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Serum very-long-chain fatty acids levels determined by gas chromatography in the diagnosis of peroxisomal disorders in Poland

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Abstract

Peroxisomal disorders are a large group of genetically determined metabolic diseases in which the biogenesis of peroxisomes is defective or there is a deficiency of only a single enzyme activity or substrate transporter.
The objective of this report is to present ten years of experience in the diagnostics of peroxisomal disorders in Poland.
Very-long-chain fatty acid (VLCFA) levels as a biomarker for peroxisomal defects were determined by gas chromatography in 1264 subjects with suspicion of peroxisome disease.
Peroxisome biogenesis disorders (PBD) were diagnosed in 8 patients, bifunctional protein deficiency in 3 and X-linked adrenoleukodystrophy (X-ALD/AMN) in 127 hemi- or heterozygotes.
The frequency of PBD was estimated as 0.20 : 100 000, and that of X-ALD/AMN 2.9 : 100 000 in Poland. Mean total delay time (onset of symptoms and diagnosis) for X-ALD/AMN was 2.2 years (range 0.25-13). High correlation of serum C26:0 concentration and survival for PBD patient ($r^2 = 0.822$; $p < 0.001$) was found.

Key words: *peroxisomal disorders, Zellweger syndrome, neonatal adrenoleukodystrophy, bifunctional protein deficiency, adrenoleukodystrophy, very long chain fatty acids (VLCFA).*

Introduction

The single-membrane organelles discovered in 1950 by Rhodene in mouse kidney cells and characterized biochemically by De Duve in the 1960's are called peroxisomes. They contain both catalase and hydrogen peroxide-producing oxidase and are present in all eukaryotic cells [6]. Peroxisomes exhibit unique morphological and metabolic variability, depending on the organism, development stage, cell type and environment. Changes in the enzyme content of peroxisomes occur dynamically through the membrane and involve an ATP-dependent step of peroxisomal matrix protein import; cycling receptors shuttle the proteins between the cytosol and peroxisome lumen [18].

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Biogenesis of peroxisomes in humans is associated with the function of genes belonging to the *PEX* family. To date, 13 *PEX* genes have been identified. Their products are necessary for peroxisome formation, which occurs in three stages: assembly of the peroxisome membrane, import of matrix proteins, and proliferation of peroxisomes [18,24].

These organelles are the site of more than 50 biochemical reactions connected mainly with lipid pathways, among which oxidation processes predominate.

The metabolic functions occur in peroxisomes include biosynthesis as well as catabolism processes. Analysis of four from numerous biochemical pathways are particularly helpful targets in diagnostics of peroxisomal disorders: fatty acid α-oxidation, fatty acid β-oxidation, synthesis of the plasmalogen, and glyoxylate detoxification [3,21].

The degradation of saturated, unbranched very-long-chain fatty acids (VLCFA), with C24:0 (lignoceric acid), C26:0 (hexacosanoic acid) and longer chains proceeds via β-oxidation process. The transport VLCFA into peroxisome by ABC half transporter membrane protein is initial stage of this process. Decomposition molecule goes through the same reactions as in mitochondrial β-oxidation (dehydrogenation, hydration, oxidation, and thiolysis). It is catalyzed by different enzymes, however, and includes the gradual shortening of the carbon chain. Disorders of β-oxidation VLCFAs leads to their accumulation in cells and body fluids [15].

Peroxisome dysfunctions resulting from either defective peroxisome biogenesis or deficiency in single biochemical reactions constitute a group of inborn errors of metabolism termed peroxisomal disorders [29].

Although the first peroxisomal diseases were described in the 1930's and 1960's, it was not possible to begin to classify them until their pathomechanism was linked to peroxisomes in 1972, when Goldfisher demonstrated morphologically that hepatocytes and renal tube cells of infants with Zelweger syndrome lacked these organelles [7].

Detection elevated VLCFA in serum and/or cultured fibroblasts became basis of biochemical diagnostics in peroxisomal diseases [15].

Peroxisomal disorders can be divided into three basic groups: diseases related to (I) peroxisomal biogenesis disorders, PBD, (II) a defect of a single enzyme or protein and (III), a recently described contiguous gene deletion syndrome [4,23].

