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Laboratory diagnosis of disorders of peroxisomal biogenesis and function: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

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Peroxisomal disorders are a clinically and genetically heterogeneous group of diseases caused by defects in peroxisomal biogenesis or function, usually impairing several metabolic pathways. Peroxisomal disorders are rare; however, the incidence may be underestimated due to the broad spectrum of clinical presentations. The inclusion of X-linked adrenoleukodystrophy to the Recommended Uniform Screening Panel for newborn screening programs in the United States may increase detection of this and other peroxisomal disorders. The current diagnostic approach relies heavily on biochemical genetic tests measuring peroxisomal metabolites, including very long–chain and branched-chain fatty acids in plasma and plasmalogens in red blood cells. Molecular testing can confirm biochemical findings and identify the specific genetic defect, usually utilizing a multiple-gene panel or exome/genome approach. When next-generation sequencing is used as a first-tier test, evaluation of peroxisome metabolism is often necessary to assess the significance of unknown variants and establish the extent of peroxisome dysfunction. This document provides a resource for laboratories developing and implementing clinical biochemical genetic testing for peroxisomal disorders, emphasizing technical considerations for sample collection, test performance, and result interpretation. Additionally, considerations on confirmatory molecular testing are discussed.

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BACKGROUND

Peroxisome structure and function

Peroxisomes are ubiquitous single membrane-bound organelles responsible for several anabolic and catabolic reactions.[1] The most well-characterized peroxisomal pathways are those used in clinical evaluation, including β-oxidation of very long–chain fatty acids (VLCFAs), α-oxidation of methyl-branched phytanic acid, and the synthesis of mature bile acids (BAs) and plasmalogens[1] (Fig. 1 Supplemental).

VLCFAs, defined as having a carbon chain with 22 carbons or more, are "tethered" to the peroxisome membrane by ACBD5[2] and transported into the peroxisome by the integral peroxisome membrane protein ABCD1.[3] Once in the peroxisome, fatty acid shortening occurs by a mechanism similar to mitochondrial β-oxidation although producing H_2O_2, which is further catabolized into water and oxygen by peroxisomal catalase.[4] Phytanic acid and C27-bile acid intermediates, dihydroxycholestanoic acid (DHCA) and

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trihydroxycholestanoic acid (THCA), require auxiliary modifications before they can undergo β-oxidation.[1,5] These substrates enter the peroxisome by ABCD3.[6] Phytanoyl-CoA requires α-oxidation by phytanoyl-CoA hydroxylase (PhyH);[7] the resulting pristanoyl-CoA, as well as DHCA and THCA-CoA derivatives, require racemization from R to S forms by α-methylacyl-CoA racemase (AMACR).[1,5,8] The first step in peroxisomal β-oxidation utilizes an oxidase: acyl-CoA oxidase (ACOX) 1[9] for straight-chain fatty acids, ACOX2[5,10] for bile acids, and ACOX2 and ACOX3 for pristanic acid. The next steps, hydration and dehydrogenation, are accomplished by the bifunctional enzyme (DBP),[11] followed by thiolysis (Sterol Carrier Protein X [SCPx][12] or ACAA1.[5]) After several β-oxidation cycles, fatty acids shortened to approximately 16 carbons exit the peroxisome as carnitine derivatives. The C27-bile acid intermediates, DHCA and THCA, and the docosahexaenoic acid (DHA) precursor (C24:6n-3) undergo only one cycle of β-oxidation, which results in the production of the C24 mature bile acids, 3α,7α,12α-trihydroxy-5β-cholanoic acid (CA) and 3α,7α-dihydroxy-5β-cholanoic acid (CDCA)[5] and DHA (C22:6n-3).[13] Subsequently, CA and CDCA are conjugated to glycine or taurine by the bile acid-CoA:amino acid N-acyltransferase (BAAT).[14]

An additional oxidative pathway occurring in peroxisomes is the oxidation of L-pipecolic acid, an intermediate of lysine catabolism, to Δ^1-piperidine-6-carboxylate (P6C).[15] The main pathway for lysine degradation occurs in liver mitochondria with conversion to saccharopine, and then to α-aminoadipic semialdehyde (AASA).[16] The two pathways converge on AASA, since the P6C ring spontaneously opens forming AASA. AASA is a substrate for the enzyme AASA dehydrogenase, encoded by *ALDH7A1* and found both in the cytosol and within mitochondria. Pathogenic variants in *ALDH7A1* cause pyridoxine-dependent epilepsy (PDE; OMIM 266100),[17,18] an infantile epilepsy unresponsive to standard anticonvulsants.

Peroxisomes are also responsible for the initial steps of plasmalogen biosynthesis. Plasmalogens are a subset of glycerophospholipids distinguished by the presence of a vinyl-ether bond at the *sn*-1 position and enriched polyunsaturated fatty acids at the *sn*-2 position. They are abundant in cellular and subcellular membranes of many tissues. Synthesis of plasmalogens begins with two enzymes associated with the peroxisomal luminal membrane, dihydroxyacetonephosphate acyltransferase (DHAPAT)[19] and alkyl-dihydroxyacetonephosphate synthase (AGPS).[20] In forming a vinyl-ether bond, AGPS utilizes fatty alcohols made by fatty acyl-CoA reductase (FAR1)[21] from fatty acyl-CoAs specifically of chain-lengths C16:0, C18:0, and C18:1. FAR1 is a membrane protein located on the cytoplasmic surface of the peroxisome membrane; it displays feedback inhibition to cellular plasmalogen levels and thus regulates this pathway. The 1-alkyl-DHAP product, generated from the reaction catalyzed by AGPS, is reduced to alkyl-glycerophosphate by an acyl/alkyl-DHP reductase. Further modifications occur in the endoplasmic reticulum to generate a mature vinyl-ether glycerophospholipid (plasmalogen).

