

Untargeted Metabolomics, Targeted Care: The Clinical Utilities of Bedside Metabolomics



Joshua Manor and Sarah H. Elsea

Abstract The second decade of the twenty-first century saw a quiet revolution in the field of inborn errors of metabolism. Decades of extensive research into metabolic pathways of physiologically active cells and tissues, along with an improved resolution of high-throughput screening capabilities, brought forth the clinical metabolome. Clinicians can now take a metabolic snapshot while assessing their patients and receive invaluable information on pathological processes, rule in or rule out a proposed diagnosis, highlight early signs of decompensation, assess response to treatment, explore new disease biomarkers, and even suggest novel treatment options. In this chapter, we review the major strengths of clinical metabolomics as a diagnostic aid and its capabilities in promoting novel biomarker discovery. We also provide an outlook for how next-gen interpretation modalities (such as machine learning) are expected to revolutionize this field further to benefit patients worldwide.

Keywords Clinical metabolomics · Inborn error of metabolism (IEM) · Mass spectrometry (MS) · Dried blood spot (DBS) · Newborn screening (NBS) · Biomarker discovery

J. Manor
Metabolic Unit, Haim Sheba Medical Center, Edmond and Lily Safra Children's Hospital,
Tel Hashomer, Israel

S. H. Elsea (✉)
Department of Molecular and Human Genetics, Baylor College of Medicine,
Houston, TX, USA
e-mail: Sarah.Elsea@bcm.edu

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2023

A. M. Abdel Rahman (ed.), *Clinical Metabolomics Applications in Genetic Diseases*, https://doi.org/10.1007/978-981-99-5162-8_6

1 Introduction

In recent years, clinical practice has seen a dramatic increase in the utilization of targeted and untargeted metabolomics (TM and UM, respectively) [1, 2]. Metabolomics has been termed “the stethoscope of the twenty-first century” with broad applications in many fields of medicine: In oncology, it is utilized for biomarkers; in neurology, for severity stratification of neurodegenerative disorders; in endocrinology for diabetes modulators; in rheumatology and cardiology where certain metabolites may improve prognostication of chronic diseases (e.g., osteoarthritis and atherosclerosis); and in gastroenterology, where metabolomics may assist in differentiating between Crohn’s disease and ulcerative colitis [3]. UM has been used in multifactorial disorders to assist in risk assessment and early diagnosis, such as the observation that increased plasma levels in three out of five aromatic and branched-chain amino acids (isoleucine, leucine, valine, tyrosine, and phenylalanine) confers a fivefold increased likelihood for developing type 2 diabetes [4] or that increased plasma levels of glycocholate, taurocholate, and glycochenodeoxycholate are associated with nonalcoholic fatty liver disease (NAFLD). In contrast, decreased plasma levels of free carnitine, butyrylcarnitine, methyl-butryl carnitine, and cysteine-glutathione are seen in nonalcoholic steatohepatitis (NASH) [5]. Similarly, breast cancer showed a pattern of increased total choline-containing substances and decreased glycerophosphocholine in the plasma [6], correlating malignancy to a glycerophosphocholine-to-phosphocholine ratio switch [7].

Naturally, UM can highlight alterations in complex “metabolic disorders” and refine our understanding of metabolic flux in health and disease. The untargeted global assessment can further provide a high-resolution cellular homeostasis map and multivariant perturbation metrics that can assist in prognostication, the need for intervention, and the effect of a therapeutic modality. From a clinical perspective, a rare disease is a primary focus, and areas of influence for untargeted metabolomics include improving diagnostic rates, allowing affordable high-throughput disease screening, and identifying novel disease biomarkers that can be translated to the clinic. UM can contribute significantly toward a facilitated diagnosis and direct targeted treatment, thus enabling a reduction of the traditionally high morbidity and mortality associated with the under-recognition and undertreatment of metabolic disorders.

