Profiles of Very-Long-Chain Fatty Acids in Plasma, Fibroblasts, and Blood Cells in Zellweger Syndrome, X-Linked Adrenoleukodystrophy, and Rhizomelic Chondrodysplasia Punctata

Ruud B. H. Schutgens,1 Idwin W. Bouman, Alie A. Nijenhuis, Ronald J. A. Wanders, and Marielle E. J. Frumau

Profiles of saturated very-long-chain (>C22) fatty acids were studied in plasma, fibroblasts, erythrocytes, platelets, and leukocytes of patients affected by peroxisomal disorders such as Zellweger syndrome, X-linked adrenoleukodystrophy (X-ALD), and classic rhizomelic chondrodysplasia punctata (RCDP) and in controls. In Zellweger patients, the concentration of hexacosanoic acid (C26:0) and the C26:0/C22:0 ratio is greatly increased in plasma and fibroblasts. However, the plasma concentration of docosanoic acid (C22:0) is greatly decreased. Also in platelets, erythrocytes, and to a lesser extent erythrocytes, the C26:0 concentrations and both the C26:0/C22:0 and C24:0/C22:0 ratios are greatly increased. The C24:0/C22:0 ratio is significantly increased in plasma, platelets, and leukocytes, but not in erythrocytes. In X-ALD, the C26:0 concentration and the C26:0/C22:0 and C24:0/C22:0 ratios are significantly increased in plasma, fibroblasts, platelets, and leukocytes, but the erythrocytes show substantial overlap in the 5–90% ranges between controls and patients. In RCDP, slightly increased C26:0 and C26:0/C22:0 ratios are found in erythrocytes, platelets, and leukocytes, but not in plasma and fibroblasts. We conclude that plasma and fibroblasts are the specimens of choice for biochemical diagnosis of Zellweger syndrome and X-ALD, respectively. The slight increase in C26:0 in blood cells of RCDP patients suggests a decreased flux of very-long-chain fatty acids through the peroxisomal beta-oxidation pathway in liver in this genetic disorder.

Indexing Terms: heritable disorders · peroxisomes · erythrocytes · leukocytes · hexacosanoic acid · docosanoic acid

Peroxisomal disorders are genetic diseases resulting from the dysfunction of one or more peroxisomal enzymes (1–4). Peroxisomes (microbodies) are organelles present in virtually every human cell except for the mature erythrocyte, and vary in size between the larger peroxisomes in liver and kidney and the smaller organelles (microperoxisomes) in other tissues. Peroxisomes form an indispensable compartment in which specific metabolic reactions occur, including peroxisomal oxidation and respiration, biosynthesis of ether lipids, metabolism of glyoxylate, biosynthesis of cholesterol and dolichols, and beta-oxidation of saturated long-chain and saturated very-long-chain fatty acids, mono- and polyunsaturated fatty acids, di- and trihydroxycholestanoic acid, prostaglandins E2 and F2α, leukotrienes, and several xenobiotics (see review in 5).

Because acyl-CoAs rather than free fatty acids are the substrates for the peroxisomal beta-oxidation pathway, conversion of fatty acids to their CoA esters is the first obligatory step in the oxidative breakdown of fatty acids. This is brought about by various acyl-CoA synthetases, which differ with regard to substrate specificity. A long-chain fatty acyl-CoA synthetase is located at the cytosolic face of the peroxisomal membrane (6). An identical enzyme is present on the membrane of the endoplasmic reticulum and the outer mitochondrial membrane. Liver peroxisomes clearly contain a second acyl-CoA synthetase, which preferentially activates very-long-chain (>C22) fatty acids (VLCFAs) such as tetracosanoic acid (C24:0) and hexacosanoic acid (C26:0) (7, 8). A similar activity has been detected in the endoplasmic reticulum but not in mitochondria (8). The topographical localization of the peroxisomal enzyme (cytosolic or luminal face of peroxisomal membrane) is still debated, but experimental evidence supports the idea that VLCFAs are activated to acyl-CoA derivatives by a specific VLCFA-CoA synthetase located in the peroxisomal membrane. The acyl-CoAs are subsequently catabolized via beta-oxidation in successive steps of dehydrogenation, hydration, dehydrogenation, and thiolytic cleavage catalyzed by a specific set of peroxisomal enzymes (5, 7).

