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Identification and diagnostic value of phytanoyl- and pristanoyl-carnitine in plasma from patients with peroxisomal disorders

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ABSTRACT

Phytanic acid is a branched-chain fatty acid, the level of which is elevated in patients with a variety of peroxisomal disorders, including Refsum disease, and Rhizomelic chondrodysplasia punctata type 1 and 5. Elevated levels of both phytanic and pristanic acid are found in patients with Zellweger Spectrum Disorders, and pristanic acid is elevated in patients with α-methylacyl-CoA racemase deficiency. For the diagnosis of peroxisomal disorders, a variety of metabolites can be measured in blood samples from suspected patients, including very long-chain fatty acids, phytanic and pristanic acid. Based on the fact that very long-chain fatty acylcarnitines are elevated in tissues and plasma from patients with certain peroxisomal disorders, we investigated whether phytanoyl- and pristanoyl-carnitine are also present in plasma from patients with different peroxisomal disorders. Our study shows that phytanoyl- and pristanoyl-carnitine are indeed present in plasma samples from patients with different types of peroxisomal disorders, but only when the total plasma levels of their corresponding fatty acids, phytanic acid and pristanic acid, are markedly elevated. We conclude that the measurement of phytanoyl- and pristanoyl-carnitine is not sensitive and specific enough to use these acylcarnitines as conclusive diagnostic markers for peroxisomal disorders.

1. Introduction

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain fatty acid, which is derived from dietary sources, including dairy products, fish and meat. In patients with Refsum disease, phytanic acid metabolism is disturbed due to mutations in the gene encoding phytanoyl-CoA hydroxylase (PHYH) (Fig. 1) [1,2]. This results in the impaired α-oxidation of phytanic acid, which subsequently accumulates in tissues and plasma of these patients [3]. Phytanic acid levels are also elevated in patients with the peroxisomal disorder Rhizomelic chondrodysplasia punctata (RCDP) type 1 [4] and type 5 [5]. These patients have mutations in the *PEX7* and *PEX5* gene, respectively, which code for two cytosolic receptor proteins involved in the import of PTS2-targeted proteins into peroxisomes [3]. Furthermore, α-oxidation is impaired in patients with a Zellweger Spectrum Disorder (ZSD), due to mutations in any of 13 different *PEX* genes, causing a defect in peroxisome biogenesis [3].

Phytanic acid is converted to pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) via α-oxidation in the peroxisome and, subsequently, pristanic acid is further broken down via peroxisomal β-oxidation (Fig. 1) [1]. Both phytanic and pristanic acid levels are elevated in ZSD patients depending on the diet and age of the patient. Elevated levels of pristanic acid are also found in plasma from patients with α-methylacyl-CoA racemase (AMACR) deficiency. AMACR is required for the conversion of the R-isomer of pristanic acid into the S-isomer, which is the only isomer that can be broken down via β-oxidation in the peroxisome [6].

Acylcarnitine analysis is an important diagnostic tool especially for the detection of patients suffering from a defect in mitochondrial fatty acid oxidation and/or a defect in amino acid degradation [7]. Acylcarnitines are formed from the corresponding acyl-CoA esters by a variety of different carnitine acyltransferases [7]. Abnormalities in acylcarnitine profiles, notably C26:0-acylcarnitine, have also been described in patients with peroxisomal disorders [8]. Besides the implementation of several acylcarnitines as biomarkers into newborn screening programs for a number of inborn errors of metabolism [9], acylcarnitine metabolites have also been reported in untargeted metabolomics approaches [10]. The use of high-throughput "omics" techniques is becoming increasingly important in clinical diagnostics, and

Abbreviations: ABCD3, ABC transporter D3; AMACR, α-methylacyl-CoA racemase; CoA, Coenzyme A; CROT, carnitine octanoyl-transferase; GC–MS, gas chromatography–mass spectrometry; MRM, multiple reaction monitoring mode; PHYH, phytanoyl-CoA hydroxylase; RCDP, Rhizomelic chondrodysplasia punctata; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry; VLCFA, very long-chain fatty acid; ZSD, Zellweger Spectrum Disorder; 4–8-DMN-CoA, 4,8-dimethylnonanoyl-CoA.

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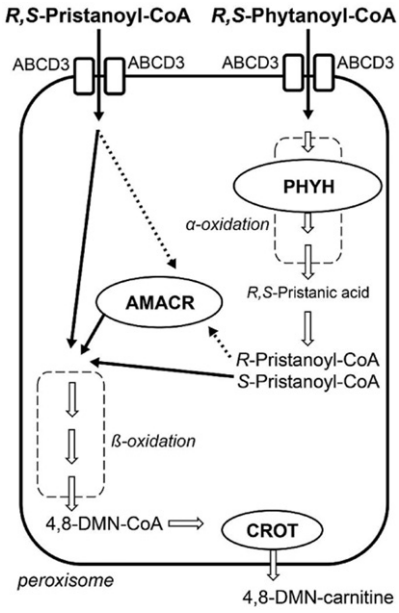

Fig. 1. Schematic overview of branched-chain fatty acid catabolism in peroxisomes. The branched-chain fatty acids phytanic and pristanic acid are transported into the peroxisome as Coenzyme A (CoA)-ester via the membrane protein ABC transporter D3 (ABCD3). Phytanic acid first undergoes α-oxidation, resulting in the formation of pristanic acid. In Refsum disease, the enzyme phytanoyl-CoA 2-hydroxylase (PHYH) is defective, and α-oxidation is blocked. The CoA-ester of pristanic acid first needs to be converted from the (*R*)-configuration into its (*S*)-configuration by the enzyme α-methylacyl-CoA racemase (AMACR) before entering the β-oxidation pathway. Pristanic acid then undergoes chain-shortening via three rounds of β-oxidation producing 4,8-dimethylnonanoyl-CoA (4,8-DMN-CoA). Carnitine octanoyl-transferase (CROT) converts 4,8-DMN-CoA into the corresponding carnitine ester (4,8-DMN-carnitine), which is exported to the mitochondrion for further oxidation.

untargeted metabolomic approaches using mass spectrometry have recently been proposed to be potentially useful in the screening for inborn errors of metabolism [10].