2 Metabolomics Joins the Diagnostic Front Seat

Clinicians justifiably consider exome sequencing (ES) to be the panacea of all diagnostic dilemmas, particularly when routine laboratory studies and tissue biopsies fail to establish a diagnosis. Indeed, ES has ushered in a new diagnostic era. Thanks to the reduced cost of sequencing, massive utilization of ES is now widely available, turning it into first-tier clinical testing for diagnostic evaluation of developmental

delay and other congenital anomalies [8–10]. ES is perhaps most heavily relied upon in the neonatal intensive care unit (ICU), in which a third of admitted patients are due to genetic causes [11]. ES is also heavily relied upon for primary mitochondrial disorders (PMD) [12], for which there is a notorious lack of biomarkers with adequate specificity [13]. This is further exemplified by the staggering finding of normal muscle respiratory chain enzymes in 10–20% of cases with mitochondrial myopathy undergoing invasive diagnostic procedures [14].

Nonetheless, as intriguing as it may appear, a diagnosis relying solely on ES without additional laboratory results lowers the test's pretest probability and increases the likelihood of false-positive results [15]. The American College of Medical Genetics and Genomics (ACMG) recommends that effort should be made to avoid using the pathogenicity of a variant as the *sole evidence* of a Mendelian disease but rather should be used in conjunction with other clinical information [16]. Perhaps, the best demonstration of the chasm between the diagnostic promise of ES and reality is variants of uncertain significance (VUS) [17], perceived as a “challenge” in ~1/3 of the articles reporting ES results [18]. With inconclusive molecular data, generating a snapshot of metabolites during illness creates a functional bioassay for candidate metabolic pathways. It provides an independent tool for ruling in or ruling out suspected diagnoses. Following the ACMG variant interpretation algorithm [16], metabolomic data fall into the functional data category by providing “well-established functional studies to show deleterious [or non-deleterious] effect,” which is considered strong supportive information for the pathogenic or benign nature of a variant. Metabolomic data, therefore, can push the needle from the neutral zone (a VUS) to the actionable zone [19, 20]. It is, nevertheless, of utmost importance to report only significant variations in metabolites to avoid reporting fluctuations stemming merely from dietary changes, environmental factors, drug exposure, and normal daily changes in metabolism. However, the definition of significance is still laboratory dependent [21]. It is also of equal importance to provide detailed clinical information and the nutritional and therapeutic status of the patient to the performing laboratory to optimize the data analysis. Given that a VUS is a common finding in ES, estimated to occur in 30–80% of clinically indicated ES tests [22, 23], and comprises ~30% of variants found by targeted sequencing for suspected inborn errors of metabolism (IEMs) [24], a variant validation and classification tool alongside molecular diagnostics is critical. UM serves as a valuable biochemical, functional validation instrument that can be integrated into clinical care.

2.1 *Monoamine Synthesis*

The utilization of UM for VUS reclassification was demonstrated by Atwal et al. in a diagnostic odyssey of an 11-month-old boy presenting with intellectual disability and hypotonia with episodes of generalized stiffening [25]. Initially diagnosed with cerebral palsy, exome sequencing showed two missense VUS in *DCC*, which

encodes aromatic-L-amino acid decarboxylase (AADC), a key enzyme in monoamine synthesis. AADC deficiency (AADCD) results in a severe neurometabolic disorder due to the combined deficiency of serotonin, dopamine, norepinephrine, and epinephrine. The onset of the disease is typically in the first months of life. However, the phenotypic spectrum of disease is broad and includes hypotonia, oculogyric crises, ptosis, dystonia, hypokinesia, developmental delays, and autonomic dysfunction [26]. Diagnosis is typically made by abnormal monoamine metabolites in CSF, followed by molecular confirmation or enzyme analysis in plasma. Elevation of 3-O-methyldopa (3-OMD), also termed 3-methoxytyrosine (3-MT), a catabolite of dihydroxyphenylalanine (L-DOPA), indicating a blockage in the conversion of L-DOPA into dopamine. For the patient described in Atwal et al., UM showed a plasma level of 3-MT 69 times higher than the control population.