Lastly, glyoxylate detoxification to glycine by the enzyme alanine-glyoxylate aminotransferase (AGT) takes place predominantly in liver peroxisomes. Deficiencies in AGT cause the kidney condition primary hyperoxaluria type 1 (OMIM 259900).[22]

Peroxisome biogenesis

Peroxisomes are versatile compartments that can increase their capacity in response to the metabolic needs of the cell. Peroxisome biogenesis refers to the formation of new peroxisome membranes, division of existing peroxisomes, and enzyme import into the peroxisome matrix.[1,23] These processes require the coordinated action of 16 PEX proteins, or peroxins, encoded by their cognate *PEX* genes (Table 1 Supplemental). Peroxisomal enzymes are synthesized on cytosolic ribosomes and contain one of two peroxisome-targeting sequences (PTS). The majority of enzymes contain the targeting sequence PTS1, recognized by PEX5 receptor.[24,25] A few enzymes, notably ACAA1, PhyH,[7] and AGPS,[20] contain a PTS2 sequence recognized by PEX7 receptor.[26] PEX5 and PEX7 receptors bind to their respective PTS1 or PTS2 targeted enzymes in the cytosol; in addition, PEX7 binds the PEX5 long isoform (PEX5L),[25] which is critical for PEX7-mediated import of PTS2-tagged proteins.

The receptor–enzyme complexes dock at the peroxisome membranes using PEX13[27,28] and PEX14[29] (importomer complex) and release their cargo enzymes into the matrix. Three integral membrane proteins PEX2,[30] PEX10,[31] and PEX12[32] participate in monoubiquitination of PEX5, which allows PEX5 (presumably with PEX7) to be recycled into the cytosol by PEX1–PEX6–PEX26[33–36] (exportomer complex) for another round of import. PEX3, PEX16,[37] and PEX19[38] are required to form new peroxisome membranes from the endoplasmic reticulum and from mitochondrial membranes.[39] PEX11 (there are 3 isoforms, PEX11 alpha, beta,[40] and gamma) is required for fission of existing peroxisomes, a process requiring mitochondrial fission proteins, MFF, FIS1, and DLP1[41] (*DNM1L*).

Clinical description of peroxisomal disorders

Peroxisomal disorders are a heterogeneous group of inherited diseases due to defects in peroxisomal biogenesis (PBD, Table 1 Supplemental) or single peroxisomal proteins (SPPD; Table 2 Supplemental). PBDs are divided into two groups: Zellweger spectrum disorders (ZSD) and rhizomelic chondrodysplasia punctata (RCDP). ZSDs are a highly heterogeneous group of disorders due to pathogenic variants in one of several *PEX* genes (Table 1 Supplemental). Core features of ZSDs include liver dysfunction, neurological abnormalities including developmental delays, adrenocortical dysfunction, and vision and hearing impairment.[42,43] RCDP is characterized by proximal shortening of the long bones, a distinctive facial appearance, cataracts, congenital contractures, and intellectual disability. Punctate calcification within the epiphyses of long bones (chondrodysplasia punctata) is apparent on radiographs, particularly in infancy.[44]

X-linked adrenoleukodystrophy (X-ALD), caused by pathogenic variants in ATP-binding cassette transporter type D1 (*ABCD1*),[3] typically presents with one of three phenotypes:[45] (1) childhood cerebral form with progressive neurological impairment (cognition, behavior, vision, hearing, motor function) and adrenal insufficiency; (2) late-adolescent or early-adulthood adrenomyeloneuropathy (AMN) with progressive paraparesis and adrenocortical dysfunction; (3) isolated adrenal insufficiency at any age. Approximately 20–50% of adult females with an *ABCD1* variant may develop symptoms of AMN. Frequently, X-ALD is misdiagnosed as attention deficit–hyperactivity disorder (ADHD), multiple sclerosis, or idiopathic adrenal insufficiency. Other SPPDs are less common than X-ALD (Table 2 Supplemental). While some features are common to patients with RCDP, ZSDs, or X-ALD, others are not. For example, ulcerating oral gangrene is typically observed in patients with catalase deficiency. Accumulation of calcium oxalate in the kidney progressing to renal failure is common in patients with hyperoxalurias, but also described in ZSD patients. Retinitis pigmentosa, peripheral neuropathy, cerebellar ataxia, and elevated protein levels in the cerebrospinal fluid are seen in Refsum disease (neurological and visual abnormalities are also seen in ZSD patients). Adult-onset neurodegeneration is present in AMACR deficiency. Elevated serum BA concentrations, itching, and fat malabsorption are seen in familial hypercholanemia. Encephalopathy, developmental delay, and hypotonia are present in patients with encephalopathy due to defective mitochondrial peroxisomal fission 1 (EMPF1).