The first group, peroxisomal biogenesis disorders (PBD), includes Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsume disease (IRD), and rhizomelic chondrodysplasia punctata (RCDP). Clinically the most severe phenotype is Zellweger syndrome, characterized by dysmorphy, severe psychomotor delay, CNS development disorders, dysmyelination. Neonatal adrenoleukodystrophy and infantile Refsum disease are similar to Zellweger syndrome, but have a milder course and longer survival. The clinical picture of rhizomelic chondrodysplasia differs from the above and is characterized by proximal shortening of extremities, marked dysmorphy and severe developmental delay [24].

The second group of peroxisome disorders encompasses those due to single-enzyme deficiencies, of which 10 have been identified in the fatty-acid β- and α-oxidation pathways, phospholipid biosynthesis, hydrogen peroxide metabolism, and glyoxylate detoxification. They include X-linked adrenoleukodystrophy (X-ALD/AMN), D-bifunctional protein deficiency (DBP), the classic adult form of Refsum disease, rhizomelic chondrodysplasia type II and III, acatalasemia, hyperoxaluria, and the recently identified deficit of sterol carrier protein-X [8,19,30].

A deficiency of D-bifunctional protein, an enzyme involved in two steps the oxidation of fatty acids, is an important member of this group. The clinical manifestation is similar to that in PBD and its severity has been shown to correlate with the residual activity of the enzyme [9,17].

The third group mentioned above is related to both a defect in the *ABCD 1* gene (similarly as in X-ALD/AMN) and in the *DXS1357E* gene, located on chromosome X (Xq28). The defect manifests from birth with profound hypotonia, severe psychomotor delay, cholestasis and a generally severe course [4].

The most common peroxisome disease is X-linked adrenoleukodystrophy. This is a severe, progressive central- and peripheral nervous system demyelinating disease, which also affects adrenal function [15,16,32]. The disease is related to a mutation in the *ABCD1* gene that encodes protein ALD [5] located in the peroxisome membrane. It probably plays a role in the transport of VLCFA or the aceto-Co-A form of these acids (VLCFA Co-A) into peroxisomes, where they undergo β-oxidation. The mechanism leading to demyelination, spinal cord axonal loss, and adrenal insufficiency is still unknown. High VLCFA concentration are probably a pathogenic factor but it couldn't be rouled out that it is only a biochemical marker of the disease. It is believed that in X-ALD with a ful-

minant, inflammatory course, acylation of gangliosides and phospholipids by VLCFA is disturbed, which evokes an immune response in macrophages and astrocytes [11]. A wide spectrum of nine clinical phenotypes has been described, from a severe, rapidly progressing one in which symptoms appear as early as 4-7 years of age, through an adolescent form with a slower course, but also including adrenal failure, to one with late onset in the 3rd-5th decade of life, termed adrenomyeloneuropathy (AMN) and characterized by a different location of leukodystrophic lesions. There are also forms in which only the adrenal glands are affected. Aubourg distinguishes, however, two basic phenotypes: a demyelinating cerebral one, and adrenomyeloneuropathy one [1].

Determining VLCFA levels is the method of choice in the diagnostics and identification of X-ALD hemi- and heterozygotes. Serum VLCFA levels are always elevated in hemizygotes, and in 80-85% heterozygotes [30]. Certain differences in serum VLCFA levels depending on age are observed in heterozygotes [25]. About 8% of mutations in gene *ABCD1* arise *de novo*, molecular analysis is thus recommended for confirmation of heterozygosity [1].

In addition to a low-fat diet, a 4 : 1 (v : v) mixture of glyceroltrioleate (GTO) and glyceroltrierucate (GTE), known as Lorenzo oil (LO), is used as diet therapy of X-ALD patients. VLCFA levels normalize 6-8 weeks after LO is introduced. The profile of monounsaturated long-chain fatty acids also changes. Moser believes that the use of LO in the presymptomatic stage may delay the onset of early-stage inflammatory X-ALD [16]. Another proposed therapeutic option is umbilical cord blood cell or hematopoietic stem cell transplantation (HSCT). It is believed that a successful transplant performed before symptoms develop may slow down demyelination [1,22].

The objective of this paper is to present ten years of experience in the diagnostics of peroxisomal disorders based on assaying serum VLCFA levels.

Material and Methods

Patients

Between 1994 and 2004, a total of 1264 samples from patients from 35 medical centers in Poland and 7 from abroad (Lithuania) were analyzed at the chromatography laboratory of the CMHI. Samples were referred mainly from patients with suspicion of Zellweger syndrome and other PBD syndromes (495), or X-ALD/AMN (460), in the latter case, samples from family members were also submitted (183). The remaining accessed samples (126) were from 15 patients who underwent various therapeutic managements. One patient with PBD in whom GTO was administrated and 10 patients with z X-ALD/AMN who received Lorenzo Oil and low fatty acids diet. Four patients underwent HSCT.