Further confirmation of the UM findings was achieved by demonstrating absolute levels of 3-MT in CSF > three-fold the upper limit of normal, along with undetectable levels of the dopamine and serotonin derivatives, homovanillic acid (HVA), and 5-hydroxy indoleacetic acid (5-HIAA), respectively. Neurotransmitter analysis in CSF has been considered the gold standard for diagnosing AADC deficiency. Yet, this work presents that UM can attain similar results in plasma without requiring an invasive procedure. At times of atypical presentation and a wide differential diagnosis, UM can cast a wide net in a single test and may render specific diagnostic tests and procedures, each for a single suspected disease, inessential. The presentation can be atypical for the late-onset subgroup of patients with AADCD, including milder symptoms of hypotonia, dystonia, and fatigue [27]. As symptoms of AADC uniformly present in the first years of life [28, 29], a differential diagnosis may include “dopa-responsive dystonia,” with a trial of L-DOPA initiated as part of the diagnostic workup [30]. In light of this treatment approach [31], an additional work examined the ability of UM to differentiate an AADCD metabolic profile from other L-DOPA-treated conditions, as 3-MT will be elevated in both cases [32]. Two patients with AADCD before initiation of L-DOPA showed similar elevations of 3-MT as five patients with non-AADCD pathology (Z-scores of +5.88 and +7.65, respectively). However, reduced levels of dopamine 3-O-sulfate (D3OS) and vanillylmandelic acid (VMA) downstream of the AADC enzyme were seen in AADCD patients. In contrast, non-AADCD patients had elevated levels of D3OS and 3-methoxytyramine sulfate (3-MTS), showing a surplus of dopamine following treatment with L-DOPA. As these results were obtained from plasma and not from CSF, these data again demonstrate the robustness of the metabolic profile of AADCD obtained noninvasively.

2.2 *Ornithine Metabolism*

Diagnosis by variant reclassification was also made in a 7-year-old male with global developmental delay (GDD), ADHD, epilepsy, and ectodermal abnormalities [33]. The boy was found to have a novel, presumed splice-site VUS in *ODC1*. This gene

encodes ornithine decarboxylase, converting ornithine into putrescine, the first step of the spermidine pathway. UM showed excessive N-acetylputrescine, a metabolite of putrescine, confirming a gain-of-function variant in this gene, consistent with a diagnosis of Bachmann-Bupp syndrome (MIM 619075), an autosomal dominant disorder of neurodevelopment characterized by GDD, macrocephaly, white matter and callosal abnormalities, spasticity, seizures, and ectodermal defects (alopecia, cutaneous vascular malformations) [34, 35].

2.3 *NAD(P)HX Repair System*

VUS reclassification can also direct treatment for metabolic disorders amenable to the intervention. The NAD(P)HX repair system is a highly conserved two-enzyme system that restores damaged NAD(P)H [36]. In an acidic and hyperthermic environment, NADH and NADPH can both undergo hydration into NAD(P)HX nonenzymatically [37]. Without the repair system, the inactive NAD(P)HX accumulates and depletes the NAD⁺ pool under cellular stress. Biallelic pathogenic variants in either of the genes coding for the two enzymes, *NAXD* and *NAXE*, cause a rapidly progressive neurometabolic disorder triggered by inflammatory stress (mostly febrile illness), bearing high mortality in the first decade of life (MIM 618321 and 617186, respectively) [38–40]. A case of a fever-triggered encephalomyopathy crisis in a 16-year-old adolescent demonstrated a small deletion encompassing the first two exons of *NAXD*, *in trans* to a missense VUS in the exon 1-intron 1 splice donor, suspected to alter both splicing and the mitochondrial localization of *NAXD*, which contains the mitochondrial targeting sequence in its first exon [41]. Plasma UM during metabolic crisis demonstrated NAD⁺ depletion and led the medical team to initiate niacin therapy. Under therapy, clinical status improved, and baseline metabolomics demonstrated repletion of NAD⁺. Not only did UM biochemically support the pathogenicity of the variant, but it also provided monitoring data for the targeted treatment. These results were further validated in a case of *NAXE* deficiency, for which evidence of depletion of NAD⁺ derivatives was presented during a crisis. Niacin treatment appeared to prevent metabolic decompensation during a subsequent febrile illness while on niacin supplementation (Fig. 1).

