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A metabolomic map of Zellweger spectrum disorders reveals novel disease biomarkers

Michael F. Wangler, MD[1,2,3,4], Leroy Hubert, PhD[1], Taraka R. Donti, PhD[1,11], Meredith J. Ventura, MD[5], Marcus J. Miller, PhD[1], Nancy Braverman, MD[6], Kelly Gawron, MS[7], Mousumi Bose, PhD[7], Ann B. Moser, BA[8], Richard O. Jones, PhD[8], William B. Rizzo, MD[9], V. Reid Sutton, MD[1], Qin Sun, PhD[1], Adam D. Kennedy, PhD[10] and Sarah H. Elsea, PhD[1]

Purpose: Peroxisome biogenesis disorders–Zellweger spectrum disorders (PBD-ZSD) are metabolic diseases with multisystem manifestations. Individuals with PBD-ZSD exhibit impaired peroxisomal biochemical functions and have abnormal levels of peroxisomal metabolites, but the broader metabolic impact of peroxisomal dysfunction and the utility of metabolomic methods is unknown.

Methods: We studied 19 individuals with clinically and molecularly characterized PBD-ZSD. We performed both quantitative peroxisomal biochemical diagnostic studies in parallel with untargeted small molecule metabolomic profiling in plasma samples with detection of > 650 named compounds.

Results: The cohort represented intermediate to mild PBD-ZSD subjects with peroxisomal biochemical alterations on targeted analysis. Untargeted metabolomic profiling of these samples revealed elevations in pipecolic acid and long-chain lysophosphatidylcholines, as well as an unanticipated reduction in multiple sphingomyelin species. These sphingomyelin reductions observed were consistent across the PBD-ZSD samples and were rare in a population of > 1,000 clinical samples. Interestingly, the pattern or "PBD-ZSD metabolome" was more pronounced in younger subjects suggesting studies earlier in life reveal larger biochemical changes.

Conclusion: Untargeted metabolomics is effective in detecting mild to intermediate cases of PBD-ZSD. Surprisingly, dramatic reductions in plasma sphingomyelin are a consistent feature of the PBD-ZSD metabolome. The use of metabolomics in PBD-ZSD can provide insight into novel biomarkers of disease.

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Key Words: metabolomics; peroxisome; PBD-ZSD; peroxisome biogenesis disorder; sphingomyelin

INTRODUCTION

Peroxisome biogenesis disorders–Zellweger spectrum disorders (PBD-ZSD) are a group of autosomal recessive diseases affecting primarily neonates and infants due to loss of function of the peroxisome biogenesis machinery of the cell.[1] PBD-ZSD describes a clinical range of mild, intermediate, and severe forms of disease.[2] Individuals with severe forms (Zellweger or cerebrohepatorenal syndrome) are typically diagnosed at birth with congenital malformations including neuronal migration defects, renal cysts, and chondrodysplasia punctata. They are severely hypotonic, develop neonatal seizures, and have dysmorphic features such as bitemporal narrowing, large anterior fontanelle, and depressed supraorbital ridges. Significant hepatic dysfunction and cholestasis can also occur.[2,3] These individuals have a poor prognosis and typically die in the first year of life. Intermediate and milder forms are characterized by the absence of congenital malformations and a progressive disorder of peroxisome dysfunction. This includes hypotonia, feeding problems, sensorineural hearing loss, vision loss due to retinal dystrophy, leukodystrophy, peripheral neuropathy, adrenal insufficiency, hyperoxaluria, and hepatic dysfunction. Milder forms of PBD-ZSD classically exhibit slower progressive hearing loss and retinopathy, milder cognitive delay or even normal intellect, and longer survival. A relatively frequent hypomorphic allele in *PEX1* (p.G843D) is observed in the majority of these cases.[4,5]

Recently, next-generation sequencing has revealed a wider phenotypic spectrum for many Mendelian disorders.[6] With widespread whole-exome sequencing comes the recognition of additional phenotypes for previously known syndromes, or "phenotypic expansion." PBD-ZSD has recently been recognized to include several phenotypic expansions particularly for mild or atypical cases. A number of cases have been reported with phenotypes that are more consistent with autosomal recessive ataxia but related to PBD-ZSD

[1]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, USA; [2]Texas Children's Hospital, Houston, Texas, USA; [3]Jan and Dan Duncan Neurological Research Institute, Texas Children's Hospital, Houston, Texas, USA; [4]Developmental Biology Program, Baylor College of Medicine, Houston, Texas, USA; [5]School of Medicine, Baylor College of Medicine, Houston, Texas, USA; [6]Research Institute of the McGill University Health Center, Montreal, Quebec, Canada; [7]Department of Nutrition and Food Studies, Montclair State University, Montclair, New Jersey, USA; [8]Division of Neurogenetics, Kennedy Krieger Institute, Johns Hopkins School of Medicine, Baltimore, Maryland, USA; [9]University of Nebraska Medical Center, Omaha, Nebraska, USA; [10]Metabolon, Morrisville, North Carolina, USA. Correspondence: Michael F. Wangler or Sarah H. Elsea (michael.wangler@bcm.edu or elsea@bcm.edu or sarah.elsea@bcm.edu)
[11]Current affiliation: Greenwood Genetic Center, Greenwood, South Carolina, USA

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mutations.[7] In addition, the *PEX1* p.G843D allele, which has been known for decades, has also been uncovered in individuals with milder phenotypes not initially recognized to have PBD-ZSD.[5] Finally, seemingly distinct diagnostic entities such as Heimler syndrome have been reclassified by whole-exome sequencing to represent the mildest end of the PBD-ZSD spectrum.[8] In many of these cases, the classical biochemical defects observed in β-oxidation of very-long-chain fatty acids, plasmalogen synthesis, and peroxisomal enzyme localization in the cell are reportedly either not present or are very mild.[7,8] These findings suggest the possibility of additional undetected peroxisomal disorders.[2] Moreover, newborn screening of X-linked adrenoleukodystrophy has been developed and permits identification of PBD-ZSD at earlier stages.[9,10]

Global metabolomic profiling is a novel method for utilizing untargeted metabolomics on clinical samples.[11–16] This metabolic screening methodology can detect abnormalities in amino acid, fatty acid, lipid, nucleotide, bile acid, and other small molecule metabolism in a single test.[13] The power of the broad detection platform has been utilized in individual samples for diagnostic purposes,[14,15] resulting in the successful diagnosis of a number of inborn errors of metabolism, including adenylosuccinate lyase deficiency[15] and aromatic amino acid decarboxylase deficiency,[17] among others.[12,13,16] Utilizing this methodology as a screening approach to identify individuals on the mild end of the phenotypic spectrum, who might not have otherwise been diagnosed or would have had significant delay in diagnosis, illustrates the power of this technique for earlier diagnosis and the potential contribution to expand our understanding of the range of severity across metabolic disorders.