At least in liver and fibroblasts, the oxidation of long-chain fatty acids is believed to take place predominantly in mitochondria. However, the initial chain shortening of VLCFAs via the beta-oxidation proceeds exclusively in peroxisomes rather than in mitochondria. Consequently, impairment of the peroxisomal beta-oxidation results in an accumulation of VLCFAs but not long-chain fatty acids.

At present, classification of the peroxisomal disorders is based on the degree of peroxisomal dysfunction (Table 1). Three categories are distinguished.

In the first category—disorders of peroxisome biogenesis—the organelle fails to form or to be stable, resulting in an almost general impairment of peroxisomal functions, including peroxisomal beta-oxidation. Zellweger syndrome and very-long-chain acyl-CoA dehydrogenase deficiency (X-linked adrenoleukodystrophy) are two examples.

Because these organs have a basically different function and perhaps different requirements for their substrate, we have included adrenoleukodystrophy in this category.

The second category consists of disorders in which a single peroxisomal enzyme is missing but the remaining enzymes of the beta-oxidation pathway are intact. Several examples have been identified, including the Rhizomelic chondrodysplasia punctata syndrome (RCDP).

In the third category, the peroxisomal pathways are intact but the enzyme activities are significantly diminished because of low substrate levels or the presence of metabolites that inhibit the enzyme reactions. This results in the accumulation of VLCFAs. Very-long-chain acyl-CoA dehydrogenase deficiency (X-linked adrenoleukodystrophy) and Rhizomelic chondrodysplasia punctata are two examples.

The accumulation of VLCFAs in the peroxisomes of patients with peroxisomal disorders is the major factor that determines their clinical presentation. In Zellweger syndrome and X-linked adrenoleukodystrophy, VLCFAs accumulate in the peroxisomes of many organs, resulting in a metabolic crisis. In Rhizomelic chondrodysplasia punctata patients, VLCFAs accumulate only in fibroblasts and skin, and the patients show a milder phenotype.

In Zellweger syndrome and X-linked adrenoleukodystrophy, VLCFAs accumulate in many organs, such as the brain, liver, and skin. The distribution of VLCFAs in Rhizomelic chondrodysplasia punctata patients is more restricted, and VLCFAs are only found in fibroblasts and skin. Therefore, VLCFAs are not found in the liver of Rhizomelic chondrodysplasia punctata patients, and their accumulation in fibroblasts is less pronounced compared to Zellweger syndrome and X-linked adrenoleukodystrophy.

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Because VLCFAs are not found in the liver of Rhizomelic chondrodysplasia punctata patients, and their accumulation in fibroblasts is less pronounced compared to Zellweger syndrome and X-linked adrenoleukodystrophy.

2 Nonstandard abbreviations: X-ALD, X-linked adrenoleukodystrophy/adrenomyeloneuropathy; RCDP, rhizomelic chondrodysplasia punctata; VLCFAs, saturated very-long-chain fatty acids; ZS, Zellweger syndrome; and FAME, fatty acid methyl ester.

1 Corresponding author.
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syndrome (ZS) belongs to this category. VLCFAs accumulate in plasma and tissues and in cultured cells from ZS patients (9–11)—a phenomenon used in prenatal and postnatal biochemical diagnosis of this disorder (2, 12).

In the second category, peroxisomes are present in the cells, at least in fibroblasts, but several peroxisomal enzymes are deficient (13–16). The classic rhizomelic type of chondrodysplasia punctata (RCDP) and a pheno-typic variant (17) belong to this category. In this disorder, besides a defective metabolism of plasmalogens and phytic acid, the maturation of the peroxisomal 3-oxo-acyl-CoA thiolase protein is impaired. A recent study suggests that the overall activity of the beta-oxidation system in fibroblasts of RCDP patients is only ~50% of controls (18). However, normal VLCFA concentrations were reported in plasma and fibroblasts of these patients (14, 15, 18).