Incidence

Collectively, peroxisomal disorders have an estimated combined incidence of 1:5,000 individuals.[1] The most common SPPD is X-ALD[3,45] with an estimated incidence of 1:17,000.[1] The most common PBDs are ZSDs,[43] with an estimated frequency of 1:50,000 in North America.[23] Although ZSDs are pan-ethnic, the incidence varies by region, ranging from 1:12,000 in the French Canadian region of Quebec[46] to 1:500,000 in Japan.[47]

Mode of inheritance

Most PBDs and SPPDs are autosomal recessive (AR) conditions. The most notable exception to this is X-ALD, which is X-linked; hemizygous males will develop a clinical phenotype and heterozygous females may develop symptoms over time. Other exceptions include autosomal dominant (AD) de novo variants in *DLP1* in patients with EMPF1[41] and a recently described autosomal dominant form of ZSD due to allelic imbalance for the PEX6-p.Arg860Trp allele (NM_000287.4:c.2578C>T).[48]

Peroxisomal disorders treatment

Several symptomatic or supportive interventions are available to treat peroxisomal disorders, such as feeding tubes to ensure adequate nutrition, eye glasses and hearing aids, antiepileptic medications, and treatment for nephrolithiasis.[42,43,49] Adrenal hormone replacement for those with adrenal insufficiency is lifesaving. Specific interventions are available for selected diagnoses. Prevention of oxalate calculi with urine alkalinization and hydration preserves renal function in patients with hyperoxaluria type I;[49] clinical trials using RNA-mediated drugs are ongoing (Alnylam Pharmaceuticals 2016, NCT02706886). In patients with Refsum disease, dietary intake of phytanic acid is restricted to reduce accumulation, and fasting is avoided to prevent its mobilization from stored fat.[50] Cholic acid therapy has been used in specific disorders of bile acid synthesis, such as AMACR deficiency, to reduce bile precursor accumulation and normalize liver function.[49] Cholic acid therapy reduces the production of bile acid intermediates in patients with ZSD; however, its use may be limited due to possible hepatotoxicity.[51,52]

Patients with X-ALD and cerebral involvement can benefit from hematopoietic stem cell transplantation (HSCT) if performed prior to significant involvement.[53,54] HSCT is associated with up to a 20% mortality rate due to transplant-related complications.[53,54] Early results of the STARBEAM study suggest that transplantation with autologous hematopoietic stem cells transfected with elivaldogene tavalentivec (Lenti-D) lentiviral vector is as effective as conventional allogeneic transplantation.[55] Currently, there is no targeted therapy for AMN.

Newborn screening for peroxisomal disorders

In February 2016, the US Secretary of Health and Human Services approved the inclusion of X-ALD in the Recommended Uniform Screening Panel (RUSP) for state newborn screening (NBS) programs.[56] Attempts to screen for this condition by analyzing VLCFAs in dried blood spots (DBS) were carried out in the late 1980s and early 1990s; however, cumbersome extraction procedures, long run times, high false positive rates, and the discovery of endogenous VLCFAs in filter paper used for NBS made analysis of VLCFAs in DBS unreliable.[57] For these reasons, other lipid fractions were evaluated and the lysophosphatidylcholine (LPC) species were found not only to be elevated in DBS of patients with X-ALD, but could be measured accurately and efficiently using standard NBS technology.[58] Current NBS protocols utilize measurement of hexacosanoyl-lysophatidylcholine (C26:0 LPC); other markers, such as the ratio of C26:0 LPC to C20:0 LPC, and hexacosanoylcarnitine (C26-carnitine) may also be useful. New York State began screening for X-ALD at the end of 2013; its experience, together with data on the efficacy of early treatment, was instrumental in obtaining RUSP approval.[56] To date, several methods are available to measure C20:0-C26:0 LPCs, either alone or in combination with other analytes,[59–65] and additional states have enacted X-ALD screening (for a list of states, refer to www.babysfirsttest.org). Guidelines on follow-up evaluation of a positive screen are available.[66,67] Although NBS was designed to identify newborns with X-ALD, LPCs may detect other conditions, such as ZSDs, and ACOX1 and DBP deficiencies. In addition, some neonates with Aicardi Goutières syndrome have elevated levels of C26:0 LPC.[68]

METHODS

The laboratory technical standard was informed by a review of the literature, including current guidelines, and expert opinion. Resources consulted included PubMed (search terms: peroxisomes; peroxisome biogenesis; peroxisomal disorders [AND newborn screening]; very long–chain fatty acids [AND liquid chromatography OR gas chromatography]; plasmalogen [AND liquid chromatography OR gas chromatography]; pipecolic acid [AND liquid chromatography OR gas chromatography]; bile acid synthesis [AND peroxisomal disorders]; X-linked adrenoleukodystrophy; Zellweger spectrum disorder; rhizomelic chondrodysplasia), the American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines for Clinical Genetics Laboratories, Clinical and Laboratory Standards Institute (CLSI) guidelines, CLIA regulations, and the Centers for Disease Control and Prevention Morbidity and Mortality Weekly Report (MMWR) on Good Laboratory Practices for Biochemical Genetics Testing and Newborn Screening for Inherited Metabolic Disorders. When the literature provided conflicting evidence about a topic or when there was insufficient evidence, the authors used expert opinion to inform the recommendations. Expert opinion included the coauthors of the document, members of the ACMG Laboratory Quality Assurance Committee, as well as the experts consulted outside of the Committee but acknowledged in this document. The standard Human Genome Variation Society (HGVS) nomenclature[69] was used to describe the sequence variants cited in this document.

