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Citation for published version (APA):
Gootjes, J. (2004). Molecular, biochemical and clinical aspects of peroxisomes biogenesis disorders
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Biochemical markers predicting survival in peroxisome biogenesis disorders

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Summary

Objective: To identify prognostic markers reflecting the extent of peroxisome dysfunction in primary skin fibroblasts from patients with peroxisome biogenesis disorders (PBD).

Background: The PBDs are a genetically heterogeneous group of disorders due to defects in at least 11 distinct genes. Zellweger syndrome (ZS) is the prototype of this group of disorders with neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD) as milder variants. Common to these three disorders are liver disease, variable neurodevelopmental delay, retinopathy and perceptive deafness. Since genotype-phenotype studies are complicated by the genetic heterogeneity among PBD patients, we evaluated a series of biochemical markers as a measure of peroxisome dysfunction in skin fibroblasts.

Methods: Multiple peroxisomal functions including de novo plasmalogens synthesis, DHAPAT activity, C26:0/C22:0 ratio, C26:0 and pristanic acid β-oxidation and phytanic acid α-oxidation were analyzed in fibroblasts from a series of patients with defined clinical phenotypes.

Results: A poor correlation with age of death was found for de novo plasmalogens synthesis, C26:0/C22:0 ratio and phytanic acid α-oxidation. A fairly good correlation was found for pristanic acid β-oxidation, but the best correlation was found for DHAPAT activity and C26:0 β-oxidation. A mathematic combination of DHAPAT activity and C26:0 β-oxidation showed an even better correlation.

Conclusions: DHAPAT activity and C26:0 β-oxidation are the best markers in predicting life expectancy of PBD patients. Combination of both markers gives an even better prediction. These results contribute to the management of PBD patients.

Introduction

Peroxisomes harbor a variety of metabolic functions including fatty acid β-oxidation, etherphospholipid biosynthesis and fatty acid α-oxidation.¹ Peroxisomal disorders are subdivided into two groups including the peroxisome biogenesis disorders (PBDs)² and the single peroxisomal enzyme deficiencies.³ The PBDs, which comprise the Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD), represent a spectrum of disease severity with ZS being the most, and IRD the least severe disorder. Common to all three PBDs are liver disease, variable neurodevelopmental delay, retinopathy and perceptive deafness.² Patients with ZS are severely hypotonic from birth and die before one year of age. Patients with NALD experience neonatal onset of hypotonia and seizures and suffer from progressive white matter disease, dying usually in late infancy.³ Patients with IRD may survive beyond infancy and some may even reach...
adulthood. Clinical differentiation between these disease states is not very well-defined and patients can have overlapping symptoms.

There is also genetic heterogeneity among PBDs. Cell fusion complementation studies using patient fibroblasts led to the identification of 11 distinct genetic groups. So far 10 of the corresponding (PEX) genes have been identified. Most complementation groups are associated with more than one clinical phenotype.

PBD patients have an impaired synthesis of plasmalogens, due to a deficiency of the two enzymes dihydroxyacetonephosphate acyltransferase (DHAPAT) and alkyl-dihydroxyacetonephosphate synthase. Peroxisomal fatty acid β-oxidation is also defective, leading to the accumulation of very-long chain fatty acids (VLCFAs), notably C26:0, the branched chain fatty acid pristanic acid and the bile acid intermediates di- and trihydroxycholestanoic acid (DHCA and THCA). Phytanic acid α-oxidation and L-pipeolic acid oxidation are also impaired. In contrast, some peroxisomal enzymes show normal activity including catalase, D-amino acid oxidase, L-α-hydroxy acid oxidase A and alanine:glyoxylate aminotransferase.

Since genotype-phenotype studies are complicated by the marked genetic heterogeneity among patients with a PBD, we evaluated a number of different biochemical markers as a measure of peroxisome dysfunction in order to identify the best marker predicting the survival of patients with peroxisome biogenesis disorders.

