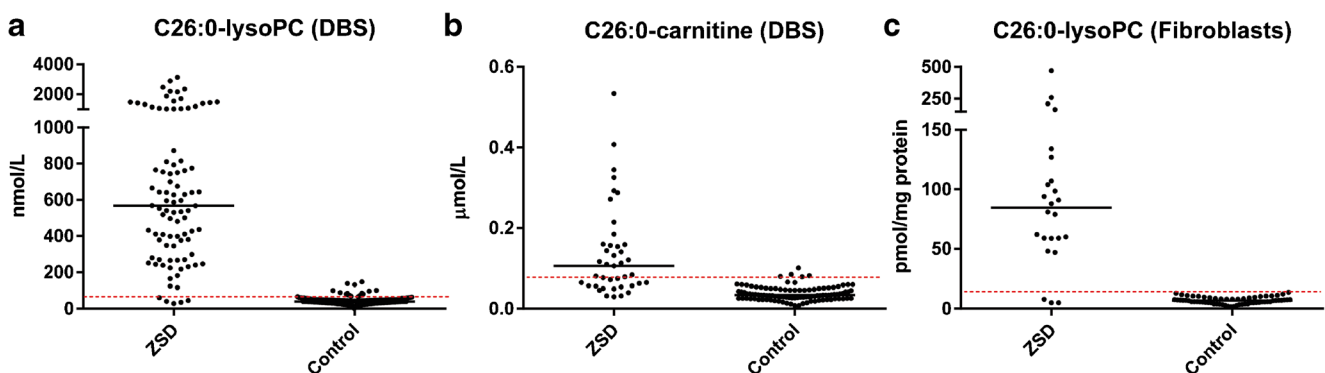


**Fig. 1** **a** Scatterplot of C26:0-lysoPC levels in DBS from ZSD patients and control individuals. The upper control reference range (72 nmol/L) is indicated by the dotted red line, the median is shown as a solid line. **b** Scatterplot of C26:0-carnitine levels in DBS from ZSD patients and control individuals. The upper control reference range (0.077 μmol/L)

is indicated by the dotted red line, the median is shown as a solid line. **c** Scatter plot of C26:0-lysoPC levels in skin fibroblasts from ZSD patients and control individuals. The upper control reference range (14 pmol/mg protein) is indicated by the dotted red line, the median is shown as a solid line

**Table 3** Individual biochemical profiles of four patients with elevated C26:0-lysoPC levels

| Patient number | #1 | #2 | #3 | #4 |
|---|---|---|---|---|
| Diagnosis | ZSD | ZSD | DBP | DBP |
| Biochemical parameter | | | | |
| C26:0-lysoPC (DBS) | **279 nmol/L** | **410 nmol/L** | **92 nmol/L** | **226 nmol/L** |
| Ref: 29–72 nmol/L | | | | |
| C26:0 (plasma) | 1.28 µmol/L | 1.24 µmol/L | 0.55 µmol/L | 1.25 µmol/L |
| Ref: 0.45–1.32 µmol/L | | | | |
| Ratio C24:0/C22:0 (plasma) | **0.95** | **1.03** | 0.8 | 0.83 |
| Ref: 0.57–0.92 | | | | |
| Ratio C26:0/C22:0 (plasma) | 0.02 | **0.03** | 0.01 | 0.02 |
| Ref: 0.0–0.02 | | | | |
| C16:0-dimethylacetal (erythrocytes) | **6.5%** | 7.9% | nm | nm |
| Ref: 6.8–11.9% | | | | |
| C18:0-dimethylacetal (erythrocytes) | 16.3% | 18.9% | nm | nm |
| Ref: 10.6–24.9% | | | | |
| Phytanic acid (plasma) | 0.9 µmol/L | 5.7 µmol/L | 1.7 µmol/L | 1.1 µmol/L |
| Ref: 0.5–9.9 µmol/L | | | | |
| Pristanic acid (plasma) | 0.1 µmol/L | 1.7 µmol/L | 0.1 µmol/L | 0.1 µmol/L |
| Ref: 0.1–3.0 µmol/L | | | | |
| Pipecolic acid (plasma) | 5.5 µmol/L | **19 µmol/L** | nm | 1.3 µmol/L |
| Ref: 0.1–7.0 µmol/L | | | | |
| DHCA (plasma) | 0.0 µmol/L | **0.2 µmol/L** | 0.0 µmol/L | 0.0 µmol/L |
| Ref: 0.0–0.0 µmol/L | | | | |
| THCA (plasma) | 0.0 µmol/L | **0.4 µmo/L** | 0.0 µmol/L | 0.0 µmol/L |
| Ref: 0.0–0.1 µmol/L | | | | |

Values outside the control reference range are presented in bold. Abbreviations: nm: not measured, ref.: control reference range, ZSD: Zellweger spectrum disorder, DBP: D-bifunctional protein deficiency, C26:0-lysoPC: C26:0-lysophosphatidylcholine, DHCA: $3\alpha,7\alpha$-dihydroxycholestanoic acid, THCA: $3\alpha,7\alpha,12\alpha$-trihydroxycholestanoic acid

0.534 µmol/L) (Fig. 1b). The current control reference range for C26:0-carnitine in our laboratory is 0.014–0.077 µmol/L. Thirteen out of 29 ZSD patients had normal levels of C26:0-carnitine ($\leq 0.077$ µmol/L) corresponding to a sensitivity of 55.2% in this cohort (median 0.056 µmol/L, range 0.030–

0.077). However, three of these patients had elevated levels of C26:0-carnitine in DBS from different time points.

