HHS Public Access

Author manuscript

Genet Med. Author manuscript; available in PMC 2020 November 22.

Published in final edited form as:

Genet Med. 2020 September ; 22(9): 1560–1566. doi:10.1038/s41436-020-0827-0.

Integrated Analysis of Metabolomic Profiling and Exome Data Supplements Sequence Variant Interpretation, Classification, and Diagnosis

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Abstract

Purpose: A primary barrier to improving exome sequencing diagnostic rates is the interpretation of variants of uncertain clinical significance. We aimed to determine the contribution of integrated untargeted metabolomics in the analysis of exome sequencing data by retrospective analysis of patients evaluated by both whole exome sequencing and untargeted metabolomics within the same clinical laboratory.

Methods: Exome sequencing and untargeted metabolomic data were collected and analyzed for 170 patients. Pathogenic variants, likely pathogenic variants, and variants of uncertain significance in genes associated with a biochemical phenotype were extracted. Metabolomic data were evaluated to determine if these variants resulted in biochemical abnormalities which could be used to support their interpretation using current ACMG guidelines.

Results: Metabolomic data contributed to the interpretation variants in 74 individuals (43.5%) over 73 different genes. The data allowed for the re-classification of 9 variants as likely benign, 15 variants as likely pathogenic, and 3 variants as pathogenic. Metabolomic data confirmed a clinical diagnosis in 21 cases, for a diagnostic rate of 12.3% in this population.

Conclusion: Untargeted metabolomics can serve as a useful adjunct to exome sequencing by providing valuable functional data that may not otherwise be clinically available, resulting in improved variant classification.

Keywords

metabolomics; whole exome sequencing; genome; variant interpretation; functional analysis

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DISCLOSURES

Dr. Alaimo and Dr. Xiao declare no other potential conflicts of interest.

INTRODUCTION

The utilization of exome sequencing has substantially increased our capacity to identify disease-causing variants across a variety of clinical indications; however, exome sequencing has a variable diagnostic rate, and many patients remain without a clear diagnosis for a variety of reasons.[1–5] Methods of supporting a definitive diagnosis remain a cardinal challenge for clinical exome sequencing, and a significant limitation is observed at the level of variant interpretation and classification. Though the American College of Medical Genetics and Genomics (ACMG), the Association for Molecular Pathology (AMP) and Clinical Genome Resource (ClinGen) have attempted to streamline and standardize this process through the publication and dissemination of official guidelines and various recommendations,[6,7] there is ample evidence that inter-laboratory variation continues to exist.[8] Many of these discrepancies may be due to the lack of compelling and validated "functional" evidence as outlined in the ACMG framework. While in some cases such evidence can be easily obtained through complementary targeted biochemical testing or model organism studies, this is not always feasible or easily accomplished in a time-sensitive manner.

One approach to address this limitation would be to integrate untargeted metabolomic profiling with genomic analysis. Metabolomic profiling refers to the systematic identification and quantification of all substrates, intermediates, and metabolites in a given organism or biological sample at a point in time. In this way, a single test is able to assay multiple biochemical pathways at once and may lead to clear evidence of perturbations due solely to abnormalities in the variant in question. We have previously described the use of untargeted metabolomics in the screening and diagnosis of a variety of inborn errors of metabolism (IEMs)[9] and the identification of novel disease biomarkers.[10,11] We have also demonstrated that untargeted metabolomics may be useful in the diagnosis of individuals at either end of a phenotypic spectrum, as in the case of several individuals with adenylosuccinate lyase deficiency[12] and in patients with GABA-transaminase deficiency[13]. Here, metabolomic data were not only able to suggest a clinical diagnosis but also informed the choice of targeted molecular testing for additional confirmation. Untargeted metabolomics may, therefore, serve as a convenient and informative piece of strong functional data, and we have thus sought to use this combined approach in the analysis of a cohort of patient samples at our clinical laboratory.