While these successes confirm the overall utility of global metabolomics, the possible wider phenotypic range of PBD-ZSD supported by recent sequencing studies suggests that an orthogonal method for detecting peroxisomal abnormalities in plasma could be a valuable tool in pediatric diagnostics to identify additional cases and better understand peroxisomal biochemistry in the pediatric population.

Here, we undertook global metabolomic profiling in 19 subjects with mild or intermediate PBD-ZSD. We demonstrate that the population under study exhibits mild or intermediate peroxisomal biochemical defects using peroxisomal targeted diagnostics. On paired plasma samples, we performed untargeted metabolomic profiling and established a "PBD-ZSD metabolome" that includes new biomarkers for PBD-ZSD.

MATERIALS AND METHODS

Clinical recruitment

All subjects were recruited to an institutional review board–approved study, "The Biochemical and Cell Biology of Peroxisomal Disorders Study," at Baylor College of Medicine (H-32837).[5,7,18] All subject caregivers/parents provided informed consent, including consent to publish patient photos. All participants were examined, and samples were collected on a single day in the setting of the Global Foundation for Peroxisomal Disorders Family Conference. Ascertainment was based on the presence of molecularly confirmed mutations in the *PEX* genes and/or biochemical confirmation of a defect in peroxisomal biogenesis. Blood samples were collected by a trained phlebotomist and immediately plasma and red blood cells were isolated by ultracentrifugation. Each plasma sample was divided into aliquots for targeted and untargeted analyses and immediately frozen at − 80 °C. Clinical data were gathered from a clinical information tool administered to the parents (completed by 18 of 19 participants), and clinical details were verified through a detailed exam and evaluation the following day, along with medical record review (for 13 of 18 subjects) or medical record review alone (for 5 of 18 subjects).

Targeted peroxisomal diagnostic studies

Targeted analyses were performed in the Peroxisomal Disorders Laboratory (Kennedy-Krieger Institute, Baltimore, MD). Briefly, pipecolic acid was measured by electron capture negative ion mass fragmentography of the pentafluorobenzyl ester of pipecolic acid.[19] Plasma very-long-chain fatty acid analysis was performed using capillary gas chromatography/mass spectroscopy of pentafluorobenzyl fatty acid esters.[20] Full total lipid fatty acid profiles were also performed on plasma and red blood cells.[21]

Untargeted metabolomic analysis

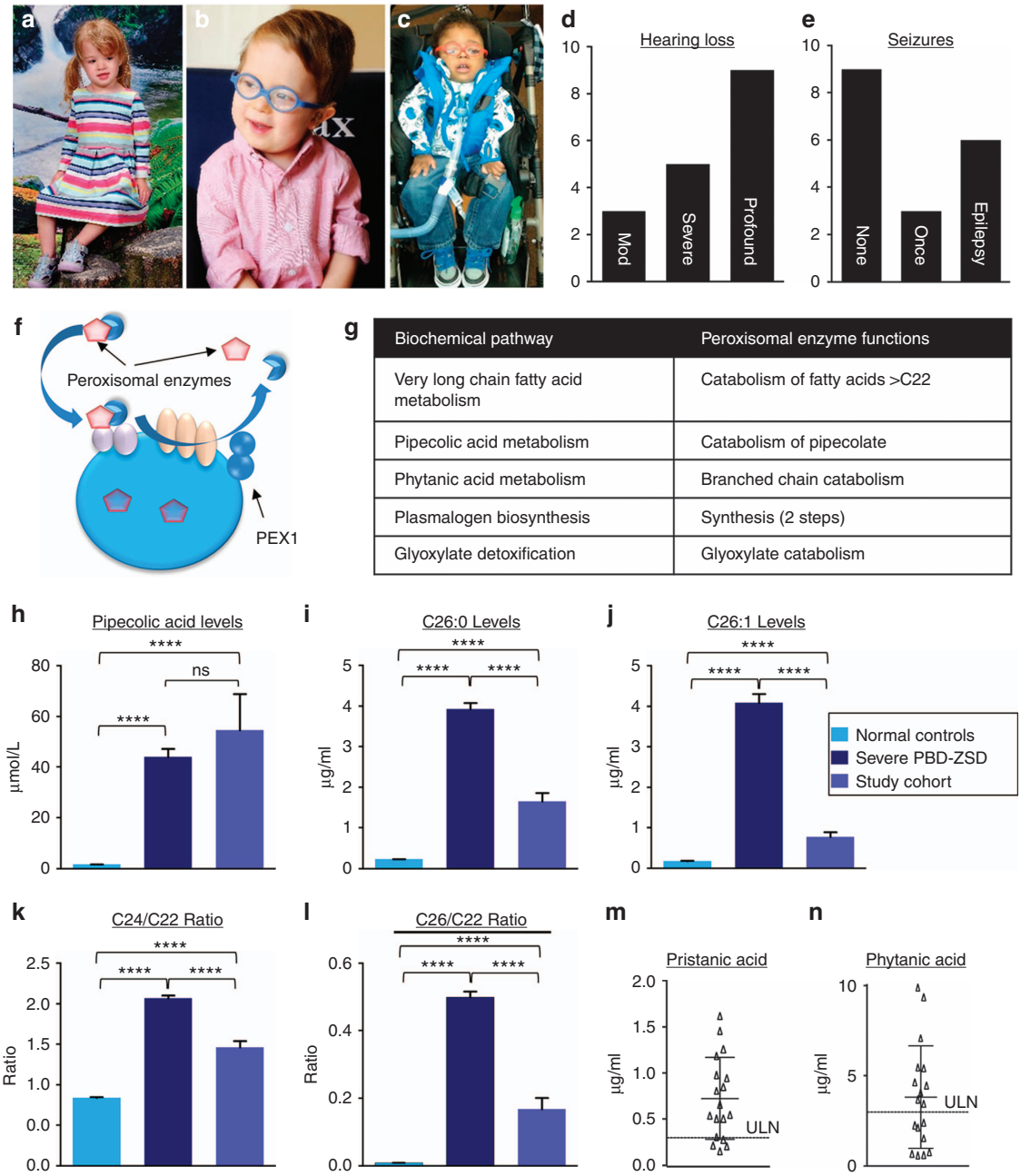
Metabolomics was performed as described previously and is provided in detail (see **Supplementary Materials** online).[22,23] Briefly, small molecules were extracted from the plasma samples in an 80% methanol solution containing recovery standards.[24] The resulting extract was divided into five aliquots (for four separate individual liquid chromatography–tandem mass spectrometry analyses and one spare sample). These five aliquots per plasma sample were briefly evaporated to remove the organic solvent and stored overnight under nitrogen before preparation for analysis. On the day of analysis, the dried sample extract aliquots were reconstituted in solvent containing standards at fixed concentrations. The aliquots were analyzed using a Waters ACQUITY ultraperformance liquid chromatography and a Thermo Scientific (Waltham, MA) Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution.[23] The four separate aliquots were resolved by four methods.[23] Metabolites were identified by matching the ion chromatographic retention index, accurate mass, and mass spectral fragmentation signatures with a reference library consisting of over 4,000 entries from standard metabolites.[22] Metabolite *z*-scores were generated using the method described.[13,15] Semiquantitative analysis was achieved by comparing subject samples with a set of invariant anchor specimens included in each batch. Raw spectral intensity values were normalized to the anchor samples, log transformed, and compared with a normal reference population to generate *z*-score values.