**Subjects and Methods**

**Subject**
Thirty-five patients with a PBD, collected during the past 20 years, were enrolled in this study. The diagnosis was confirmed in our laboratory based on biochemical studies in plasma and fibroblasts. Most patients were Dutch, but some originated from other parts of Europe. Patients were divided into two groups: 1. patients who died before one year of age, representing the classical ZS group, and 2. patients who survived for more than five years, representing the relatively milder phenotypes of NALD and IRD.

**Biochemical assays**
DHAPAT activity, de novo plasmalogen synthesis, concentrations of VLCFAs, C26:0 and pristanic acid β-oxidation and phytanic acid α-oxidation were assayed in primary skin fibroblasts cultured in DMEM or HAM-F10 medium as previously described. Inter- and intraassay CVs are 15% and 4.4% for DHAPAT activity, 8.8% and 2.3% for VLCFA ratios, 18% and 5.4% for C26:0 β-oxidation, 22% and 5.3% for pristanic acid β-oxidation and 22% and 4.3% for phytanic acid α-oxidation. All presented data are the means of two individual measurements.

**Numerical and statistical analysis**
Combination of DHAPAT activity and C26:0 β-oxidation was done using the formula: (DHAPAT activity/control value DHAPAT activity + C26:0 β-oxidation/control value C26:0 β-oxidation) x 0.5 x 100%. Control values were 10.9 nmol/2hr.mg protein for DHAPAT activity and 1350 pmol/hr.mg protein for C26:0 β-oxidation. The correlation of the different markers and survival between the two groups was evaluated using the Mann-Whitney U test.
Chapter 2

Results

In this study we included thirty-five patients divided into two groups: patients who died before one year of age, representing the classical ZS group (group 1), and patients who survived for more than five years, representing relatively mild phenotypes including NALD and IRD (group 2). Patients that died between one and four years of age were excluded because this study seeks to distinguish between severe and mild cases. Six markers of peroxisome function were measured in cultured skin fibroblasts of the patients: 1. DHAPAT activity, 2. de novo plasmalogen synthesis, 3. C26:0/C22:0 ratio, 4. C26:0 β-oxidation, 5. pristanic acid β-oxidation and 6. phytanic acid α-oxidation.

Plasmalogen biosynthesis

Two markers of plasmalogen biosynthesis were determined including DHAPAT activity and de novo plasmalogen synthesis. DHAPAT activity clearly differed between the two groups (P<0.001) (figure 1) as illustrated by the numbers shown above the graph. Only two of nine patients in the classical ZS group, have DHAPAT activities that fall within the standard deviation found for DHAPAT in group 2, whereas the DHAPAT activity found in four of the 20 patients in group 2 fall within the standard deviation of group 1. Thus, DHAPAT activity appears a very good marker in predicting survival of PBD patients. This is in contrast to de novo plasmalogen biosynthesis (data not shown) in which there is a large overlap between the two groups of patients (P=0.491).

![Figure 1](image)

**Figure 1** DHAPAT activity in fibroblasts from PBD patients, who died before one year of age (group 1) and patients who survived for more than five years (group 2). Each circle represents the activity of DHAPAT as measured in fibroblasts from each individual patient (mean of duplicate experiments). The individual values were used to calculate the mean (group 1: 0.5 and group2: 2.1) plus standard deviation (0.20 and 1.4) as shown in the graph. Mann-Whitney U test showed that the two groups were different from each other (P<0.001).

Peroxisomal β-oxidation

For peroxisomal β-oxidation we evaluated the C26:0/C22:0 ratio, C26:0 β-oxidation and pristanic acid β-oxidation. The C26:0/C22:0 ratios determined in fibroblast homogenates show extensive overlap between the two groups (data not shown), indicating that this ratio has no prognostic value in terms of patient survival (P=0.059). Figure 2 reveals a clear distinction between the two groups for C26:0 β-oxidation (P<0.001). Only one of seven classical ZS patients belonging to group 1 falls within the standard deviation of group 2.
Biochemical markers predicting survival

and none of the milder patients fall within the standard deviation of group 1. Thus, also C26:0 β-oxidation appears a very good marker predicting the survival of PBD patients. Pristanic acid β-oxidation shows less correlation with survival (P=0.009) (figure 3) than C26:0 β-oxidation, but still appears informative.