MATERIALS AND METHODS

Sample and data acquisition

Clinical whole exome sequencing and global untargeted clinical metabolomic testing were ordered through a physician's office or institution through standard procedures. Samples were sent to the Baylor Genetics Laboratory (Houston TX - www.baylorgenetics.org) for processing and analysis. We surveyed our internal databases to identify cases where both metabolomic profiling and whole exome sequencing were requested for the same patient between May 2012 and August 2017. These studies were approved with a waiver of consent by the Baylor College of Medicine Institutional Review Board.

Untargeted metabolomic profiling

Metabolomic profiling (Global MAPS®) was performed by Baylor Genetics (Houston, TX) (www.baylorgenetics.com), in collaboration with Metabolon, Inc. (Durham, NC) (www.metabolon.com), as described previously using two different platform configurations in plasma, urine and/or CSF.[9,14] On the first platform configuration, samples were subjected to four chromatographic analyses: GC-MS, LC-MS/MS in positive mode (LCMS Pos), LC-MS/MS in negative mode (LCMS Neg), and LC-MS/MS Polar method (LCMS Pol). On the second platform, samples were subjected to the same mass spectrometry methods with the following chromatographic methods: LCMS Neg, LCMS Pol, and LCMS positive ion method focusing on lipophilic compounds (LCMS Pos Lipid) and LCMS positive ion method focusing on polar compounds (LCMS Pos Polar). The chemical structures of known metabolites were identified by matching the ions' chromatographic retention index, nominal mass, and mass spectral fragmentation signatures with reference library entries created from authentic standard metabolites under the identical analytical procedure as the experimental samples. Raw spectral intensity values were normalized to the anchor samples, log transformed, and compared to a normal reference population to generate z-scores. Results were considered abnormally low if the z-score for a compound was equal or less than two standard deviations below the mean (i.e. ≤2.00) or abnormally high if the z-score was equal or greater than two standard deviations above the mean (i.e. ≥2.00) of the control reference population.

Whole exome sequencing

Whole exome sequencing data were acquired using previously described protocols[1] developed by the Human Genome Sequencing Center at Baylor College of Medicine and adapted for clinical testing. Classification criteria for likely pathogenic and pathogenic variants were based on the current ACMG-AMP guidelines, and a case was classified as molecularly diagnosed if pathogenic or likely pathogenic variants were detected in Mendelian disease genes that overlapped with described phenotypes of the patient in the appropriate inheritance pattern (i.e. biallelic for recessive disorders, etc.).[3] Variant data were submitted to ClinVar, submission SUB7249674.

Integrated exome and metabolomics analyses

Integrated analysis of exome and metabolomic data was performed for each patient by extracting all pathogenic variants, likely pathogenic variants and variants of uncertain significance in genes associated with known inborn errors of metabolism (Supplementary Tables S1 - S5).[15] Untargeted metabolomic data were treated as functional evidence based on the ACMG and AMP guidelines for the interpretation of sequence variants (PS3/BS3). Metabolomic data were determined to confirm or upgrade a variant in the homozygous state or if the variant was *in trans* with another VUS, pathogenic or likely pathogenic variant for an autosomal recessive disorder if there were characteristic analyte or metabolic pathway abnormalities. Metabolomic data supported the downgrade of a variant in the homozygous or hemizygous state if there were no abnormalities consistent with the IEM associated with that variant. The contribution of a variant was designated as ruled out in cases where there was no clear evidence of metabolic disturbances for a single VUS in the heterozygous state

or in the case of compound heterozygous variants, and in these cases, no change in variant classification was made. Finally, metabolomic data were considered non-contributory or uninformative to variant interpretation if results were non-specific, contradictory to a clear molecular or clinical diagnosis, if a subject had no variants in a known IEM, or if the assay was unable to detect the biochemical signature of the IEM in question.