Control groups categories

1. Control healthy children's establish 30 persons (14 males and 16 females) aged from 2 months to 5 years. 2. Females' control – 25 healthy women's aged 18-50 years. 3. Males' control – 35 healthy males (4-52 years).

Methods

The studied material was serum samples (0.25 ml). Blood was from which serum was obtained was sampled after a 12-hour fast and centrifuged for 4 min at 3000 g, preferably within 40 min of sample collection. Separated serum was stored frozen at –20°C until analysis to three month.

Gas chromatographic analysis

Total fatty acids (C22:0, C24:0, C26:0) were determined according procedure described early [26]. The serum VLCFAs as methyl esters derivatives were identified and assayed by gas chromatography (GC) method. Separation was performed on a OV-1 (30 m, 0.25 mm I.D.) capillary column using a Hewlett Packard 5890 series II GC equipped with an FID and moving needle glass injector. VLCFA concentration were calculated with ChemStation Software.

Statistical analysis

For analysis of variance of VLCFA levels between study and control groups, the Student-test was used. The differences between X-ALD/AMN phenotypes were assessed with analysis of variance (ANOVA), homogeneity of variance was checked with Levene-"s test. $P < 0.05$ was considered significant. Regression analysis according to least squares method was used for estimation a relationship between survival and the C26:0 concentration.

The frequency of peroxisomal disorders was estimated on the basis of demographic data from Central Statistical Office of Poland.

Results

The number of referred samples, confirmed diagnoses of PBD, bifunctional protein deficiency, and adrenoleukodystrophy is presents in Table I.

From a total number of 495 referrals, PBD was confirmed in 8, bifunctional protein deficiency, in 3 persons. These data allow to estimate the frequency of PBD in Poland as 0.20 cases per 100 000 live births.

In a total of 460 assays of samples from patients with suspicion of X-ALD/AMN, an elevated VLCFA level characteristic of adrenoleukodystrophy was found in 46. Overall, 127 patients with adrenoleukodystrophy, including both hemi- and heterozygotes, were identified. Of these, 81 were identified through the family screening. The frequency of X-ALD/AMN in Poland is 2.9 cases per 100 000 live-born boys, for both hemi and heterozygotes the frequency is estimated as 1 : 31 000 live births.

Table II presents the VLCFA concentration in patients with PBD and DBP. In this group, enzymatic and microscopic studies were performed in one patient with Zellweger syndrome and in three with bifunctional protein deficiency. Molecular analysis were performed in two patients one with NALD and Zellweger-like syndrome. In the remaining patients, differentiating among ZS, NALD and ZS-like was based on clinical presentation.

In this study we investigated a relationship between serum C26:0 concentration and the disease severity (defined as survival) for 8 patients with PBD and DBP (ZS, NALD, DBP).

It was found the high correlation of the parameters (r^2 = 0.822; $p < 0.001$). Survival depending on the C26:0 concentration is expressed by the power equation:

$t = a * (C26:0)^{b}$; where, a = 3.404, b = –1.310.

Serum VLCFA levels in X-ALD/AMN patients for different phenotypes showed in (Table III). It's not found statistically significant differences for particularly studied parameters ($p > 0.259$). Independently of phenotype in all analyzed patients C22:0 level is lower versus healthy control ($p < 0.001$).

In all patients, who were unaware of existence of X-ALD in their family, we estimated delay time between onset of symptoms and diagnosis. The time (*T*) is lowest in the childhood form of the disease, with a mean ±SD = 1.1 ±0.6 years, and longest for AMN, 5.8 ±4.2 years (Table III).

The results of serum VLCFA levels in obligatory X-ALD/AMN heterozygotes in different age range are presented in Table IV.