Metabolite *z*-scores were also analyzed for correlation to the targeted quantitative values (see **Supplementary Materials**). Pathway analysis was performed using MetaboAnalyst (http://www.metaboanalyst.ca/).[25,26] The clinical parameters and analyte *z*-scores were compared using Pearson *r* correlations. Principal component analysis was performed using all the metabolomic data in the data set for this cohort and a set of controls from a clinical diagnostic laboratory.

RESULTS

Clinical characteristics of PBD-ZSD cohort

Nineteen participants representing a group of mild or intermediate PBD-ZSD were recruited (**Figure 1a–c**). Full clinical data were available for 18 of the 19 participants (**Table 1**,**Supplementary Table S1**). The participants were ascertained through the Global Foundation for Peroxisomal Disorders, and all participants had clinically and biochemically confirmed diagnoses. All but one subject (24-1) had molecular confirmation of the specific *PEX* mutations (**Table 1**). More than half the participants were male (10/19), and the average age was 10 years (range 2–34 years). Nearly all participants exhibited hypotonia as a neonate and hearing loss (**Figure 1d**,**Supplementary Table S2**). Interestingly, half the subjects exhibited some degree of ataxia, and half had experienced a seizure, although only 6 subjects had epilepsy (**Supplementary Table S2**,**Figure 1e**). Medications at the time of sample collection and a subjective assessment of severity were also gathered (**Supplementary Table S2**).



Biochemical pathway	Peroxisomal enzyme functions
Very long chain fatty acid metabolism	Catabolism of fatty acids >C22
Pipecolic acid metabolism	Catabolism of pipecolate
Phytanic acid metabolism	Branched chain catabolism
Plasmalogen biosynthesis	Synthesis (2 steps)
Glyoxylate detoxification	Glyoxylate catabolism

Biochemical and molecular studies in the PBD-ZSD cohort

Peroxisome biogenesis disorders affect the assembly of peroxisomes leading to a mislocalization of peroxisomal enzymes in severe cases and generalized peroxisomal enzyme dysfunction (**Figure 1f,g**,**Supplementary Figure S1**). Consistent with the clinical evaluations, the subjects exhibited clear biochemical evidence of peroxisomal dysfunction in targeted PBD-ZSD diagnostic assays; however, they exhibited less dramatic biochemical defects than individuals with more severe forms of PBD-ZSD (**Figure 1h–n**,**Table 1**,**Supplementary Table S3**).

We also obtained or determined the genotype for 18 of the 19 subjects in the study (**Table 1**). Seventeen of the 18 individuals had biallelic variants in *PEX1*, the most common locus for PBD-ZSD, while one subject had variants in *PEX6*, another common locus.[27] Four of the 18 subjects were homozygous for the most common European missense alleles in *PEX1* (NM_000466.2: c.2528G > A: p.Gly843Asp), a genotype associated with particularly mild manifestations in some subjects,[5,28,29] and another 5 subjects were compound heterozygotes for this allele along with a common European frameshift allele (NM_000466.2: c.2097dupT: p.Ile700TyrfsTer42). An additional five probands were compound heterozygous for the p.Gly843Asp allele and another *PEX1* variant. Overall, this cohort's genotypes were, therefore, consistent with a group of mild to intermediate cases of PBD-ZSD, with a predominance of the p.Gly843Asp allele.[5,28,29]

A comprehensive metabolomic profile for PBD-ZSD: the "PBD-ZSD metabolome"