RESULTS

Cohort characteristics and indications

Our cohort consisted of 170 patients referred to our diagnostic laboratory for exome sequencing and contemporaneous untargeted metabolomics between May 2012 and August 2017 (Supplementary Table S1). Patients were, on average, 7.61 years of age at the time of testing, with approximately equal sex-distribution (56% male: 44% female). The majority of patients were referred for a primarily neurologic indication (91%). Of the 170 cases, 65 underwent trio whole exome sequencing (proband with both biological parents) while the remaining 105 underwent proband exome sequencing (with or without a parental control).

Integrated analyses

Out of 170 patients, 74 (74/170 = 43.5%) were found to have at least one variant in an IEM gene amenable to assessment by our metabolomics platform (Figure 1). These 74 individuals carried 145 variants in 73 different IEM genes, and metabolomic data contributed to the interpretation of 131 of these variants (131/145 = 90.3%) (Supplementary Table S2).

For 87 (87/145 = 60.0%) single, heterozygous variants in autosomal recessive conditions, metabolomic data were able to rule out any contribution in terms of being a biochemically symptomatic carrier or possessing a second disease causing allele not detected due to the constraints of WES.

Metabolomic data were able to contribute functional evidence to the interpretation of 42 (42/145 = 29%) identified variants, resulting in changes to their classification. Characteristic metabolomic profiles upgraded 18 variants (3 from LP to P and 15 from VUS to LP), while also downgrading 9 variants from VUS to LB. In addition, profiles also revealed confirmatory perturbations for 17 (17/145 = 11.7%) pathogenic variants and therefore resulted in no classification changes. These assertions were based on identifying biochemical signatures in (a) autosomal recessive disorders with corresponding homozygous or compound heterozygous variants, (b) autosomal dominant disorders with corresponding heterozygous variants, and (c) X-linked disorders with corresponding hemizygous or heterozygous variants.

The variant reclassification due to metabolomic data resulted in confirmed diagnoses in 21 individuals based on clear biochemical evidence of an IEM and the identification of homozygous and/or compound heterozygous variants in autosomal recessive conditions, hemizygous variants in an X-linked condition or single, heterozygous variants in autosomal dominant conditions. This approach resulted in an overall diagnostic rate of 12.3% (21/170 = 12.3% [Table 1]).[10,13,16–18]

DISCUSSION

With the increasing clinical use of exome sequencing and the implementation of clinical whole genome sequencing, there continues to be a need for tools capable of aiding in the interpretation of variants of uncertain significance and complex genetic backgrounds. While improvements in bioinformatics[19] and functional assays[20] have helped to alleviate some of this burden, there can still be significant limitations. Often these analyses result in imprecise and sometimes conflicting conclusions or require significant time in order to design and conduct an appropriate assay that may not have established clinical validity (i.e. research only testing). Of the 73 unique genes assayed by our metabolomic platform, for example, only 21 (21/73 = 28%) had readily available functional or confirmatory biochemical testing available in the United States (https://www.ncbi.nlm.nih.gov/gtr/).

Our results indicate that untargeted metabolomics can serve as a rapid and comprehensive way to screen variants of uncertain clinical significance. The test has the advantage of being able to detect aberrations in hundreds of molecules indicative of many different metabolic pathways not easily assayed through standard biochemical assays. In this study, when assessed in conjunction with exome sequencing, untargeted metabolomic data were found to contribute to variant interpretation in 43.5% of cases (Figure 1). For most of these variants, metabolomic profiling helped to rule out any likely clinical contribution, while in 22 cases, the data allowed for classification upgrade or confirmation of pathogenicity and diagnosis. Though our study population appears biased towards patients with neurologic disorders, this is consistent with previously reported pediatric referral indications to our clinical laboratory.[1,3] Similarly, the majority of untargeted metabolomic samples were obtained from plasma, likely due to the ease with which this may be obtained, particularly in a pediatric population. Our results also highlight how useful metabolomic data can be in instances where an individual is found to carry only a single pathogenic or likely pathogenic variant in an IEM, either ruling out disease or supporting the need to look further for a second pathogenic variant. Caution should be taken, however, in the interpretation of variants associated with diseases in which patients may only intermittently manifest with metabolic abnormalities. It is well recognized, for example, that individuals with intermittent forms of maple syrup urine disease may exhibit normal biochemical markers outside of acute decompensations. For example, in the present study for Patient 145 with biallelic variants in *ALDOB* consistent with hereditary fructose intolerance, metabolomic analysis showed only mildly elevated fructose, though it is possible that the patient had been treated at the time of sampling making it difficult to detect more characteristic abnormalities. This case highlights one important facet of metabolomic and other biochemical analyte analyses that should always be considered – the analysis reflects a patient's biochemical features at a single point in time, reflecting diet, supplements, and medications at the time the sample is taken.