Table I. Referrals samples from patients with suspicion of PBD or patients with X-ALD and number of confirmed diagnoses, as serum VLCFA levels (1994-2004)

Number of referrals with suspicion of Zellweger syndrome/PBD	Number of confirmed diagnoses	Number of referrals with suspicion of X-ALD and AMN		Number of confirmed diagnoses of X-ALD and AMN		Number of confirmed diagnoses among screened family members hemizygotes and heterozygotes	
490	11	460		46		81	
		X-ALD	AMN	X-ALD	AMN	hemizygotes	heterozygotes
		419	41	39	7	12	69

Table II. Serum VLCFA levels in patients with PBD (ZS, NALD, ZS like) and DBP. The values represent means ±SD

Number of patients (n)	C24:0 [mg/mL]	C26:0 [mg/mL]	C24:0/C22:0	C26:0/C22:0
ZS (4)	19.28 ±6.64	5.40 ±3.22	2.130 ±0.193	0.564 ±0.137
NALD (2)	15.73 ±1.86	1.93 ± 0.59	1.722 ±0.068	0.345 ±0.134
ZS-like (2)	10.66 ±1.12	0.37 ±0.025	1.150 ±0.050	0.040 ±0.008
DBP (3)	22.22 ±3.00	1.99 ±0.68	2.073 ±0.104	0.185 ±0.043
Control (30)	14.13 ±2.65	0.15 ±0.05	0.782 ±0.060	0.008 ±0.003

Table III. Serum VLCFA levels in X-ALD hemizygotes with different phenotypes. Delay time between symptoms onset and diagnosis

Disease phenotype	Symptoms onset – age [years] (range)	C22:0 [mg/mL]	C24:0 [mg/mL]	C26:0 [mg/mL]	C24:0/C22:0	C26:0/C22:0	T [years] (range)
X-ALD childhood onset(n=27)	7.6 ±1.3 (4.9-9.8)	13.47 ±2.67	20.71 ±3.74	0.76 ±0.19	1.551 ±0.14	0.061 ±0.025	1.1 ±0.6 (0.25-2.4)
X-ALD adolescence onset (n=8)	11.5 ±1.6 (10.1-14.5)	12.74 ±2.58	19.70 ±4.89	0.82 ±0.15	1.540 ±0.12	0.067 ±0.015	2.8 ±2.5 (0.3-7.0)
Addison disease (n=4)	9.4 ±1.7 (8.2-12)	13.49 ±2.75	20.53 ±5.53	0.81 ±0.25	1.506 ±0.11	0.060 ±0.014	1.8 ±0.8 (1.0-3.0)
AMN (n=7)	24.8 ±5.4 (19-30)	13.73 ±1.93	22.54 ±4.19	0.72 ±0.14	1.634 ±0.096	0.053 ±0.010	5.8 ±4.2 (2.0-13)
Presymptomatic form*							
- infants (3-12 m.) (n=3)	-	12.07 ±3.12	19.96 ±4.09	0.73 ± 0.05	1.668 ± 0.107	0.063 ±0.013	
- older children (9–14 y) (n=9)		14.62 ±3.10	21.86 ±4.05	0.77 ±0.13	1.506 ±0.10	0.055 ±0.014	
Males' control	-	18.31 ± 3.98	14.51 ± 2.76	0.17 ± 0.04	0.797 ±0.045	0.009 ±0.003	

** Patients identified through family screening; T – time from symptom onset to biochemical confirmation of disease; the values represent means ±SD.*

Table IV. Serum VLCFA levels in X-ALD heterozygotes. The values represent means ±SD

Age [years]	C24:0 [mg/mL]	C:26:0 [mg/mL]	C24:0/C22:0	C26:0/C22:0
22-50	18.17 ±3.85	0.382 ±0.13	1.144 ±0.129	0.036 ±0.053
50-70	14.95 ±1.92	0.149 ±0.04	0.915 ±0.079	0.008 ±0.003
Females' control	13.88 ±2.59	0.13 ±0.05	0.776 ±0.058	0.007 ±0.003

The means values of C24:0 and C26:0 are lower in female carriers than in hemizygotes. Similarly for heterozygotes the C24:0/C22:0 and C26:0/C22:0 ratios stand about 70% and 50% of hemizygotes values respectively.

In one patient with the classical form of Zellweger syndrome, who followed the GTO diet (glyceroltrioleate), VLCFA levels were monitored for 9 months. The diet reduced C26:0 and C24:0 concentrations by ~50% and 30%, respectively (Fig. 1) and lowered the C24:0/C22:0 and C26:0/C22:0 ratios by about 50%.

In ten patients with X-ALD following LO administration with, low-fat diet serum C26:0 and C24:0 concentrations normalized within 1.2 to 1.5 months. The concentration of the unsaturated fatty acids, C22:1 and C24:1, rose, however, to even ca. 220 % (Figs. 2, 3).