The third category contains a large group of genetic disorders with a deficiency of only a single peroxisomal enzyme and probably an intact peroxisome structure. X-linked adrenoleukodystrophy (X-ALD) and its pheno-typic variants belong to this category (19, 20). Biochemical studies suggest that the VLCFA accumulation results from a deficiency of the peroxisomal VLCFA-CoA synthetase (21–24). However, Mosser et al. (25) recently identified a candidate gene for X-ALD. This gene encodes a protein homologous to a 70-kDa peroxisomal mem-brane protein that is involved in peroxisome biogenesis and belongs to the (ATP-binding cassette) superfamily of transporters, rather than the expected VLCFA-CoA synthetase. Presumably, this protein may be involved in importing (or) anchoring VLCFA-CoA synthetase protein.

Here we report the results of our studies on the VLCFA profiles in specimens from controls and patients affected by either ZS, X-ALD, or RCDP. We analyzed VLCFA profiles not only in plasma and fibroblasts, but also in erythrocytes, platelets, and leukocytes to determine whether VLCFA analyses in blood cells could contribute to the biochemical diagnosis of these genetic disorders.

Materials and Methods

Patients. Zellweger patients, X-ALD patients, and RCDP patients were diagnosed on the basis of their clinical and biochemical characteristics (1–4).

Isolation of blood cells. Blood (5 mL) was sampled after an overnight fast into tubes containing sodium-EDTA, then without delay centrifuged for 10 min at 800 × g at room temperature, thus separating the erythrocytes from the platelet-rich plasma and theuffy coat. Erythrocytes were washed three times in isotonic NaCl solution and centrifuged at 800 × g for 5 min each time. Erythrocyte “ghosts” were isolated as described (26) and stored at −20 °C.

Leukocytes were isolated from EDTA-blood as described earlier (24) and stored as a pellet at −20 °C.

Platelets were isolated from EDTA-blood essentially as described (27) and stored as a pellet at −20 °C.

Analyses of VLCFA. Frozen pellets of fibroblasts, erythrocyte ghosts, leukocytes, and platelets were thawed, suspended in 0.5 mL of distilled water, and sonicated for 10 s. Portions were taken for the measurement of the protein concentrations by the method of Lowry et al. (28).

Fatty acids were extracted by the Folch et al. procedure (29), and 10 μg of nonadecanoic acid (C19:0) and 1.3 μg of heptacosanoic acid (C27:0) were added to each extract as internal standards. The internal standards were obtained from Phase Separation, Waddingxveen, The Netherlands. Methyl esters of both the free and esterified fatty acids were prepared by reacting with methanolic 1 mol/L hydrochloric acid for 16 h at 80 °C. The methyl esters of the fatty acids (FAME) were purified by thin-layer chromatography on Silica 60 plates (Merck, Darmstadt, Germany) by developing with a toluene/ether mixture (97/3, by vol). After making the different spots visible with iodine vapor, the FAMEs were scraped from the plates and extracted from the silica with hexane. Subsequently the FAMEs were injected onto a 25 m × 0.2 mm fused-silica capillary column (HP-101; Hewlett-Packard, Palo Alto, CA) mounted in a Model 5880A Hewlett-Packard gas chromatograph equipped with a splitless capillary injection system. Gas-chromatographic conditions were as described earlier (30). The identity of the different FAMEs was verified by mass-spectrometric detection (Hewlett-Packard MS detector type MSD 5970).

Statistical analyses. The statistical significance of the VLCFA analyses was evaluated by a Kruskal–Wallis one-way analysis of variance. P-values were corrected by applying the Bonferroni algorithm.
Results

The distribution of the C26:0 fatty acid values in plasma of 54 ZS patients (Figure 1) was skewed to the left and deviated from a normal distribution. Consequently, we tested the statistical evaluation of the data by the Kruskal–Wallis one-way analysis of variance with correction for simultaneous interference at the P-levels. Essentially the same phenomenon was found for both the C26:0 and C24:0 fatty acid concentrations in ZS fibroblasts and in plasma and fibroblasts of X-ALD patients.

The detailed results of the fractionation of the VLCFAs in plasma of controls, ZS patients, X-ALD patients, and RCDP patients are summarized in Figure 2 and in Table 2.