2.4 *Riboflavin Metabolism*

Diagnosis and treatment guidelines were also provided in a case of a 2-year-old male diagnosed with hypoplastic macrocytic anemia (hemoglobin of 5.5 g/dL and mean corpuscular volume of 107.1 fL, range 10.5–14.0 g/dL and 76–90 fL, respectively) who later developed ataxia and nystagmus in the context of respiratory syncytial virus (RSV) infection [42]. Brain magnetic resonance imaging (MRI) showed enhancement of cranial nerves III and V, diffuse intramedullary T2 hyperintensity of

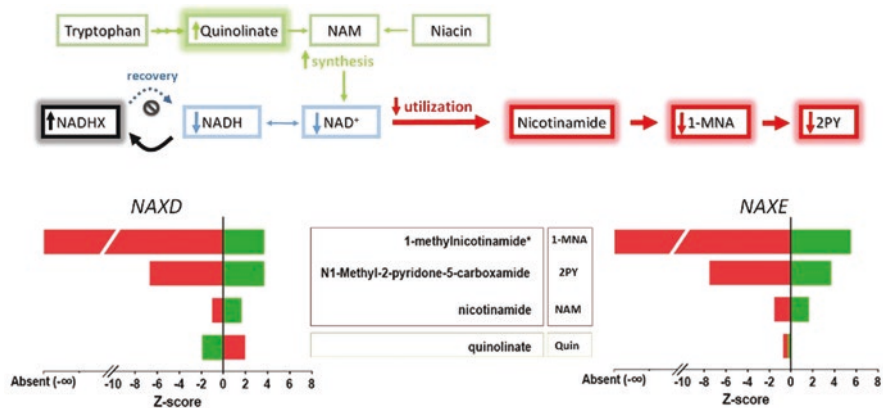


Fig. 1 NAD(P)HX repair system deficiency and plasma untargeted metabolomics. **Top:** NAD⁺ utilization (red) and repletion by biosynthesis (green) pathways are shown. When the repair system is defective (left, black), correction of spontaneously converted NADH to NADHX back to NADH (blue) is impaired, lowering available NAD⁺ and, thus, lowering utilization products while increasing upstream biosynthesis markers (quinolinate). **Bottom:** Plasma metabolomic profiles of deficiency in the NAD(P)HX repair system when patients are under inflammatory stress. Two patient profiles are shown, one for each repair system enzyme (NAXD, NAXE), with the effect of NAD⁺ depletion on selected analytes (red bars) and after supplementation with niacin (green bars). Taken together, in both NAXD and NAXE deficiencies, these results point to NAD⁺ depletion during inflammatory stress that is amenable to correction with substrate repletion. **Bottom: Left panel—**At the time of acute inflammatory stress, the red bars show the metabolomic profile from a patient with NAD(P)HX dehydratase deficiency (NAXD, EC 4.2.1.93), demonstrating the absence of 1-methylnicotinamide, marked deficiency in N1-methyl-2-pyridone-5-carboxamide, and increased quinolinate (red bars). At 11 months' post-crisis, the patient underwent another UM profiling (green bars), showing a reversal of these alterations while under niacin treatment. **Bottom: Right panel—**Shown are the same key molecules in plasma from a patient with NAD(P)HX epimerase deficiency (NAXE, EC 5.1.99.6) demonstrating similar trends during an acute inflammatory crisis (red bars). Repeat UM profiling at 9 months' post-crisis while on niacin supplementation showed a reversal of these alterations (green bars)