Four Polish patients with X-ALD received HSCT. In two of them, VLCFA levels remain at heterozygote levels. In the remaining patients, VLCFA levels remain at hemizygote levels (Table V).

Discussion

Presented data allow us to estimate the frequency of peroxisomal disorders in Polish population. Frequency of PBD is 0.20 cases per 100000 births, which is similar to that in Japan, i.e., 0.22 : 100 000. At the University of Gifu, the only center in Japan diagnosing peroxisomal diseases, over a period of 20 years, 40 cases of PBD and 11 of single-enzyme deficit were identified; these assays included determining plasmalogen, pipecolic acid, and molecular analyses [23].

In the 90-ties the frequency X-ALD/AMN was described at least one per 100 000 [15], at present data found, that it is the most common peroxisomal disorder.

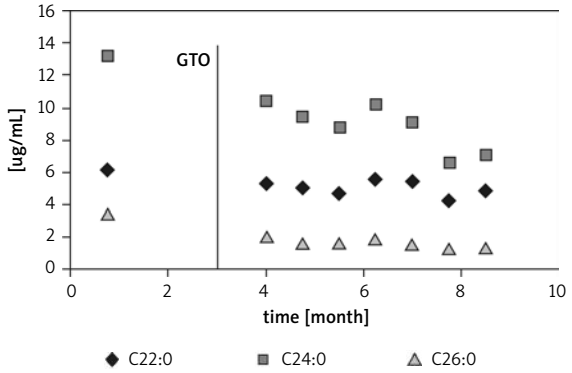

Fig. 1. Serum VLCFA levels in a ZS patent before and during GTO milk formula therapy.

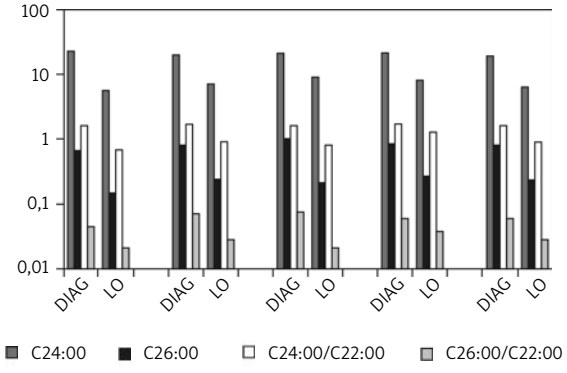

Fig. 2. Serum VLCFA levels in X-ALD/AMN patient's at diagnosis and LO administration

The frequency of X-ALD/AMN in Poland, 2.9 cases per 100 000 live-born boys, is within the range found in Japan, 2-3.3 : 100 000 [27], but a higher rate than found Australian studies: 1.6 : 100 000 [12]. Bezman [2] reports a very high rate of both hetero- and hemizygotes, 1 : 16 800, and according to this classification, Poland has a frequency of 1 : 31 000 births.

The delay, time (*T*) between symptoms onset and diagnosis for X-ALD/AMN (Table III) is different, the shortest in the childhood form of the disease, with a mean ±SD = 1.1 ±0.6 years, and longest for AMN, 5.8 ±4.2 years. Similar values, 0.9 and 5.8 years, respectively, have been reported by Takemoto [27]. However, the mean total delay was 2.2 (range 0.25 – 13) years, in compare to the studies by van Geel (9.9; range 1-33) is definitely lower [31].

Measurement of VLCFA levels is basic diagnostics method for PBD and the best biomarker in X-ALD/AMN diagnosis [30]. For estimating dysfunction peroxisomal β-oxidation process mainly C26:0, C26:0/C22:0 levels is used, more rarely C24:0/C22:0.

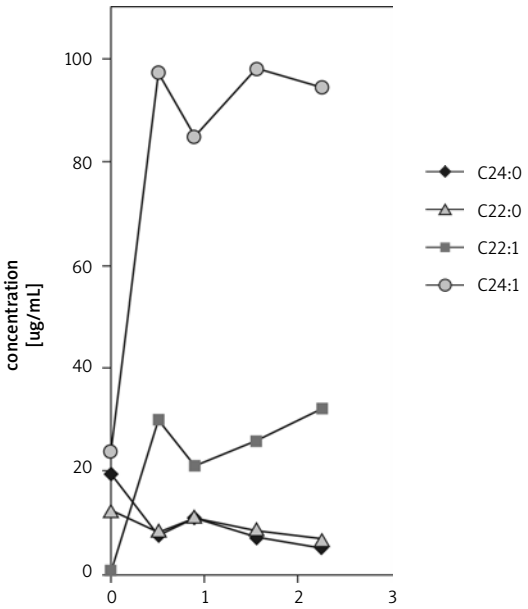

Fig. 3. Effect of treatment LO of saturated and unsaturated VLCFA in X-ALD patient.