Our study, however, has some limitations. While our current metabolomic platform is able to detect the characteristic metabolic signatures of >100 inborn errors of metabolism, there are many disorders not currently reliably detectable by this method. These include mitochondrial respiratory complex deficiencies, glycogen storage disorders, most lysosomal storage disorders, and rare enzyme defects not previously assayed in our laboratory that require additional study and validation. Additionally, while untargeted metabolomic data in

combination with whole exome/genome data may suggest or point to a specific diagnosis, in some cases, these findings may need to be confirmed via traditional assays, such as enzyme activity or assessment of additional body fluids, until associations are more clearly understood.

Our study also, however, highlights deficiencies in the way functional studies are treated in the current ACMG/AMP guidelines. At present, functional data may only be categorized as "PS3" or "BS3", that is strongly supportive of either a variant's pathogenicity or benign nature. Amended categorizations based on differing levels of support would be useful given the variable degrees of enzyme defects and the effects of treatment regimens or dietary interventions on biochemical phenotypes. Patient 1 (Table 1), for example, displayed minor abnormalities only modestly consistent with their molecular diagnoses which may or may not be due to hypomorphic alleles or a specific treatment intervention. This stands in stark contrast to Patient 92 where metabolomics identified the diagnostic signature[10] of a peroxisomal disorder quite clearly. As the use of metabolomics continues to grow, there will be a need for additional ways of categorizing such data in a clear, standardized manner in the context of clinical outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors would like to thank Drs. Leroy Hubert and Charul Gijavanekar and Susan M. Toon for their assistance with data collection and helpful discussions.

All authors were, at the time of this study, employees of Baylor College of Medicine, which has a partnership with Baylor Genetics that derives revenue from clinical testing. Dr. Liu was supported by 5T32GM007526 Medical Genetics Research Program. Dr. Glinton was supported by the Takeda Pharmaceuticals/ACMG Foundation Next Generation Medical Biochemical Fellowship Award. Drs. Sutton, Elsea and Yang receive salary support from Baylor Genetics, a for profit company that offers clinical testing referenced in this manuscript. Dr. Sutton is a paid advisory board member for PTC Therapeutics and CoA Therapeutics; he is also a non-compensated advisor for Mereo Biopharma. Dr. Yang is the founder of AiLife Diagnostics, Inc., a for profit company that provides interpretation and mining services. Dr. Elsea's work is funded by the NIH, Smith-Magenis Syndrome Research Foundation, PRISMS Inc., Shire Genetic Therapies, and Fondation Jerome Lejeune, and she is Chair of the Professional Advisory Board for PRISMS, and has received consulting fees from Enzyvant, Speragen, and Takeda.