Next, we performed untargeted metabolomic profiling of the cohort. We generated a comprehensive data set of > 650 named compounds detected in the plasma of these subjects. *Z*-score values for each compound in each sample were generated versus a population database[13] and compounds in which the cohort as a group had average *z*-scores of > 2 or < −2 were identified (**Supplementary Table S4**). We then identified a pattern of abnormalities associated with PBD-ZSD. Principal component analysis revealed a separation between the PBD-ZSD samples and a population of controls (**Figure 2a**). The classes of analytes with consistent alterations in the subjects included elevated levels of long-chain dicarboxylic acids, and reductions in phosphatidylcholines and phosphatidylethanolamines as well as plasmalogens (**Figure 2b**). Pipecolic acid, a standard biomarker for PBD-ZSD diagnosis, which was also quantitatively measured (see **Figure 1h**), had an average *z*-score of 3.67 (range −0.47–6.3; **Figure 3a**). The most dramatically elevated metabolite in the cohort was 1-lignoceroyl-GPC (24:0) with an average *z*-score of 3.69 (range −0.41–6.8; **Figure 3a**). Plasmalogens are typically assessed in erythrocytes as plasmalogen phosphatidylethanolamines in the diagnostic workup of PBD-ZSD,[30,31] and several plasmalogen derivatives, plasmalogen phosphatidylcholines, were consistently observed with average *z*-scores < −2 in plasma (**Figure 3a**).

Other alterations were also anticipated from peroxisomal biochemistry, although to our knowledge these had not been previously characterized in a mild PBD-ZSD population (**Figure 3a**). For example, 7α-hydroxy-3-oxo-4-cholestenoic acid, a biochemical precursor of bile acids that accumulates in the plasma of individuals with bile acid synthesis defects, had an average *z*-score of 2.11 in our cohort. In addition, several dicarboxylic acids were elevated including hexadecanedioate (average *z*-score 3.13), octadecanediaote (2.77), eicosanodiaote (2.4), and docosadioate (3.66) (**Figure 3a**). Taken together, the metabolomic alterations provide a global profile or a "PBD-ZSD metabolome," which

Figure 1 Clinical characteristics of study subjects with mild or moderate peroxisome biogenesis disorders–Zellweger spectrum disorders (PBD-ZSD). (**a**) A 3 $\frac{1}{2}$-year-old female. (**b**) A 3-year-old male subject. (**c**) A 2 $\frac{1}{2}$-year-old male subject. (**d**) Spectrum of hearing loss in the subjects; number of subjects is shown on the *y*-axis. (**e**) Presence of seizures in the subjects; number of subjects is shown on the *y*-axis. Three subjects had experienced one seizure only at the time of the study. (**f**) Schematic model of the process of peroxisomal biogenesis showing the role for PEX1 protein; 18 of 19 subjects had mutations in *PEX1*. (**g**) Summary of biochemical pathways implicated by peroxisomal functions (see also **Supplementary Figure S1 online**). (**h**) Pipecolic acid levels measured by electron capture negative ion mass fragmentography of the pentafluorobenzyl ester of pipecolic acid, shown in μmol/L. Normal controls shown in light blue, a group of subjects with severe PBD-ZSD (not included in our study and more severe subjects[20]) shown in dark blue, and our study cohort of mild or moderate PBD-ZSD shown in royal blue. ****$P < 0.0001$, ns indicates not significant. Pipecolic acid was elevated, although variable in the cohort (mean ± SD 54.6 ± 60.7 μmol/L in our cohort versus 1.7 ± 1.1 μmol/L in clinical controls over 5 years). (**i**) C26:0 levels measured by capillary gas chromatography/mass spectroscopy of pentafluorobenzyl bromide fatty acid esters; μg/ml quantity is shown on *y*-axis. Normal controls shown in light blue, a group of subjects with severe PBD-ZSD (not included in our study and more severe subjects[20]) shown in dark blue, and our study cohort of mild–intermediate PBD-ZSD shown in royal blue. ****$P < 0.0001$. C26:0 levels are elevated in the cohort (1.65 ± 0.88 μg/ml versus 0.23 ± 0.09 μg/ml in controls). (**j**) C26:1 levels measured by capillary gas chromatography/mass spectroscopy of pentafluorobenzyl bromide fatty acid esters, μg/ml quantity is shown on *y*-axis. Normal controls shown in light blue, a group of individuals with severe PBD-ZSD (not included in our study and more severe subjects[20]) shown in dark blue, and our study cohort of mild or moderate PBD-ZSD shown in royal blue. ****$P < 0.0001$. C26:1 levels are elevated in the cohort (0.78 ± 0.46 μg/ml versus 0.18 ± 0.09 in controls). (**k**) C24/C22 fatty acid ratios. Normal controls shown in light blue, a group of individuals with severe PBD-ZSD (not included in our study and more severe subjects) shown in dark blue, and our study cohort of mild or moderate PBD-ZSD shown in royal blue. ****$P < 0.0001$. C24/C22 (1.46 ± 0.32 versus 0.84 ± 0.10, (**l**)) fatty acids were also elevated, although not to the degree of severe PBD-ZSD (C26/C22: 0.50 ± 0.16). (**l**) C26/C22 fatty acid ratios. Normal controls shown in light blue, a group of individuals with severe PBD-ZSD (not included in our study and more severe subjects) shown in dark blue, and our study cohort of mild or moderate PBD-ZSD shown in royal blue. ****$P < 0.0001$. (**m**) Pristanic acid levels in μg/ml in the subjects exhibited elevated pristanic acid levels (0.73 ± 0.44 μg/ml). The dashed line (ULN) marks the upper limit of normal. (**n**) Phytanic acid levels in μg/ml in the subjects who exhibited elevated phytanic acid levels (3.8 ± 2.8). The dashed line (ULN) marks the upper limit of normal.