Table V. VLCFA levels in Polish X-ALD patients before and after HSCT

Patient	*Onset*	*VLCFA*						*Outcome (clinical status)*
		before HSCT		*after HSCT*				
				8 m		*14 m*		
		C24:0/C22:0	C26:0/C22:0	**C24:0/C22:0**	**C26:0/C22:0**	**C24:0/C22:0**	**C26:0/C22:0**	
1.	(-)	1.556	0.043	1.145	0.018	1.115	0.015	asymptomatic
2.	(-)	1.564	0.048	1.200	0.050	1.580	0.064	asymptomatic
3.	(+) (7y 9m)	1.542	0.036	1.414	0.031	(-)	(-)	death (sepsis)
4.	(+) (8,5 y)	1.492	0.034	1.274	0.020	(-)	(-)	stable

Analysis of the range of serum VLCFA levels in patients with peroxisome biogenesis disorders and adrenoleukodystrophy shows that in PBD patients, means VLCFA ratio are significantly higher than in X-ALD (134% for C24:0/C22:0 and as much as 472% for C26:0/C22:0).

Gootjes et al. [10] showed that the level of C26:0 (in fibroblasts) is good marker to predict the disease severity as determined by survival in PBD [23]. Our studies support the high correlation of these parameters ($r^2 = 0.822$; $p < 0.001$) in patients with PBD and DBP, for serum C26:0 concentrations.

It doesn't concern adrenoleukodystrophy were patient's serum VLCFA levels (together with C26:0) do not differ among hemizygotes with diverse courses including patients with significantly advanced disease and those still symptomless. VLCFA levels are similar, regardless of age and disease form, which supports the rule that VLCFA levels cannot be used to predict disease phenotype (Table III). Similar results were presented early [14].

However, our data showed, decreased C22:0 levels for X-ALD/AMN patients ($p< 0.001$). This observation, allows understand the results, which we presented in previous studies, where by discriminative analysis showed that the most precise diagnostic assessment for hemi- and heterozygotes with X-ALD is obtained by taking into account 5 analytical parameters, together with C22:0 (C22:0, C24:0, C26:0, C24:0/C22:0, C26:0/C22:0) [25].

In peroxisomal disorders we haven't got treatment nearly at all, the remedies which can be applied conduct only to decrease the VLCFA levels. GTO administration in ZS patient decreased serum VLCFA levels; it had no influence on the clinical condition of the patient [28]. Similar reduction of the C24:0/C22:0 and C26:0/C22:0 ratios were shown, in other ZS patient, after following a diet containing another unsaturated acid, DHA (docosahexaenoic acid) [13].

All X-ALD patients who was LO administrated, VLCFA levels decreased, but unsaturated acids, C22:1 and C24:1 increased. It is not clear how high levels of these fatty acids affect the course of the diseases. It still too has not been possible to elucidate the exact connection between high VLCFA levels and demyelination in X-ALD patients [11]. As mentioned above, the usage of LO can be delay disorders process [16]. On the other hand, postmortem examinations by Poulos [20] have shown that the consumption of LO leading to normalization of serum VLCFA has little effect on their level in the central nervous system. Some reports deal with symptom amelioration using anti-inflammatory and immunosuppressive treatment in patients with the inflammatory form of X-ALD [11]. However, because of the heterogeneity of clinical presentations in X-ALD and AMN and the resulting impossibility of predicting the form and course of the disease, the efficacy of this, or any other therapy, either LO or HSCT, cannot be reliably assessed.

Ten years of experience in the diagnosis of peroxisomal disorder show that the frequency of PBD and that of X-ALD/AMN in Poland is similar to the other countries.

Determining serum VLCFA levels is a sensitive diagnostic assay as well is the basic screening test for peroxisomal disorders. In patients undergoing various methods of treatment i.e. diet with GTO, LO or bone marrow transplantation serum VLCFA should be systematically monitored.

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