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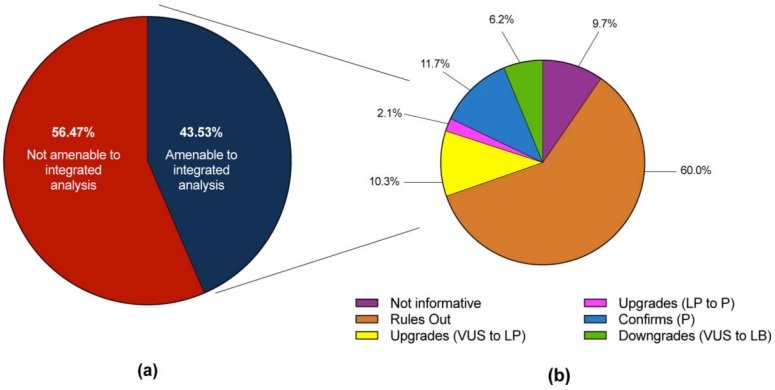

Fig. 1. Relative contribution of metabolomic data to variant interpretation.

(a) Metabolomic data contributed to the interpretation of a variant in 44% of clinical cases. **(b)** In these cases, 74 individuals carried 145 variants amenable to interpretation via untargeted metabolomics. Of these variants, metabolomics helped to rule out the contribution of 60%, confirmed the pathogenicity of 11.7%, upgraded 12.4%, and downgraded 6.2%, according the ACMG variant interpretation guidelines.

Table 1

Cases for which integrated analysis of metabolomic and WES data supported the diagnosis of inborn errors of metabolism

Patient	Sex	Age (years)	Gene	Disease	Inheritance	Variant (Amino Acid Change)	Zygosity	Category (ACMG)	Key Analyte (z-Score)	Contribution of Metabolomic Data
1	M	16.19	*TJP2*	Cholestasis, progressive familial intrahepatic 4 [MIM:615878]; Hypercholanemia, familial [MIM:607748]	AR	c.3371C>T (p.Thr1124Met)	Het	VUS	Cholate (+2.87) Taurochenodeoxycholate (−2.30)	Upgrades (VUS to LP)
			TJP2		AR	c.185C>T (p.Thr62Met)	Het	VUS		Upgrades (VUS to LP)
17	M	8.72	*SLC13A5*	Epileptic encephalopathy, early infantile, 25 [OMIM:615906]	AR	c.997C>T (p.Arg333Ter)	Het	VUS	Citrate (+3.86, +2.81)	Upgrades (VUS to LP)
			SLC13A5	Epileptic encephalopathy, early infantile, 25 [OMIM:615906]	AR	c.680C>T (p.Thr227Met)	Het	VUS		Upgrades (VUS to LP)
25	M	0.04	*SLC13A5*	Epileptic encephalopathy, early infantile, 25 [OMIM:615905]	AR	c.655G>A (p.Gly219Arg)	Het	P	Citrate (+ 3.12, +3.53, +2.36)	Confirms (P)
			SLC13A5	Epileptic encephalopathy, early infantile, 25 [OMIM:615905]	AR	c.1475T>C (p.Leu492Pro)	Het	LP		Upgrades (LP to P)
44	M	2.26	*DDC*	Aromatic L-amino acid decarboxylase deficiency [MIM:608643]	AR	c.286G>A (p.Gly96Arg)	Het	VUS	3-methoxytyrosine (+6.08) Vanillylmandelate (−2.71) 5-hydroxyindoleacetate (−1.93)	Upgrades (VUS to LP)
			DDC	Aromatic L-amino acid decarboxylase deficiency [MIM:608643]	AR	c.260C>T (p.Pro87Leu)	Het	VUS		Upgrades (VUS to LP)
48[a]	F	1.22	*PAH*	Phenylketonuria [MIM:261600]	AR	c.842+1G>A (N/A)	Het	P	Phenylalanine (+3.45) Phenylpyruvate (+2.80) γ-glutamylphenylalanine (+2.33)	Confirms (P)
			PAH	Phenylketonuria [MIM:261600]	AR	c.805A>C (p.Ile269Leu)	Het	VUS		Upgrades (VUS to LP)
55	M	14.97	*GAMT*	Cerebral creatine deficiency syndrome 2 [MIM:612736]	AR	c.797T>C (p.Tyr27His)	Hom	VUS	Guanidinoacetate (+2.80) Creatine (−3.05)	Upgrades (VUS to LP)
58[b]	F	1.55	*ACADS*	Acyl-CoA dehydrogenase, short-chain, deficiency of [MIM:201470]	AR	c.934–5T>A (N/A)	Hom	VUS	Ethylmalonate (+6.39) Methylsuccinate (+6.15) Butyrylcarnitine (+6.19)	Upgrades (VUS to LP)
62	F	1.82	*ABHD5*	Chanarin-Dorfman syndrome [MIM:275630]	AR	c.550C>T (p.Arg184Ter)	Hom	P	12,13-DiHOME (+2.02) 2-hydroxypalmitate (+2.22) α-hydroxycaproate (+2.36) Azelate (+2.03) Tetradecanedioate (+2.10)	Confirms (P)