Table 1 Clinical cohort of subjects with mild peroxisome biogenesis disorder–Zellweger spectrum disorder (PBD-ZSD)

Identifier	Age	Gender	Severity (parent-reported)	Pipecolic acid in plasma (μmol/L)	C26:0 – Hexacosanoic in plasma (μg/ml)	Genotype
PEX_FAM10-1	4	M	Mild to intermediate	225	3.44	*PEX1*-del exon24 c.2097_2098insT
PEX_FAM11-1	6	M	Mild to intermediate	36.2	1.34	*PEX1*-c.2528G > A (p.G843D)/p.Q128X
PEX_FAM12-1	4	F	Mild to intermediate	ND	ND	*PEX1*-c.2528G > A (p.G843D)/ c.1961insCAGTGTGAA
PEX_FAM13-1	3	M	Intermediate to severe	111.5	1.68	*PEX6* c.2663G > A (p.R888H), c.1220C > A (p. T407N)
PEX_FAM14-1	16	F	Mild	7	0.69	*PEX1*-c.2528G > A (p.G843D) homozygous
PEX_FAM14-4	16	M	Mild	4.5	0.62	*PEX1*-c.2528G > A (p.G843D) homozygous
PEX_FAM15-1	18	F	Mild to intermediate	9.7	1.01	*PEX1*-c.2528G > A (p.G843D) homozygous
PEX_FAM16-1	8	F	Mild to intermediate	12.6	1.44	*PEX1*-c.2528G > A (p.G843D), c.2097_2098insT
PEX_FAM17-1	34	F	Intermediate to severe	8.3	2.27	*PEX1*c.2528G > A (p.Gly843Asp), c.2097_2098insT
PEX_FAM18-1	6	M	Mild to intermediate	23.6	1.44	*PEX1*-c.1777G > A (p.G593R)/c.2916delA (p.G973fs)
PEX_FAM19-1	7	F	Mild	62.9	2.71	*PEX1*-c.2528G > A (p.G843D), c.2097_2098insT
PEX_FAM20-1	5	F	Mild to intermediate	135.7	2.96	*PEX1*-del exon 24/c.2097dupT
PEX_FAM21-1	16	M	ND	52.2	0.75	*PEX1*-c.2528G > A (p.G843), c.2097_2098insT
PEX_FAM22-1	8	M	Mild to intermediate	20.2	1.41	*PEX1*-c.2528G > A (p.G843D)/c.484C > A (p.P162T)
PEX_FAM23-1	3	F	Mild to intermediate	72.7	1.58	*PEX1*-c.2528G > A (p.G843D)/c.2383C > T (p. R795X)
PEX_FAM24-1	19	M	Intermediate to severe	8.4	1.17	ND
PEX_FAM25-1	ND	M	ND	6.98	0.35	*PEX1*-c.2528G > A (p.G843D) homozygous
PEX_FAM26-1	7	F	Mild to intermediate	55.5	2.11	*PEX1*-c.2528G > A (p.G843D)/, c.2097_2098insT
PEX_FAM27-1	2	M	Mild to intermediate	130.3	2.76	*PEX1* c.2528G > A (p.G843D)/p.Ile370Leufs*17

ND, not done.