**Phytanic acid α-oxidation**
There was no clear distinction between the two groups (P=0.359) with respect to phytanic acid α-oxidation indicating that this is not a good predictive marker (data not shown).

**Combination DHAPAT activity and C26:0 β-oxidation**
Although DHAPAT activity and C26:0 β-oxidation were best in predicting survival of the patients, both showed some overlap between the two groups (see figures 1 and 2). Combining both markers, however, led to a complete separation of the two groups (P=0.001, figure 4).
Figure 4 Combination of DHAPAT activity and C26:0 β-oxidation ((DHAPAT activity/control value DHAPAT activity + C26:0 β-oxidation/control value C26:0 β-oxidation) x 0.5 x 100%) in fibroblasts from patients belonging to group 1 (death before one year of age) and group 2 (survival beyond 5 years of age) as percentage of control values. Each circle represents a patient and mean values (7 and 24) and standard deviations (2.7 and 12.3) are shown in the graph. Mann-Whitney U test showed that the two groups were different from each other (P=0.001).

Discussion

This study investigated biochemical markers to predict the survival of patients with a peroxisome biogenesis disorder. The relationship between the extent of peroxisomal dysfunction and the patient survival has not been defined. Previous studies have shown a clear genotype-phenotype correlation for only two mutations in PEX1, the gene affected in the majority of PBD patients. Unfortunately the correlation only holds for either the mild 2528G>A or the severe 2097insT mutation. In case of heterozygosity or defects in one of the other PEX genes the correlation is not clear. This implies that at present molecular analysis will only be helpful for a subset of patients. For this reason we evaluated the consequences of mutations, rather than the mutations themselves in cultured skin fibroblasts. Our results show that of the six biochemical markers analyzed, DHAPAT and C26:0 acid β-oxidation were the only markers showing a good correlation with disease severity, whereas pristanic acid β-oxidation activity correlates reasonably well. The predictive power of DHAPAT and C26:0 β-oxidation was even better when the two markers were combined.

Previous studies have shown that, on average, plasmalogen biosynthesis is less impaired in NALD and IRD fibroblasts than in ZS fibroblasts. However, when the individual values were considered, there was a large overlap indicating that plasmalogen synthesis is an adequate diagnostic tool but not a good predictive test, which is in agreement with our results.

As an alternative one could study the temperature sensitivity of the cell lines. It was shown recently that in some PBD cell lines the defect in peroxisome biogenesis can be (partly) corrected by growth of the cells at a lower temperature. This phenomenon correlates with the milder phenotype. Although we did not evaluate this in this article, it may be a useful approach for the future.

In this study, peroxisomal functions were evaluated in cultured skin fibroblasts only. However, the dysfunction of peroxisomes is reflected also in plasma by elevated levels of VLCFAs, DHCA, THCA, phytanic and pristanic acid and in erythrocytes, by lowered
plasmalogen levels. Ideally, one should measure also these metabolites to see whether there is a relation between phenotype and extent of abnormality. Unfortunately, we did not have plasma (or serum) samples from most of the patients in amounts required for such analyses, making such a comparison impossible. Earlier studies have shown that plasma VLCFA levels give a significant correlation with the three phenotypes, although the spreads were rather large.19

A limitation of this study is that it uses survival as the only marker of phenotype, whereas parents of children diagnosed with a PBD often will be interested also in the patient's quality of life and what will be achieved in terms of neurological and neurosensory development. We are currently in the process of developing a scoring system20 that may help in this respect.

Acknowledgements

The authors thank Rebecca Brauner for suggestions and critical reading of the manuscript and Dr. Guy Besley for editorial comments. Supported by Prinses Beatrix Fonds, grant 99.0220.

References