Patient	Sex	Age (years)	Gene	Disease	Inheritance	Variant (Amino Acid Change)	Zygosity	Category (ACMG)	Key Analyte (z-Score)	Contribution of Metabolomic Data
68	M	1.35	*ABAT*	GABA-transaminase deficiency [MIM:613163]	AR	c.454C>T (p.Pro152Ser)	Het	VUS	2-pyrrolidinone (+6.88, +11.35) Succinamic acid (+3.57) Homocarnosine (+1.27)	Upgrades (VUS to LP)
			ABAT	GABA-transaminase deficiency [MIM:613163]	AR	c.1393G>C (p.Gly465Arg)	Het	VUS		Upgrades (VUS to LP)
75[b]	M	5.44	*UROC1*	Urocanase deficiency [MIM:276880]	AR	c.855G>A (p.Trp285Ter)	Hom	P	Imidazole propionate (+3.95) (cis- and trans-urocanate detected in large amounts as rare analytes but not z-scored)	Confirms (P)
85	M	3.91	*ABAT*	GABA-transaminase deficiency [MIM:613163]	AR	c.168+1G>A (N/A)	Het	P	4-guanidinobutanoate (+3.03) 2-pyrrolidinone (+2.20 in plasma, +5.91 in CSF)	Confirms (P)
			ABAT	GABA-transaminase deficiency [MIM:613163]	AR	c.638T>G (p.Phe213Cys)	Het	VUS		Upgrades (VUS to LP)
87	M	3.38	*PDHA1*	Pyruvate dehydrogenase E1-alpha deficiency [MIM:312170]	X-linked	c.832G>A (p.Gly278Arg)	Hem	P	Lactate (+3.32) Alanine (+3.17)	Confirms (P)
92	M	1.00	*PEX6*	Peroxisome biogenesis disorder 4A (Zellweger) [MIM:614862]; Peroxisome biogenesis disorder 4B [MIM:614863]	AR	c.611C>G (p.Ser204Ter)	Hom	P	Pipecolate (+5.90) 1-lignoceroyl-GPC (24:0) (+6.10) Docosadioate (+4.10) Eicosanodioate (+2.80) Hexadecanedioate (+5.13) Octadecanedioate (+3.31)	Confirms (P)
116	F	0.04	*TRMU*	Liver failure, transient infantile [MIM:613070]	AR	c.117G>A (p.Trp39Ter)	Het	P	Glycochenodeoxycholate (+3.16) Glycohyocholate (+3.24) Taurocholate (+3.05) Lactate (+4.84) Pyruvate (+3.85)	Confirms (P)
			TRMU	Liver failure, transient infantile [MIM:613070]	AR	c.680G>C (p.Arg227Thr)	Het	VUS		Upgrades (VUS to LP)
126	M	0.58	*ALDH5A1*	Succinic semialdehyde dehydrogenase deficiency [MIM:271980]	AR	c.1015–2A>C (N/A)	Het	P	4-guanidinobutanoate (+3.04) 2-pyrrolidinone (+3.20) Succinylcarnitine (+2.79)	Confirms (P)
			ALDH5A1	Succinic semialdehyde dehydrogenase deficiency [MIM:271980]	AR	c.1597G>A (p.Gly533Arg)	Het	P		Confirms (P)
136	F	0.59	*DDC*	Aromatic L-amino acid decarboxylase deficiency [MIM:608643]	AR	c.714+4A>T (N/A)	Hom	P	3-methoxytyrosine (+6.06) Vanillylmandelate (−3.37) Dopamine sulfate (−2.87)	Confirms (P)
146	M	0.77	*MTR*	Homocystinuria-megaloblastic anemia, cblG complementation type [MIM:250940]	AR	c.2405+1G>A (N/A)	Het	P	Methionine (−2.15) S-adenosylhomocysteine (+2.43) Methylmalonate (0.50) Betaine (+10.55) Dimethylglycine (+4.75)	Confirms (P)