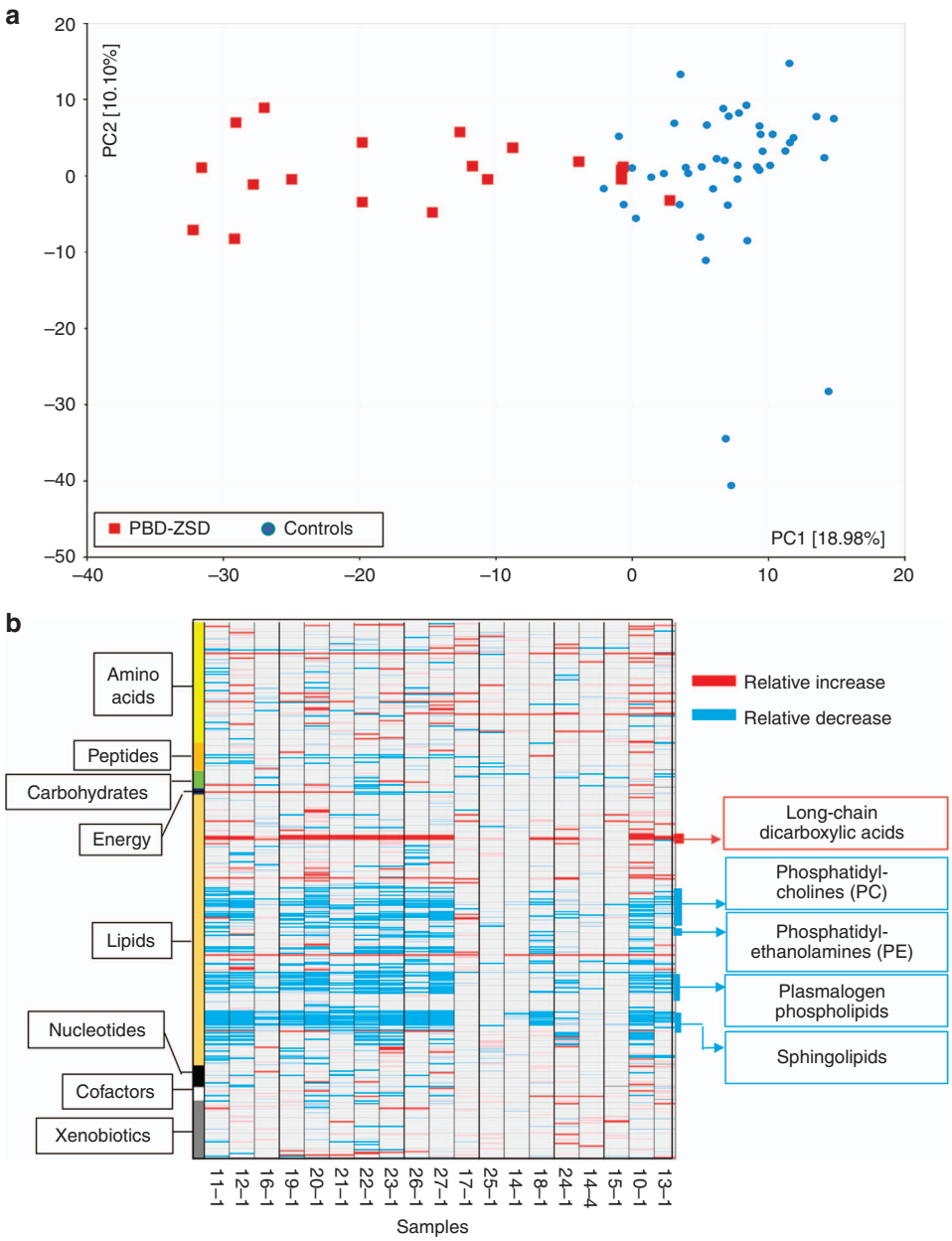


Figure 2 Metabolomic profile of our study cohort: the peroxisome biogenesis disorders–Zellweger spectrum disorders (PBD-ZSD) metabolome. (**a**) Principal components analysis is shown; red squares represent the PBD-ZSD samples, blue circles represent a collection of control samples. The principal components, PC1 and PC2, were generated using all metabolomic data. Percent signs indicate the percent of variation explained by that principal component. (**b**) Heat map of the PBD-ZSD samples; red shows relative increases, while blue shows reductions. Pathways of the analytes are shown on the left axis (individual analytes in **Supplementary Table S4 online**). Specific compounds noted to be altered are marked at right. Subjects are labeled according to family designation (see **Table 1**).

links the known biochemical defects to additional actionable molecular links.

While these findings were anticipated from knowledge of peroxisomal biochemistry, novel biomarkers were also identified. Nine sphingomyelin species were dramatically reduced in the PBD-ZSD metabolome (**Figure 3b**, **Supplementary Table S4**). Subsequent to identification of this pattern of metabolite changes in the PBD-ZSD metabolome including the changes in sphingomyelins, we were able to utilize the pattern we observed to aid in the diagnosis of other cases. For example, of 1,011 clinical samples submitted for metabolomics, excluding the PBD-ZSD samples from this study, we observed only 21 of 1,011 having z-score < -2 for multiple sphingomyelins suggesting this is a rare finding. One of these cases was an undiagnosed infant who presented to our hospital and exhibited the reductions in sphingomyelins in plasma and other features of the PBD-ZSD metabolome. This infant was subsequently found to have elevated plasma

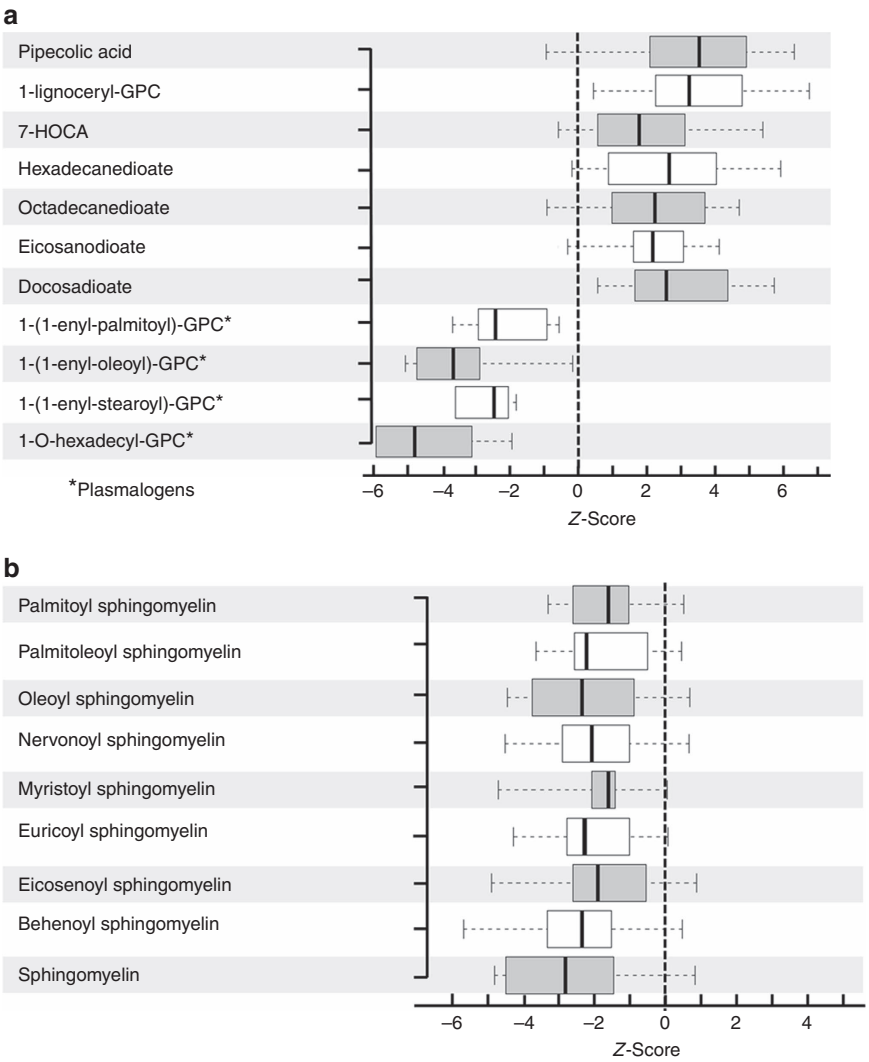


Figure 3 Dramatically altered analytes in the peroxisome biogenesis disorders–Zellweger spectrum disorders (PBD-ZSD) metabolome. (**a**) Box plots of analytes that are related to biochemical pathways known to be peroxisomal (see **Supplementary Figure S1 online**); error bars correspond to 95% confidence interval. (**b**) Box plots of alterations in sphingomyelins including palmitoyl sphingomyelin (palmitoyl sphingomyelin (d18:1/16:0)), palmitoleoyl sphingomyelin (sphingomyelin (d18:2/16:0, d18:1/16:1)*), oleoyl sphingomyelin (sphingomyelin (d18:1/18:1, d18:2/18:0)), nervonoyl sphingomyelin (sphingomyelin (d18:1/24:1, d18:2/24:0)*), myristoyl sphingomyelin (sphingomyelin (d18:1/14:0, d16:1/16:0)*), euricoyl sphingomyelin (sphingomyelin (d18:1/22:1, d18:2/22:0, d16:1/24:1)*), eicosenoyl sphingomyelin (sphingomyelin (d18:1/20:1, d18:2/20:0)*), and behenoyl sphingomyelin (behenoyl sphingomyelin (d18:1/22:0)*).

very-long-chain fatty acids and was diagnosed with D-bifunctional protein deficiency with variants in the *HSD17B4* gene, a disorder of peroxisomal β-oxidation, providing independent evidence for a role of peroxisomes in sphingomyelin metabolism, and validating the PBD-ZSD metabolome as a diagnostically useful pattern of metabolite changes.