Any conflicts of interest for workgroup members or consultants are listed. A draft was delivered to the ACMG Board of Directors for review and member comment. The draft document was posted on the ACMG website and an email link was sent inviting ACMG members to provide comment. The authors assessed all comments. When appropriate, additional evidence was included to address member comments and the draft was amended. Both member comments and author responses were reviewed by a representative of the ACMG Laboratory Quality Assurance Committee and by the ACMG Board of Directors. The ACMG Board of Directors approved the final document before submission for publication.

PREANALYTICAL REQUIREMENTS

Specimen requirements

Laboratory testing for peroxisomal disorders includes different classes of metabolites that accumulate in different metabolic pathways. Hence, a variety of assays are utilized, each being routinely performed in a certain specimen type depending on analyte abundance, ease of collection, or other analytical and clinical considerations. In general, the laboratory should evaluate sample requirements and factors influencing its collection, handling, shipping, and storage at the time of method validation. Sample requirements must be made available to ordering physicians.

Quantitative analysis of very long–chain fatty acids (VLCFAs) and branched-chain fatty acids (BCFAs; e.g., phytanic and pristanic acids) can be performed in plasma or serum[70] separated from venous blood (2–5 mL) ideally within 1 hour from collection to avoid hemolysis; sodium or lithium heparin (green top) or EDTA (lavender top) tubes can also be used.[71] Metabolite analysis, immunochemical or enzymatic assays can be performed on primary fibroblasts derived from a 2–3 mm skin biopsy under sterile conditions and cultured according to standard protocols. Free and conjugated bile acids (BA) are usually quantified in serum samples; EDTA plasma and serum (red top) contain similar BA concentrations.[72]

Although plasmalogens can be detected in plasma and serum, these specimens are less sensitive for distinguishing peroxisomal disease states from controls, due to their lower plasmalogen concentrations compared with erythrocytes and possibly due to the influence of diet on plasma/serum levels.[70,73] In general, erythrocytes are separated by centrifugation from 1–5 mL of EDTA anticoagulated blood, washed twice in phosphate-buffered saline to remove residual plasma and buffy coat, flushed with nitrogen, and stored frozen until processing.[74]

Pipecolic acid accumulates in several body fluids. For ease of collection, clinical testing is usually carried out in plasma (sodium or lithium heparin, EDTA, or ACD),[75] serum,[76] or urine. While pipecolic acid can be measured in cerebral spinal fluid (CSF), normal levels in this specimen type are very low, approximately tenfold less than plasma; thus, detection requires very sensitive methods.[77] Pipecolic acid is not consistently elevated in the urine of patients with PBDs,[76] so plasma is preferred with one exception: in neonates, plasma pipecolic acid in affected individuals may be within normal limits, thus urine is recommended (unpublished data from the Peroxisomal Diseases Laboratory, Kennedy Krieger Institute). Pipecolic acid urinary excretion is measured using random urine, and is normalized to creatinine. Urine should be collected in a clean container without preservatives.[75,76]

Molecular genetic studies are typically performed using DNA isolated from leukocytes, usually using 1–2 mL EDTA whole blood. Some laboratories offer testing of RNA by complementary DNA (cDNA) sequencing. Other tissue sources, including cultured chorionic villus sample (CVS) or amniocytes for preimplantation/prenatal diagnosis, can be used if necessary. When no pathogenic variant(s) have been previously identified, biochemical testing in CVS or amniocytes can be used in at-risk pregnancies. When testing CVS or amniocytes, maternal cell contamination must be excluded.[78]

Sample handling, shipping, and storage

Plasma, urine, and CSF should be immediately frozen upon collection, and shipped on dry ice. Stability under different conditions varies among metabolites, and laboratories should establish proper handling, shipping, and storage conditions. VLCFAs and BCFAs are stable up to 15 days at room temperature (18–25 °C) or refrigerated (2–8 °C), and at least 92 days at ≤−20 °C (unpublished data from the Biochemical Genetics Laboratory at Mayo Clinic). Plasmalogens are stable

in whole blood at room temperature (18–25 °C) or refrigerated (2–8 °C) for up to 72 hours. However, once erythrocytes have been isolated and washed, they should be stored under nitrogen at ≤−65 °C, where plasmalogens are stable for up to 12 months (unpublished data from the Peroxisomal Diseases Laboratory, Kennedy Krieger Institute). Pipecolic acid and the metabolites P6C and AASA are stable in plasma or urine up to 5 hours at room temperature (18–25 °C) and up to 24 hours refrigerated (2–8 °C); once samples are frozen, they are stable at least 40 days at ≤−20 °C and up to 1 year at ≤−65 °C.[75,79] BAs are stable in EDTA plasma up to 24 hours at room temperature (18–25 °C), at least for several weeks once samples are frozen (≤−20 °C).[72] Cultured primary fibroblasts are shipped at room temperature (18–25 °C) in T-25 flasks filled with culture media. Whole blood for DNA extraction should be shipped at room temperature if delivery is within 24 hours or refrigerated if it takes longer (2–8 °C).