In controls, the 5–90% range of plasma C26:0 fatty acid concentration was 0.11–0.62 mg/L (median 0.31). In ZS patients, the plasma C26:0 concentration was greatly increased. The 5–90% range of the plasma C26:0 concentration was 1.66–4.45 mg/L (median 2.90). Interestingly, the plasma C22:0 concentration in ZS patients was significantly (P < 0.001) decreased, resulting in a greatly increased C26:0/C22:0 ratio. Also the plasma C24:0/C22:0 ratio in these patients was increased. Fibroblasts of ZS patients displayed essentially the same abnormalities as described for plasma. The C26:0 concentration and both the C26:0/C22:0 and C24:0/C22:0 ratios were significantly (P < 0.001) increased in the membranes of these cultured cells. The C22:0 content in the cultured cells was only mildly decreased. Essentially the same abnormalities were found in the various blood cells of ZS patients, although the C24:0/C22:0 ratio in erythrocytes was only marginally increased. The C22:0 concentrations in erythrocytes and platelets were slightly but significantly decreased (P = 0.002 and 0.05, respectively). However, in leukocytes from these patients the C22:0 concentration was similar to that in the controls.

In X-ALD patients, both the plasma and fibroblast concentrations of C26:0 and the C26:0/C22:0 ratios were significantly increased. The 5–90% range for the plasma concentration of C26:0 was 0.82–1.71 mg/L (median 1.28). Also, in platelets and leukocytes of X-ALD patients, the C26:0 concentration and the C26:0/C22:0 and C24:0/C22:0 ratios were significantly increased. However,
Table 2. Concentrations of Very-Long-Chain Fatty Acids in Various Specimen Types from Controls, Zellweger Patients, X-Linked Adrenoleukodystrophy Patients (X-ALD), and Rhizomelic Chondrodysplasia Punctata Patients (RCDP)