The PBD-ZSD metabolome attenuates with age

To further characterize the clinical utility of the PBD-ZSD metabolome and the biomarkers uncovered, we examined the cohort's clinical parameters. We were interested in identification of clinical severity, genotype correlations, or manifestation biomarkers within the cohort (**Table 1**,**Supplementary Table S5**). As noted, the genotypes were fairly uniform in the study sample (with a predominance of *PEX1*, particularly missense alleles), and this limited any genotype-specific analysis. However, we noted that our cohort, representing cases of mild or intermediate PBD-ZSD, had long-term survivorship and so likely represents a diverse group of PBD-ZSD with respect to age (range 1 to 34 years).

We observed a strong correlation between age and all the biomarkers of the PBD-ZSD metabolome such that there was an attenuation of the abnormalities in older subjects (**Supplementary Figure S2**). The pipecolate levels, which were consistently elevated, for example, exhibited a significant negative correlation with age (Pearson r −0.6707, $p = 0.0017$). The sphingomyelin biomarkers uncovered in the study all exhibited a positive correlation with age

(sphingomyelin Pearson r 0.7391, $p = 0.0003$), demonstrating that in the older subjects, the sphingomyelin markers were within normal ranges (**Supplementary Figure S2B,D,F**). These age-related differences were also present in the targeted analysis of these samples; for example, in subjects under 10 years, pipecolate levels were 80.5 ± 64.7 µg/ml, versus 13.9 ± 17.0 µg/ml in the subjects over 10 years ($p = 0.018$). Likewise, in subjects under 10 years, levels of C26:0 hexacosanoic acid were 2.07 ± 0.756 µg/ml, versus 0.98 ± 0.63 µg/ml in the subjects over 10 years ($p = 0.006$). Taken together, the results suggest the sphingomyelin changes match the overall biochemical pattern observed in adults with PBD-ZSD who exhibit less dramatic biochemical changes.[4,32]