The control group showed a median C26:0-carnitine level of 0.034 µmol/L (range 0.008–0.101 µmol/L) (Fig. 1b). Five out of 97 individuals in the control group had elevated levels
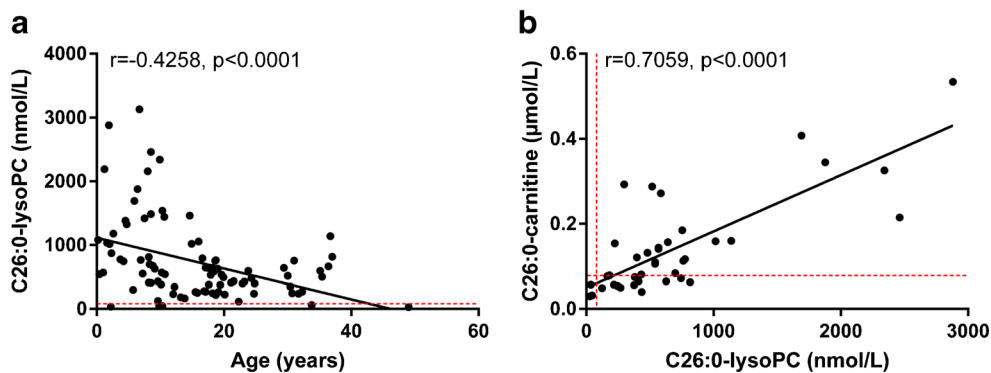


Fig 2. **a** Scatterplot showing the correlation between C26:0-lysoPC levels and the age of the patient at the time of the analysis. Statistical analysis was performed using Spearman's correlation test, the correlation coefficient is indicated by r. The upper control reference range of C26:0-lysoPC (72 nmol/L) is indicated by the dotted red line. **b** Scatterplot showing the correlation between C26:0-carnitine and C26:0-lysoPC

levels measured in the same DBS. Statistical analysis was performed using Spearman's correlation test, the correlation coefficient is indicated by r. The upper control reference range of C26:0-lysoPC (72 nmol/L) is indicated by the vertical dotted red line, the upper control reference range of C26:0-carnitine (0.077 µmol/L) is indicated by the horizontal dotted red line

of C26:0-carnitine (>0.077 μmol/L) (median 0.082 μmol/L, range 0.079–0.101 μmol/L). Two of the five individuals showed a normal C26:0-carnitine level in DBS from a different time point.

For 40 cases, C26:0-carnitine levels and C26:0-lysoPC levels measured in the same DBS were available for correlation analysis. This showed a significant positive correlation between C26:0-carnitine levels and C26:0-lysoPC levels in DBS from ZSD patients (Fig. 2b).

### C26:0-lysoPC in cultured skin fibroblasts

The median level of C26:0-lysoPC measured in ZSD skin fibroblasts was 85 pmol/mg protein (range 5–469 pmol/mg protein) (Fig. 1c). Our C26:0-lysoPC reference range in control skin fibroblasts is set at 2–14 pmol/mg protein. Three out of 24 patients had normal levels (≤ 14 pmol/mg protein) of C26:0-lysoPC in skin fibroblasts (median 5 pmol/mg protein, range 5–8 pmol/mg protein) corresponding to a sensitivity of 87.5%. One of these patients also had a normal level of C26:0-lysoPC in DBS. No C26:0-lysoPC levels in DBS were available for the other two patients. These three patients also showed normal total levels of C26:0 in skin fibroblasts. The 21 patients with elevated levels of C26:0-lysoPC in skin fibroblasts also had elevated total levels of C26:0 in skin fibroblasts.

### C26:0-lysoPC in DBP deficiency

C26:0-lysoPC levels measured in DBS of two patients with a DBP deficiency were available. Both had an increased C26:0-lysoPC level (226 nmol/L and 92 nmol/L respectively). The first patient showed normal C26:0-lysoPC levels in skin fibroblasts, measured 3 years earlier at the age of 4 years (13 pmol/mg protein). Both patients had no other abnormal peroxisomal parameters in plasma, measured in the same blood sample (Table 3 patient #3 and #4). For one DBP patient, C26:0-lysoPC was measured in skin fibroblasts only, which was normal (5 pmol/mg protein) at the age of 11 years. The VLCFA profile in skin fibroblasts of this patient was also completely normal. No C26:0-carnitine levels were determined for the DBP deficient patients.