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Zellweger</th>
<th>X-ALD</th>
<th>RCDP</th>
</tr>
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<tbody>
<tr>
<td><strong>Plasma</strong></td>
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<tr>
<td>n</td>
<td>109</td>
<td>54</td>
<td>41</td>
<td>20</td>
</tr>
<tr>
<td>C\textsubscript{26:0}/C\textsubscript{22:0} ratio</td>
<td>0.01 (0.00–0.02)</td>
<td>0.43 (0.25–0.63)</td>
<td>0.06 (0.04–0.09)</td>
<td>0.02 (0.01–0.04)</td>
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<tr>
<td>C\textsubscript{24:0}/C\textsubscript{22:0} ratio</td>
<td>0.73 (0.48–0.89)</td>
<td>1.91 (1.55–2.40)</td>
<td>1.50 (1.18–1.74)</td>
<td>0.85 (0.68–1.03)</td>
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<tr>
<td>C\textsubscript{26:0} mg/L</td>
<td>0.31 (0.11–0.62)</td>
<td>2.90 (1.68–4.45)</td>
<td>1.28 (0.82–1.71)</td>
<td>0.35 (0.02–0.03)</td>
</tr>
<tr>
<td>C\textsubscript{24:0} mg/L</td>
<td>17.4 (8.5–35.7)</td>
<td>12.0 (7.7–22.4)</td>
<td>13.7 (7.0–21.1)</td>
<td>17.3 (1.0–27.3)</td>
</tr>
<tr>
<td>C\textsubscript{22:0} mg/L</td>
<td>25.3 (10.5–51.0)</td>
<td>6.3 (4.4–11.8)</td>
<td>20.8 (11.8–29.5)</td>
<td>18.3 (1.0–30.8)</td>
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<tr>
<td><strong>Erythrocytes</strong></td>
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<td>10</td>
<td>12</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>C\textsubscript{26:0}/C\textsubscript{22:0} ratio</td>
<td>0.10 (0.06–0.15)</td>
<td>0.38 (0.22–0.56)</td>
<td>0.18 (0.08–0.23)</td>
<td>0.26 (0.12–0.37)</td>
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<tr>
<td>C\textsubscript{24:0}/C\textsubscript{22:0} ratio</td>
<td>2.06 (1.64–2.53)</td>
<td>2.16 (1.72–3.97)</td>
<td>2.58 (1.51–3.13)</td>
<td>2.65 (1.98–3.53)</td>
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<tr>
<td>C\textsubscript{26:0} mg/g protein</td>
<td>0.57 (0.46–0.67)</td>
<td>1.17 (0.82–1.72)</td>
<td>0.79 (0.47–1.62)</td>
<td>0.88 (0.43–1.53)</td>
</tr>
<tr>
<td>C\textsubscript{24:0} mg/g protein</td>
<td>9.94 (8.40–13.30)</td>
<td>7.59 (5.32–11.20)</td>
<td>10.80 (8.81–15.92)</td>
<td>8.67 (6.22–14.80)</td>
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<tr>
<td>C\textsubscript{22:0} mg/g protein</td>
<td>4.83 (3.94–8.81)</td>
<td>3.61 (2.08–4.14)</td>
<td>5.92 (2.82–10.04)</td>
<td>3.40 (2.35–5.79)</td>
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<td><strong>Platelets</strong></td>
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<td>4</td>
<td>7</td>
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<tr>
<td>C\textsubscript{26:0}/C\textsubscript{22:0} ratio</td>
<td>0.01 (0.01–0.02)</td>
<td>0.16 (0.07–0.25)</td>
<td>0.04 (0.04–0.18)</td>
<td>0.03 (0.02–0.05)</td>
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<tr>
<td>C\textsubscript{24:0}/C\textsubscript{22:0} ratio</td>
<td>0.47 (0.33–0.54)</td>
<td>1.17 (0.95–1.62)</td>
<td>0.86 (0.68–0.97)</td>
<td>0.66 (0.80–0.77)</td>
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<tr>
<td>C\textsubscript{26:0} mg/g protein</td>
<td>0.03 (0.03–0.06)</td>
<td>0.31 (0.15–0.61)</td>
<td>0.17 (0.10–0.29)</td>
<td>0.08 (0.06–0.11)</td>
</tr>
<tr>
<td>C\textsubscript{24:0} mg/g protein</td>
<td>1.30 (0.88–1.73)</td>
<td>2.36 (0.94–3.06)</td>
<td>1.84 (1.48–3.32)</td>
<td>1.73 (1.28–2.27)</td>
</tr>
<tr>
<td>C\textsubscript{22:0} mg/g protein</td>
<td>2.67 (2.22–3.66)</td>
<td>2.07 (0.75–2.53)</td>
<td>2.45 (1.62–3.44)</td>
<td>2.74 (2.14–3.31)</td>
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<td><strong>Leukocytes</strong></td>
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<td>n</td>
<td>10</td>
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<tr>
<td>C\textsubscript{26:0}/C\textsubscript{22:0} ratio</td>
<td>0.03 (0.01–0.05)</td>
<td>0.28 (0.27–0.29)</td>
<td>0.09 (0.07–0.11)</td>
<td>0.05 (0.04–0.07)</td>
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<tr>
<td>C\textsubscript{24:0}/C\textsubscript{22:0} ratio</td>
<td>0.75 (0.44–0.99)</td>
<td>1.57 (1.43–1.71)</td>
<td>1.11 (1.03–1.55)</td>
<td>0.85 (0.65–1.20)</td>
</tr>
<tr>
<td>C\textsubscript{26:0} mg/g protein</td>
<td>0.03 (0.02–0.04)</td>
<td>0.41 (0.24–0.57)</td>
<td>0.13 (0.09–0.20)</td>
<td>0.09 (0.06–0.11)</td>
</tr>
<tr>
<td>C\textsubscript{24:0} mg/g protein</td>
<td>0.64 (0.55–1.03)</td>
<td>2.15 (1.52–2.77)</td>
<td>1.42 (1.31–2.85)</td>
<td>1.35 (1.04–1.68)</td>
</tr>
<tr>
<td>C\textsubscript{22:0} mg/g protein</td>
<td>0.86 (0.63–2.36)</td>
<td>1.42 (0.89–1.94)</td>
<td>1.30 (1.18–2.69)</td>
<td>1.69 (1.00–2.34)</td>
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</table>

the 5–90% ranges for the C\textsubscript{26:0} concentration, the C\textsubscript{26:0}/C\textsubscript{22:0} ratio, and the C\textsubscript{24:0}/C\textsubscript{22:0} ratio in the erythrocytes of X-ALD patients overlapped greatly with the respective values for the controls.