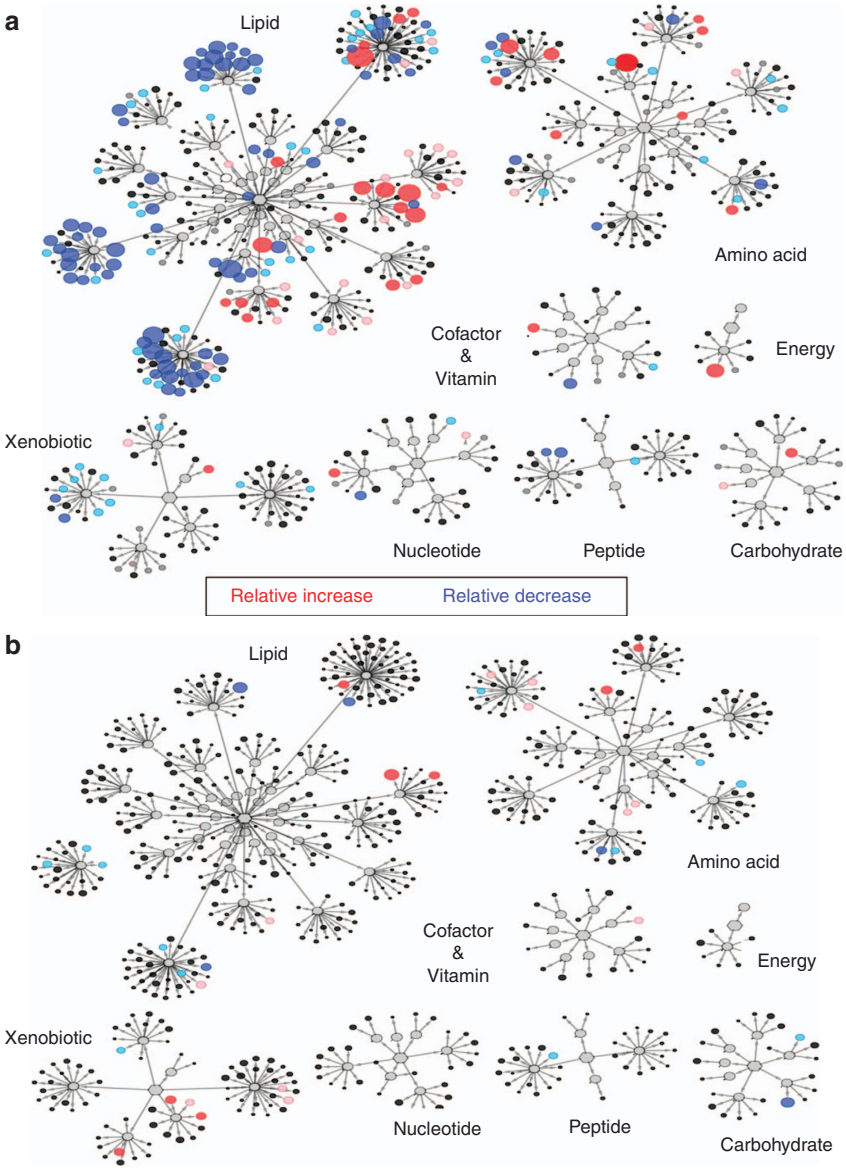


Figure 4 Comprehensive metabolomic findings are impacted by age in peroxisome biogenesis disorders–Zellweger spectrum disorders (PBD-ZSD) study cohort. Figures generated using Cytoscape to delineate biochemical pathways (http://cytoscape.org).[40] (**a**) Biochemical pathway showing metabolic perturbations in plasma from subjects diagnosed with PBD-ZSD and under 10 years of age at the time of sample collection. Red circles indicate biochemicals with positive z-scores (>2) and blue circles indicate biochemicals with negative z-scores (< -2). The diameters of the circles indicate the magnitude of the z-score. Pink circles represent biochemicals with z-score of $1.5 \leq z < 2.0$ and light blue circles represent biochemicals with $-2.0 < z \leq -1.5$. Black circles represent other biochemicals in the pathway detected in the samples but had z-scores of $-1.5 < z < 1.5$. (**b**) Biochemical pathway showing metabolic perturbations in plasma from subjects diagnosed with PBD-ZSD and over 10 years of age at the time of sample collection. Red circles indicate biochemicals with positive z-scores (>2) and blue circles indicate biochemicals with negative z-scores (< -2). The diameters of the circles indicate the magnitude of the z-score. Pink circles represent biochemicals with z-score of $1.5 \leq z < 2.0$ and light blue circles represent biochemicals with $-2.0 < z \leq -1.5$. Black circles represent other biochemicals in the pathway detected in the samples but had z-scores of $-1.5 < z < 1.5$.

Finally, visualization of the PBD-ZSD metabolomic network revealed clusters of analytes grouped by biological and metabolic pathways that were dramatic in the subjects under 10 years (**Figure 4a**), whereas the metabolic signature was more muted in those individuals over 10 years of age (**Figure 4b**).

DISCUSSION

PBD-ZSDs are rare pediatric disorders with a range of clinical severity,[3,9] and while the severe PBD-ZSD phenotype was described decades ago by Hans Zellweger, the full range of disease is still being discovered and described.[5,7] Metabolomics is a diagnostic tool that allows simultaneous detection of hundreds of metabolites, and as such, increases the richness of the information available for diagnosis. However, given the sheer amount of data, the utility of metabolomics requires the establishment of a characteristic pattern or metabolome for a disorder.[11,12] Our population of PBD-ZSD represented a clinically and biochemically mild or intermediate cohort compared with the classic Zellweger syndrome phenotype. Moreover, the subjects had molecular findings predominated by the common *PEX1* p.Gly843Asp allele, well documented to occur in milder cases. As such, this cohort might have been expected not to exhibit a characteristic profile. Therefore, it is very encouraging that we observed consistent abnormalities within this group, particularly because the milder or atypical cases are more likely to have not undergone targeted diagnostic approaches.

We also examined the metabolomics data in subgroups related to the clinical features (**Table 1**,**Supplementary Table S5**). With the exception of age, we did not identify any significant correlations, but further studies with larger sample sizes may be needed to uncover such associations. In addition, future studies looking at subjects with PBD-ZSD with a set of diverse genotypes with respect to different alleles in *PEX* genes are also needed to clarify the determinants of the biochemical changes. Our study does establish age as a clear determinant of the PBD-ZSD metabolome. While age has been recognized as having an impact on PBD-ZSD diagnostic studies,[20] we were able to catalog an entire network of metabolic changes in younger subjects that was less prominent in older PBD-ZSD subjects. There are several possible explanations for this observation. First, there could be a bias for milder individuals amongst the older subjects. However, this is not consistent with longitudinal studies that have shown adults with PBD-ZSD have less dramatic results of targeted biochemical analyses than at the time of diagnosis.[32] Our study, along with these longitudinal observations, point to a normalization of plasma biochemistry with age. Alternatively, individuals with PBD-ZSD may have changes in excretion or tissue storage of the relevant lipid molecules. Another possibility is that peroxisomes are more metabolically active at younger ages when processes such as neuronal migration, myelination, and metabolism are more active. However, the biochemical normalization observed may not impact disease severity or progression.[4,32] In any case, our analysis suggests that the diagnostic sensitivity of metabolomics in PBD-ZSD may differ with age.

Our study provides a very complete PBD-ZSD metabolome that connects to other known and more common disorders. For example, we observed elevations of long-chain dicarboxylic acids as a prominent feature. In addition, hexadecanedioate, which was also found to be elevated in our cohort, has been implicated in human blood pressure regulation.[33] This observation may relate to the renal hypertension that has been noted in older individuals with PBD-ZSD.[34] Of these novel biochemical observations, the discovery of multiple sphingomyelin species having dramatic reduction in the PBD-ZSD samples is the most significant finding of this study.

Plasma sphingomyelin has not been previously studied in PBD-ZSD. We hypothesize that this represents a novel biomarker for PBD-ZSD (**Supplementary Figure S3**). Sphingomyelin reductions are extremely uncommon in clinical samples submitted to us for metabolomics, suggesting peroxisome dysfunction is the major cause for this pattern. There are several possible explanations for the reductions in sphingomyelins in plasma. First, a defect in lipid transport could lead to a plasma reduction, an idea consistent with the lack of lipoprotein[a] in PBD-ZSD.[35] Another explanation could relate to an inability to metabolize longer chain–length sphingolipids causing a secondary defect in sphingolipid synthesis, an idea consistent with the role of sphingolipids in lipid homeostatic regulation and with the data showing increased carbon-chain phospholipid ratios in PBD-ZSD fibroblasts and mouse models,[36–38] as well as plasma acylcarnitines.[39] Very little is known about the causes or consequences of low sphingomyelin levels in plasma. Larger longitudinal studies will determine if sphingomyelin can be used as a prognostic marker in PBD-ZSD and if these markers resolve or change with treatment/therapy of disease.

In conclusion, untargeted metabolomic studies are a useful tool in detecting abnormalities of peroxisomal function in a pediatric cohort. Individuals with PBD-ZSD have reduced levels of plasma sphingomyelin, a new biomarker that will require further investigation and can be used for the identification of additional peroxisomal phenotypes in the future.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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DISCLOSURE

M.F.W., L.H., M.J.M., V.R.S., Q.S., and S.H.E. are employees of Baylor College of Medicine, which has a partnership with Baylor Genetics and derives revenue from genetic testing. A.D.K. is an employee of Metabolon and, as such, has affiliations with or financial involvement with Metabolon. T.R.D. is an employee of

Greenwood Genetics. The other authors declare no conflict of interest.

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