Based on the finding that very long-chain fatty acylcarnitines are elevated in tissues and plasma from patients with certain peroxisomal disorders [8,11–13], we investigated whether phytanoyl-carnitine is present in plasma from patients with elevated phytanic acid levels, including patients with Refsum disease, RCDP type I and ZSD patients. In addition, we investigated whether pristanoyl-carnitine can be detected in plasma from patients with AMACR deficiency.

2. Materials and methods

Anonymised plasma samples from healthy individuals (n = 15), and patients with Refsum disease (n = 8), RCDP type 1 (n = 8), ZSDs (n = 11), and AMACR deficiency (n = 4) were used for the determination of acylcarnitines. Twenty microliters of plasma were mixed with 200 μL of internal standard (20 nM [16,16,16-$^2H_3$]-C16-carnitine (D3-C16-carnitine) in acetonitrile). Subsequently, samples were sonicated for 5 min in a water bath, followed by centrifugation for 5 min at 4 °C with 20,000 g. The supernatant was evaporated at 60 °C under a nitrogen stream, and the residue was dissolved in 50 μL methanol. Ten microliters of extract were injected. Liquid chromatography was performed at 50 °C using an Acquity BEH C18 column (Waters, Milford MA). Extracts were separated by a linear gradient between solution A (0.1% HCOOH in water) and solution B (100% methanol), with a gradient from 0 to 6 min from 20% solution A to 8% solution. The flow rate was 0.5 mL/min. A Micromass Quattro Premier XE Tandem Mass Spectrometer (Waters, Milford, MA) was used in the multiple reaction monitoring mode (MRM) in positive electrospray ionisation mode. The spray voltage used was 3.5 kV, source temperature was 130 °C, and the desolvation temperature was 350 °C. Cone gas flow: 50 L/h, desolvation gas flow: 900 L/h, collision gas pressure 2.5e-3 m bar. We determined a subset of acylcarnitine species, ranging from C16-carnitine to C20-carnitine, pristanoyl-carnitine, and phytanoyl-carnitine. The common daughter ion was *m/z* 85.0. Following parent ions were used for acylcarnitine species in MRM channels: *m/z* 403.3 (D3-C16); *m/z* 442.4 (C19; pristanoyl-carnitine); *m/z* 456.4 (C20; phytanoyl-carnitine). Acylcarnitine levels were processed using Masslynx software. Phytanic acid and pristanic acid were measured using gas chromatography–mass spectrometry (GC–MS), as described previously [14]. Data in figures are presented as mean ± SD, and one-way ANOVA was used for statistical comparison between the groups.

3. Results

We measured phytanoyl-carnitine and pristanoyl-carnitine levels in plasma from patients with different peroxisomal disorders. We included plasma samples from patients with Refsum disease (n = 8), RCDP

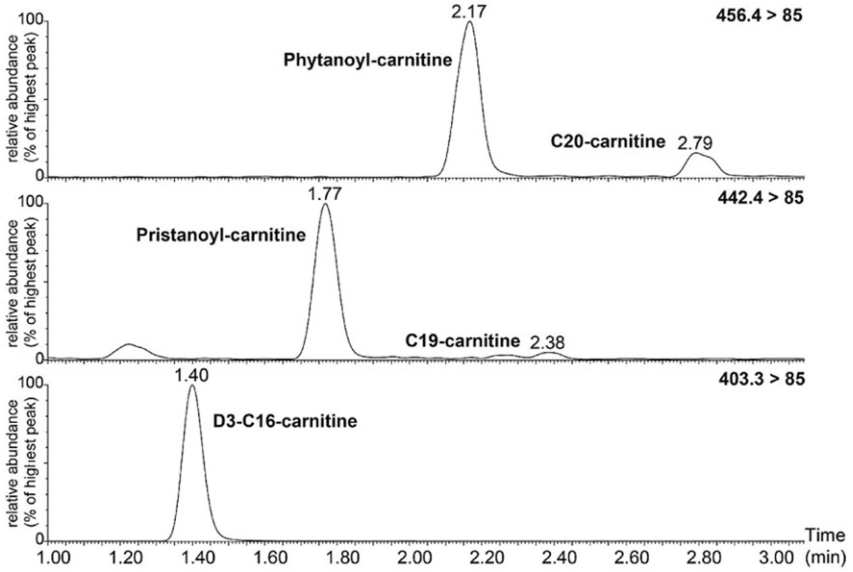

Fig. 2. Representative extracted ion chromatograms of newly identified acylcarnitine species. Transitions are shown for C20- and phytanoyl-carnitine, C19- and pristanoyl-carnitine, and the internal standard D3-C16-carnitine.