In RCDP patients, the plasma and fibroblast concentrations of C\textsubscript{26:0} fatty acid and the C\textsubscript{26:0}/C\textsubscript{22:0} and C\textsubscript{24:0}/C\textsubscript{22:0} ratios were comparable with the control values. However, in all types of blood cells studied in this disorder, the C\textsubscript{26:0} concentration and the C\textsubscript{26:0}/C\textsubscript{22:0} ratio were slightly greater than in the controls.

Discussion

We studied the concentrations of the VLCFAs in plasma, in fibroblasts, and also in blood cells of patients affected by ZS, X-ALD, or RCDP. All these disorders are peroxisomal diseases caused by a defect of one or more peroxisomal enzymes (Table 1).

Since the early 1980s, quantification of VLCFAs in plasma and fibroblasts has been used in the biochemical diagnosis of ZS and especially X-ALD, the catabolism of VLCFAs via peroxisomal beta-oxidation being defective in both disorders (1–4, 10, 11). The peroxisomal system is not just a functional duplication of the mitochondrial beta-oxidation system but is involved in the chain-shortening of a distinct set of compounds that cannot be handled by the mitochondrial system, including VLCFAs. In contrast to the mitochondrial system, the activity of the peroxisomal system towards short-chain fatty acids is negligible.

Peroxisomes do not contain any DNA. Consequently, all peroxisomal proteins are coded for by nuclear genes. This includes the peroxisomal matrix proteins, e.g., the peroxisomal beta-oxidation enzyme proteins, and a specific set of peroxisomal integral membrane proteins, including the quantitatively major 70- and 20-kDa proteins. Functional peroxisomes are absent in ZS cells.
(reviewed in 2 and 5). As a result, peroxisomal enzymes that are newly synthesized on free ribosomes in the cytoplasm, including the enzymes of the peroxisomal beta-oxidation, are degraded rapidly in the cytoplasm (31). Consequently, VLCFA degradation is impaired in ZS.

In X-ALD patients, the peroxisomal VLCFA-CoA synthetase that specifically activates the VLCFAs is deficient (21–24). However, the finding of a deficient peroxisomal VLCFA-CoA synthetase activity in X-ALD does not necessarily mean that the mutation in X-ALD involves the gene encoding this enzyme. The primary defect may well be at the level of another gene, the product of which is essential for correct expression and (or) anchoring of peroxisomal VLCFA-CoA synthetase in the peroxisomal membrane. Recent evidence supports the latter hypothesis (25).

Molzer et al. (32) reported that determination of C26:0 concentrations in leukocytes may be a diagnostic tool for detecting X-ALD patients and ALD gene carriers. We confirmed that finding and extended it to X-ALD platelets.

Martinez (33) found that ZS patients have extremely low concentrations of docosahexaenoic acid (C22:6w3) in the brain, liver, and kidney—which suggests a defect involving the desaturation of long-chain polyunsaturated fatty acids. Moreover, in determining the fatty acid composition of the erythrocytes in a ZS patient, she found increases of both C26:0 and C24:0 (34). In our studies, we found highly increased C26:0 concentrations and C26:0/C22:0 and C24:0/C22:0 ratios in plasma, fibroblasts, platelets, and leukocytes of ZS patients (Table 2 and Figure 2). In erythrocytes of ZS patients, the concentration of C26:0 and the C26:0/C22:0 ratio were both only mildly but significantly increased (P = 0.01). Interestingly, the C22:0 concentrations in plasma, fibroblasts, erythrocytes, and platelets of ZS patients were significantly (P < 0.001) decreased (Table 2). No clear explanation is available for this phenomenon.

In RCDP patients, no VLCFA accumulation was found either in plasma or in fibroblasts. However, concentrations of C26:0 and the C26:0/C22:0 ratios were mildly increased in erythrocytes, platelets, and leukocytes of these patients (Table 2). Recently, Heikoop et al. (18) found that the activity of the peroxisomal beta-oxidation in fibroblasts of RCDP patients is only ~50% of the control values. The abnormalities in the VLCFA profiles in the blood cells of RCDP patients probably reflect a decreased flux of VLCFAs through the peroxisomal beta-oxidation in the liver in patients affected by this disorder.

We conclude that fractionation and quantification of the VLCFA, not only in plasma and fibroblasts, but also in blood cells, can contribute to the biochemical diagnosis of ZS and X-ALD.

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