Preanalytical variables

In general, fasting or preprandial blood specimens are recommended for metabolite testing; the effect on metabolite concentrations following feeding has not been evaluated for all analytes and may differ among analytes. VLCFAs and BCFAs levels have been shown to increase slightly after a meal.[80] BA concentrations also increase after a meal; the increase is modest in healthy individuals, but significant in patients with various liver conditions.[81] Other factors affect VLCFA and/or BCFA concentrations leading to an abnormal result: a ketogenic diet,[82] high peanut butter consumption,[83] severe liver disease, and hyperlipidemia. Hemolysis increases VLCFAs through release from erythrocyte membranes. Hence, plasma/serum should be separated within 24 hours from collection,[74] ideally within the first hour, and conditions compromising blood integrity should be avoided. On the other hand, pipecolic acid levels in plasma/serum are not affected by hemolysis or hyperlipidemia,[75] and there is no evidence of significant changes with postprandial collection.[15]

Hemolyzed blood samples are not optimal for plasmalogen analysis, as loss of erythrocytes will artificially lower absolute levels of plasmalogens measured as C16:0 and C18:0 dimethyl acetals (DMAs). Specimens demonstrating moderate to severe hemolysis should be rejected; erythrocytes collected from slightly hemolyzed blood may be used if plasmalogen content is evaluated as a ratio to C16 and C18 fatty acids. Testing performed in blood collected following a blood transfusion could result in erroneously normal or partially reduced plasmalogen levels, as transfused erythrocytes can survive for up to 120 days.[74] Results obtained in this scenario requires clinical correlation and reanalysis at least 4 weeks post-transfusion should be considered.

METHOD VALIDATION

Protocols should be established by each laboratory to determine and periodically verify performance characteristics of methods used in peroxisomal disorder testing, in accordance with CLIA and the College of American Pathologists (CAP) regulations (see CLSI document C24[84]). The laboratory should implement procedures to address values outside of their established criteria for an assay's performance.

Gas chromatography–mass spectrometry (GC-MS) and tandem mass spectrometry (MS/MS), with or without liquid chromatography (LC), are routinely used to quantify VLCFAs/BCFAs, plasmalogens, pipecolic acid, and BAs. Quantitation with these methods requires a calibration curve with purified synthetic standards, and isotope-labeled internal standards. With few exceptions (e.g., the metabolites P6C and AASA), high-grade standards and internal standards are commercially available from several sources; however, standards may not be available for all components of a multianalyte panel. Reagent performance should be verified by the laboratory prior to clinical use.

As for any analytical assay, quality control (QC) samples should be included with each batch of patient samples processed. Ideally, QC samples should be of the appropriate sample matrix and include two levels of quality control, typically normal and abnormal. Target ranges for QC samples should be established by analyzing replicates (at least 15–20) over several runs, possibly by multiple operators. Plasma or serum specimens from several normal individuals can be pooled and used as normal QC for metabolites assayed in plasma, serum, or CSF. Similarly, urine from a normal individual is used for metabolites assayed in urine. Often, normal plasma/serum or urine samples spiked with standards are used as abnormal QC, also referred to as "high control," while the normal control can be also referred to as "low control." Ideally, the abnormal QC is around 75% of the highest calibrator used, which usually corresponds to the upper limit of quantification, and within the range of values observed in patients. A readily available source of erythrocytes with normal plasmalogen levels can be obtained from expired blood purchased from the American Red Cross. Canine blood, which has low erythrocyte plasmalogen levels relative to human blood, is a potential source for an abnormal control and may be obtained from veterinary clinics.

Proficiency testing

Laboratories should participate in an ongoing proficiency testing (PT) program, at least semiannually, as required by regulating agencies. PT outcome should be carefully reviewed to monitor performance and identify results requiring investigation; any remedial action should be carefully documented. The European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Disorders of Metabolism (ERNDIM) provides a commercially available, external proficiency testing service for the quantitative analysis of VLCFAs (C:22, C24:0, C26:0), phytanic and pristanic acids in serum, and L-pipecolic acid in serum and urine. Scheduled interlaboratory comparison (alternative PT) can be used when external proficiency testing is unavailable.

Reference intervals

Laboratory-specific reference intervals should be established for all analytes and periodically verified following guidelines (e.g., CLSI document EP28-A3c[85]). Literature-based ranges should be verified using specific methods/platforms employed by the laboratory to validate the test. Anonymized samples from the general population are acceptable once relevant conditions affecting test results have been excluded; for some analytes, reference intervals are age-specific. If possible, the range of values observed in affected individuals should also be determined. Since there are often significant differences between sample types, reference intervals should be established or verified for each specimen type used for clinical testing.