### Discussion

Here, we show that the C26:0-lysoPC concentration in DBS is a sensitive marker for VLCFA accumulation in ZSD patients. In individual cases, C26:0-lysoPC in DBS was even more sensitive than other known peroxisomal plasma markers like VLCFA, bile acid intermediates, phytanic acid, and pristanic acid. This is of great importance, since more ZSD patients with a relatively mild or atypical phenotype have been

recognized over recent years, causing diagnostic challenges (Klouwer et al 2016). Based on our results we recommend to include measurement of C26:0-lysoPC in DBS in the standard diagnostic work-up when suspecting a ZSD or clinically indistinguishable SED (i.e., DBP deficiency, Acyl-CoA oxidase type 1 deficiency).

Evidently, not all ZSD patients can be identified by measuring C26:0-lysoPC in DBS. As our results show, a small subset of ZSD patients had normal C26:0-lysoPC levels in DBS. However, it must be noted that C26:0 levels in plasma isolated from the same blood sample in all these patients were also normal. Since the underlying mechanism of elevated C26:0-lysoPC is most likely an overflow mechanism reflecting the intracellular accumulation of VLCFAs, it should be taken into account that some mild ZSD patients, or patients with certain PEX mutations, may not accumulate VLCFAs at all or not anymore at a later age (Berendse et al 2016). Specific attention seems warranted for patients with PEX10 mutations (and most likely also patients with PEX2 and PEX12 mutations), since both PEX10 patients in this cohort showed normal C26:0-lysoPC levels in DBS. Full biochemical work-up, including analyses in fibroblast, therefore will remain necessary in the diagnostics of peroxisomal disorders.

Newborn screening for ZSD could be feasible using C26:0-lysoPC as a biomarker. However, based on our dataset it can be anticipated that some ZSD patients will be left undiagnosed. Since both PEX10 patients in this cohort showed clearly elevated bile acid intermediates, we hypothesize that the combined analysis of C26:0-lysoPC and $C_{27}$-bile acid intermediates (i.e., $3\alpha,7\alpha$-dihydroxycholestanoic acid and $3\alpha,7\alpha,12\alpha$-trihydroxycholestanoic acid) could increase the sensitivity of such a screening procedure. It must be noted that the average age of ZSD patients in this study was 15.6 years and it cannot be ruled out that the sensitivity of C26:0-lysoPC measurement in DBS in the newborn period is different than reported here.

The control reference range of C26:0-lysoPC in DBS was previously set at 29–72 nmol/L in our laboratory. Based on the data from this study the upper control reference limit has been increased to 100 nmol/L. Ten out of the 14 false-positive results in the control group are below this limit, while no ZSD patients other than the already reported four patients with normal C26:0-lysoPC levels, have levels below 100 nmol/L.

Our study also shows that measurement of C26:0-carnitine in DBS is of limited value for the diagnostics of ZSD patients, since only 55% of the ZSD patients in this cohort showed elevated levels of C26:0-carnitine in DBS. This is an important finding since it was previously suggested that measuring C26:0-carnitine in DBS for ALD newborn screening would allow high-throughput screening for ALD without measuring C26:0-lysoPC in addition (van de Beek et al 2016). In the current ALD newborn screening programs measuring C26:0-lysoPC in DBS, the majority of ZSD patients will also

be identified. However, only a small part of ZSD patients will probably be identified during ALD newborn screening based on C26:0-carnitine alone.

Lastly, we analyzed C26:0-lysoPC levels in skin fibroblast of ZSD patients. It must be noted that a selection bias of relatively mild patients is likely for the skin fibroblast measurements, since fibroblast analyses in several of the cases studied were only done when diagnosis was unclear after extensive biochemical analyses in plasma. However, this study shows that C26:0-lysoPC also is a good marker for C26:0 accumulation in fibroblasts and sample work-up is faster than for VLCFA determination followed by gas chromatography-mass spectrometry.

In conclusion, our results show that C26:0-lysoPC in DBS is a sensitive and useful marker for VLCFA accumulation in patients with a ZSD. C26:0-carnitine in DBS is elevated in some patients, but is less useful as a diagnostic marker for ZSD. C26:0-lysoPC levels are also elevated in skin fibroblasts of most ZSD patients and in DBS of DBP deficient patients, but more data is needed to determine the sensitivity of these measurements.

**Compliance with ethical standards**   Approval of the Research Ethics Committee was not required, since all measurements were performed as part of diagnostic procedures or standard patient care and data were anonymized for further analysis.

**Conflict of interest**   F. C. C. Klouwer, S. Ferdinandusse, H. Lenthe, W. Kulik, R. J. A. Wanders, B. T. Poll-The, H. R. Waterham, and F. M. Vaz declare that they have no conflict of interest.

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