Patient	Sex	Age (years)	Gene	Disease	Inheritance	Variant (Amino Acid Change)	Zygosity	Category (ACMG)	Key Analyte (z-Score)	Contribution of Metabolomic Data
148	F	0.07	*MTR*	Homocystinuria-megaloblastic anemia, cblG complementation type [MIM:250940]	AR	c.2473+3A>G (N/A)	Het	LP	Pipecolate (+ 3.18, +6.05 [CSF]) 6-oxopiperidine-2-carboxylate (+6.83, +7.01 [CSF])	Upgrades (LP to P)
			ALDH7A1	Epilepsy, pyridoxine-dependent [MIM:266100]	AR	c.834G>A (N/A)	Het	P		Confirms (P)
			ALDH7A1	Epilepsy, pyridoxine-dependent [MIM:266100]	AR	c.1279G>C (p.Glu427Gln)	Het	P		Confirms (P)
153	M	0.05	*HSD17B4*	D-bifunctional protein deficiency [MIM:261515]; Perrault syndrome 1 [MIM:233400]	AR	c.936_937delTA (p.Thr313Ter)	Het	P	Sphingomyelin d18:2/23:0,d18:1/23:1,d17:1/24:1 (−2.84) Euricoyl sphingomyelin (−2.45) Behenoyl sphingomyelin d18:1/22:0 (−2.06)	Confirms (P)
			HSD17B4	D-bifunctional protein deficiency [MIM:261515]; Perrault syndrome 1 [MIM:233400]	AR	c.1210–11C>G (N/A)	Het	VUS	Sphingomyelin d18:2/23:0, d:18:1/23:1, d17:1/24:1 (−2.84) 1-lignoceroyl-GPC 24:0 (+3.70) Linoleamide (3.38) Oleamide (3.01) Palmitic Amide (3.56)	Upgrades (VUS to LP)
157	F	1.18	*ASS1*	Citrullinemia [MIM:215700]	AR	c.830A>G (p.Lys277Arg)	Hom	LP	Citrulline (+ 7.09) N-acetylcitrulline (+4.67) 2-hydroxyadipate (+3.16)	Upgrades (LP to P)
			DHTKD1	2-aminoadipic 2-oxoadipic aciduria [MIM:204750]	AD/AR	c.1118C>T (p.Lys277Arg)	Hom	VUS		Upgrades (VUS to LP)
166[a]	M	0.12	*MUT*	Methylmalonic aciduria, mut(0) type [MIM:251000]	AR	c.1218delG (p.Asn407fs)	Het	P	Methylmalonate (+10.79) Propionylcarnitine (+8.40) Propionylglycine (+4.18) Glycine (+3.51) Alanine (+2.54) Carnitine (−2.65)	Confirms (P)
			MUT	Methylmalonic aciduria, mut(0) type [MIM:251000]	AR	c.1531C>T (p.Arg511Ter)	Het	P		Confirms (P)

[a]Patient originally identified on state newborn screening (NBS) and results confirmed with exome sequencing and metabolomics.

[b]Biochemical phenotype is benign and not causative of the patient phenotype.