TESTING FOR PEROXISOMAL DISORDERS

Analysis of very long–chain fatty acids and branched-chain fatty acids in plasma

Traditionally, the measurement of very long–chain fatty acids (VLCFAs) and branched-chain fatty acids (BCFAs) in plasma/serum was carried out by GC-MS.[74] This method quantifies fatty acids containing hydrocarbon chains with 8 to 30 carbons, but only a subset is used for diagnosing peroxisomal disorders. The first step of sample preparation consists of hydrolysis of fatty acids bound to complex molecules, using an acid hydrolysis followed by basic hydrolysis and reacidification. After extraction with hexane, the fatty acids are derivatized using pentafluorobenzyl bromide (PFBBr).[74] Capillary GC-MS using negative chemical ionization with ammonia as the reagent gas and selected negative-ion monitoring allows separation and detection of the corresponding PFB-esters. Quantitation is achieved using stable isotope-labeled internal standards.[74] VLCFA/BCFA quantitation by GC-MS requires a lengthy and laborious sample preparation (approximately 8 hours), and run time of approximately 30 minutes per sample. Several methods using positive-ion electrospray ionization tandem mass spectrometry (ESI-MS/MS)[86–88] or negative-ion electrospray ionization mass spectrometry (ESI-MS)[89] have been developed to simplify sample preparation and reduce run time. The ESI-MS/MS method originally developed by Johnson,[86] detecting fatty acids as dimethylaminoethyl ester derivatives, offered a rapid sample preparation (approximately 2 hours) and a short run time (3.5 minutes/sample). However, BCFAs cannot be distinguished from their straight-chain isomers with this method. Method limitations were resolved by the inclusion of a second derivatization step to trimethylaminoethyl ester derivative, and the use of liquid chromatography;[87] however, these changes resulted in sample preparation and run time comparable with GC-MS methods. The method developed by Al-Dirbashi et al. utilizes ultraperformance liquid chromatography (UPLC) and derivatization with 4-[2-(N,N-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE) to rapidly detect (5 minutes/sample) plasma BCFAs/VLCFAs after a relatively simple sample preparation (approximately 3 hours).[88] Overall, VLCFA/ BCFA concentrations are comparable among methods.[88]

VLCFAs/BCFAs analysis is a particularly useful biochemical test for the diagnosis of peroxisomal disorders, since abnormalities in these metabolites are noted in the majority of patients. Elevations in hexacosanoic acid (C26:0) and the C26:0 to docosanoic acid (C22:0) ratio are typical of X-ALD patients. Some cases, especially adults, might also show an elevation in tetracosanoic acid (C24:0). The test is very sensitive in males, with almost all X-ALD or AMN patients presenting an abnormal profile. However, 15–20% of female heterozygotes have a normal profile, whether or not they are symptomatic.[90,91] Recently, C26:0 LPC in DBS has been shown to be a more sensitive marker to identify X-ALD heterozygotes compared with C26:0-carnitine in plasma, being abnormal in all X-ALD heterozygotes, including those with normal plasma results.[92] The degree of the VLCFA abnormality does not correlate with age of onset or severity of phenotype,[93] and cannot be used as a predictor of clinical outcome. Abnormal results for this test are not specific to X-ALD/AMN; peroxisomal biogenesis disorders, ACOX1 deficiency, and DBP deficiency have a similar VLCFA profile but different clinical features and other biochemical abnormalities. Elevated phytanic acid accompanies the abnormal VLCA profile in ZSD and some DBP deficiencies. As a consequence of impaired α-oxidation, isolated elevations in phytanic acid are seen in Refsum disease, RCDP types 1 and 5; however, the VLCFA profile is normal.[7,50,94] AMACR deficiency is characterized by elevation of BCFAs, in particular pristanic acid, and by an increased pristanic to phytanic acid ratio;[8] similar abnormalities can be seen in SCPx deficiency.[12] Since VLCFA levels do not change over time, age is not a significant factor in result interpretation.[90] In contrast, BCFAs are low at birth, and increase with dietary intake of phytanic acid; hence, affected newborns may not show BCFA abnormalities. As noted previously, nonfasting and hemolyzed specimens and samples from individuals on a ketogenic diet can produce abnormal results; the pattern of abnormalities in different VLCFA and BCFA species can assist in differentiation from peroxisomal disorders; in particular, the C24/C22 ratio is less influenced by diet. A normal result in plasma does not exclude a peroxisomal disorder, as false negative results have been reported.[95,96]

Analysis of plasmalogens in red blood cells

Historically, plasmalogen deficiency was identified by measuring plasmalogens as DMAs in erythrocytes.[73] Several analytical methods for measuring plasmalogen levels have been published and include gas–liquid chromatography (GC), GC-MS, and liquid chromatography/mass spectrometry (LC-MS) techniques.[70,73,97–100] GC and GC-MS methodologies require solvent extraction of lipids and conversion of fatty acids to fatty acid methyl esters (FAME) and plasmalogens to DMA derivatives by acid methanolysis.[74] DMA derivatives are separated by GC using a nonpolar capillary column and plasmalogen levels may be

expressed as a ratio of C16:0 DMA and C18:0 DMA compared with their corresponding FAME or quantified using a nonendogenous free fatty acid of similar chain length (e.g., C19:0 free fatty acid). Ratio analysis may have greater sensitivity, as samples from individuals with RCDP have a higher level of long-chain alcohols, which are subsequently converted to C16 fatty acid.[101] When GC alone is used, appropriate standards must be analyzed concurrently to confirm relative retention times for DMAs and FAMEs. GC-MS methods use selected-ion monitoring (SIM) mass spectrometry to increase assay specificity by utilizing mass assignments, *m/z* 255 for C16:0 DMA and *m/z* 283 for C18:0 DMA, in addition to retention time for accurate identification. More recently, LC-MS methods measuring plasmalogens have been published.[99,100] The advantages of these techniques include reduced sample processing time and more accurate quantification using synthetic plasmalogens as internal standards, although these internal standards also could be used in SIM-GC-MS analysis. The disadvantage is that not all plasmalogens are measured, only the major species of the ethanolamine and choline plasmalogens. One source of variability in analyte measurement is the GC injection port. Recovery may be reduced as a result of inadequate passage of DMAs and FAMEs through the injection port due to interaction with septal particles produced during the injection process. Thus, for GC and GC-MS analyses, a specialized septum such as the Merlin microseal septum, which is designed to eliminate septal coring, may be required. Likewise, for LC-MS assays, recovery may be influenced by carryover, as plasmalogens may adhere to LC polyetheretherketone (PEEK) tubing.

Conditions affecting plasmalogen synthesis, including PBDs and isolated deficiencies of DHAPAT, AGPS, or FAR1, can lead to reduced plasmalogen levels and ultimately result in an abnormal phenotype. Plasmalogen levels are typically low at birth, gradually increasing to plateau by 6 months of age.[102] Because unaffected neonates can have levels that overlap those in older children confirmed to have a PBD, each laboratory, based on their analytical method of choice, must establish age-specific reference intervals. Still, plasmalogen levels in PBD patients may normalize with age,[103] decreasing the utility of this assay for the investigation PBDs in older children. In RCDP, the extent of plasmalogen deficiency correlates with phenotypic severity.[104] While severely affected individuals have a significant reduction in red blood cell (RBC) plasmalogen content, those with less severe phenotypes show a more modest reduction. In very mild cases, the RBC plasmalogen level may be near normal. While RCDP is often the primary clinical indication for measuring plasmalogen levels, this assay also is performed as part of an overall investigation for inborn errors of metabolism affecting peroxisomal function or assembly. Secondary plasmalogen deficiency has been observed in a number of common conditions such as respiratory disease, inflammatory conditions, Parkinson disease, Down syndrome, and Alzheimer disease.[105]

Analysis of pipecolic acid in urine and plasma

Amino acid analysis performed with traditional methods, like ion exchange chromatography, can detect L-pipecolic acid when present in large amounts;[106] but several more sensitive methods have been developed using mass spectrometry combined with gas or liquid chromatography.[74,76,77,107–109] Pipecolic acid can be measured by GC-MS in negative[74,77] or positive[107] mode following derivatization of the amine group with methyl chloroformate, acidic ethyl acetate extraction, and further derivatization with pentafluorobenzyl bromide. Some LC-MS/MS methods do not require sample derivatization, thus requiring smaller sample volumes and less processing time.[108,109] A deuterated stable isotope, like ^{2}PA-d_{11} or ^{2}PA-d_{9}, is utilized as internal standard. Quantitation can be performed by stable isotope dilution or using a calibration curve. $^{2}H_{5}$-phenylalanine has also been used as internal standard.[108,109] A chiral column can specifically discriminate L-pipecolic acid from the D-enantiomer.[108] However, in both affected and normal individuals, the D-enantiomer represents only 2% of pipecolic acid.[110]

Interestingly, elevated pipecolic acid was the first biochemical abnormality described in ZSD patients.[111] Patients with single peroxisomal protein defects, like ACOX1 deficiency or DBP deficiency, display pipecolic acid within the normal range.[23,94,112] Pipecolic acid accumulation is not exclusive to peroxisomal biogenesis disorders. In addition to lysine, patients with hyperlysinuria type I accumulate pipecolic acid in plasma and urine.[113] Pipecolic acid accumulates and is considered a secondary biomarker in patients with PDE,[114] although normal levels have been described.[115] In PDE, the specific metabolites P6C and AASA are markedly increased,[17,116] and pipecolic acid is routinely assessed as part of multianalyte panels that also include AASA and P6C.[75,117] Elevated pipecolic acid has also been described in a patient with pathogenic variants in *NADK2*,[118] a gene encoding a mitochondrial enzyme implicated in several pathways, including lysine degradation.[118] Elevated pipecolic acid is also seen in chronic liver dysfunction[119] and has been reported in an individual without any clinical features.[120,121]

Other biochemical tests

Bile acids (BAs) are hydroxylated steroids synthetized from cholesterol in the liver and conjugated to the amino acids taurine or glycine. BAs facilitate the absorption of fat and fat-soluble vitamins in the small intestine; after being metabolized to unconjugated and dehydroxylated species by the gut bacteria, they are reabsorbed and recycled by the liver. Serum concentrations are directly affected by liver function and represent a sensitive marker for common liver conditions, including intrahepatic cholestasis of pregnancy. The final step of BA synthesis takes place in the peroxisomes, where the C27-bile acid intermediates DHCA and THCA are converted to the primary C24-bile acids. In PBD patients, the C27-bile acid intermediates accumulate and cause progressive liver disease.[122] Testing for these intermediates specifically aids in the diagnosis of peroxisomal disorders.[123–125] The

quantitative analysis of serum BAs by GC-MS is sensitive, accurate, and allows for good discrimination between species.[123] However, since GC-MS methods require a large sample volume and cleavage of conjugated bile acids followed by derivatization, they have been largely replaced by LC-MS/MS methods in the clinical laboratory.[72,124,125] BA abnormalities can be severe in ZSDs, and disease severity correlates with the impairment in BA synthesis; mildly affected individuals can have normal BA levels.[126]

Metabolite levels can also be assessed in cultured skin fibroblasts, although these tests are seldom performed using this sample type, since it requires an invasive skin biopsy and time to establish primary fibroblast cultures. In a minority of individuals (<5%) suspected of having a peroxisomal disorder, measurement of peroxisomal metabolites in fibroblasts could be useful (Steven Steinberg, personal communication); several individuals with abnormalities in fibroblast VLCFAs and BCFAs suggestive of a peroxisomal disorder, with normal plasma values, have been reported.[95,96] Assays have been described that measure peroxisomal enzyme activities in patient fibroblasts, usually using radioisotope-labeled substrates.[127] Only phytanic acid oxidase and pristanic acid oxidase are routinely offered clinically. Fibroblasts can be used to assess the cellular distribution of the enzyme catalase (catalase solubility), which is normally localized to peroxisomes; catalase localization to the cytosol is a sensitive marker of peroxisomal dysfunction.[127]

Molecular testing

Molecular testing can be used to confirm (e.g., X-ALD) or identify (e.g., ZSD) the specific gene defect in individuals with highly suggestive clinical and biochemical findings. In some individuals, molecular testing may be used to clarify equivocal laboratory findings in the presence of a nonspecific clinical presentation. Carrier testing for at-risk relatives by molecular testing is the best approach, since carrier testing by biochemical methods is not possible for most peroxisomal disorders, and has only about 80% sensitivity for X-ALD heterozygotes. The classification of variants can be challenging and may require further research-based studies for clarification, but should be made using the ACMG variant classification guidelines.[128] In families where a peroxisomal defect has been confirmed biochemically (e.g., abnormal VLCFA results in cultured skin fibroblasts from the index case), but molecular testing has identified variants of uncertain clinical significance, predictive prenatal testing using biochemical methods is best, if available. When two definitive pathogenic variants are identified in *trans* in a proband, predictive testing, including preimplantation genetic diagnosis, can be reliably offered to families. For confirmatory testing, test options range from a single-gene test to a multigene panel to exome or genome sequencing. The best approach varies depending on the clinical presentation and certainty of the biochemical profile. Testing *ABCD1* in males with a biochemical and clinical diagnosis of X-ALD should be adequate, but males with a mild or dominant presentation of a ZSD may overlap[129] (PEX6-p.Arg860Trp; NM_000287.4:c.2578C>T) and thus require follow-up of a negative *ABCD1* test with a ZSD gene panel. Likewise, individuals with a suspected diagnosis of acatalasemia or primary hyperoxaluria type 1 would be candidates for single-gene testing. For individuals with a suspected diagnosis of ZSD, but who have undergone plasma VLCFA testing only, a broader panel that includes PEX genes and genes associated with SPPD should be considered. Similarly, a patient with a suspected diagnosis of RCDP who has deficient erythrocyte plasmalogens and phytanic acid oxidation (elevated plasma phytanic acid) could have molecular testing limited to *PEX7*, but if there is uncertainty then a broader RCDP multigene panel is recommended. For individuals with a mild or equivocal biochemical profile, a comprehensive multigene panel that includes most PEX genes and genes associated with SPPDs would be ideal. Exome or genome sequencing may be considered for patients with negative single-gene and multigene panel tests or when the most appropriate single or multigene test cannot be determined. The majority of pathogenic variants associated with peroxisomal disorders involve single-nucleotide substitutions or small insertion/deletions that can be detected by sequencing (either Sanger or next-generation sequencing platforms); however, in some instances, exon-level deletions or duplications are present, and require alternative techniques for detection. Although some laboratories may be able to detect multiexon deletions or duplications using next-generation sequencing platforms, an orthogonal method such as multiplex ligation-dependent probe amplification (MLPA) will be usually needed to confirm a single exon copy-number alteration. About 5–6% of *ABCD1* pathogenic variants are large deletions that can be detected by Southern blot or MLPA; in these cases, *ABCD1* sequencing results are negative in a female heterozygote, but would fail to amplify in a male hemizygote. In both cases, further testing to detect large deletions is required. Moreover, when sequencing *ABCD1* it is important to be aware of pseudogene regions, homologous to exons 7–10, duplicated on chromosomes 2, 10, 16, and 22.[130] Both Sanger and next-generation sequencing platforms necessitate careful validation to be certain that these pseudogene regions are avoided.

For autosomal recessive disorders, parental testing is useful to be able to offer carrier testing to family members in each lineage. If a pathogenic variant is detected in apparent homozygosity, parental targeted testing will confirm that one pathogenic variant was inherited from each parent. If both parents are not carriers, the second variant may be a large deletion; alternatively, the variant could be present in two copies, but of the same parental chromosome (uniparental isodisomy has been reported in association with ZSD[131] and RCDP[132]); apparent homozygosity could also be caused by allele dropout due to technical reasons.

SUPPLEMENTARY INFORMATION

The online version of this article (https://doi.org/10.1038/s41436-019-0713-9) contains supplementary material, which is available to authorized users.

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DISCLOSURE

The following authors direct laboratories that perform laboratory testing for the diagnosis and management of peroxisomal disorders as a fee for service: I.D.B., S.T., S.S., L.K., and K.C.-O. N.B. is a member of the medical and scientific advisory board for the Global Foundation for Peroxisome Disorders.

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