the entire cord, and cauda equina nerve root thickening and enhancement. ES showed a pathogenic variant and a novel VUS predicted to cause an in-frame single amino acid deletion (p.Phe153del) in *SLC52A2*, an intestinal basolateral and a blood-brain barrier riboflavin transporter (MIM 607882). UM showed elevated C6 (hexanoylcarnitine), C8 (octanoylcarnitine), C10 (decanoylcarnitine), and C10:1 (decanoylcarnitine), supporting the pathogenicity of the in-frame deletion variant, in addition to elevations in 2-hydroxyglutarate, methyl succinate, and ethylmalonate, common secondary alterations in short- and medium-chain fatty acid oxidation defects. These perturbations can also be detected on TM, yet the value of UM was nicely demonstrated by showing additional perturbations in metabolic pathways that are related to riboflavin deficiency. Riboflavin is the precursor of flavin adenine dinucleotide (FAD), a necessary cofactor for kynurenine-3-monooxygenase, the *de novo* NAD⁺ biosynthesis pathway member; in the patient, the proximal kynurenine was increased, and the distal picolinate was decreased. Reduction of

5,10-methylene tetrahydrofolate into 5-methyltetrahydrofolate is also dependent on FAD, leading to inhibition of the one-carbon cycle and recycling of sulfur-containing amino acid, ultimately resulting in elevations in sarcosine, dimethylglycine, methionine, and glycine. These values normalized upon initiation of high-dose riboflavin (70 mg/kg/day), with a resolution of the anemia and nystagmus and improvement in ataxia. While ataxia and nystagmus are common findings, the highly suggestive upper body proximal muscle weakness with prominent neck flexion and the early symptom of dysphagia were not seen; however, macrocytic anemia is a rare finding [43], making a diagnosis based on clinical suspicion very difficult. Indeed, SLC52A2 deficiency, also called Brown-Vialetto-Van Laere syndrome 2 (MIM 614707), is a difficult diagnosis to make, with an average time to diagnosis of more than 2 years, a significant delay for a disorder manifesting in the first decade of life [43]. For example, normocytic anemia was misdiagnosed in a toddler as pure red cell aplasia overlooking an insidious weakness and areflexia and delaying the diagnosis of SLC52A2 deficiency for 3 years [44].

2.5 *Histidine Metabolism*

Not only for VUS can interpretation by UM assist in diagnostic dilemmas. Deficiency of urocanase (also called urocanate hydratase), an enzyme participating in the histidine deamination breakdown pathway, was previously considered to be associated with intellectual disability [45, 46]. Intellectual disability (ID) is a relatively common clinical finding, with a 0.8–3.7% prevalence in the pediatric population [47, 48]. Common disorders can manifest independently in rare diseases. A connection can be falsely established due to (a) small cohorts (an inherent problem of rare disorders), (b) consanguinity increasing independently the prevalence of IEM [49, 50] and ID [51, 52], and (c) publication bias, resulting from a top-down sequencing of patients presenting with a multitude of symptoms. In a recent work, plasma and urine UM showed a significant increase in both *cis*- and *trans*-urocanate and imidazole propionate in two asymptomatic patients with biallelic pathogenic variants in *UROCI* and normal intellect and with no other significant metabolic alterations [53] (Fig. 2). UM helped expand our understanding of the benign nature of urocanase deficiency (MIM 276880) and the need to pursue a genetic diagnosis for ID in patients with urocanase deficiency, per standard guidelines [10].

2.6 *The Diagnostic Rate Among Inborn Error of Metabolism*

A direct comparison of TM (plasma amino acids [PAA], acylcarnitine profile [ACP], and urine organic acids [UOA]) to UM showed that the latter has an overall ~sixfold higher diagnostic rate for IEM (7.1% vs. 1.3%) [19]. That result may not be unexpected, yet the power of UM was demonstrated by the variety of diagnoses it allows

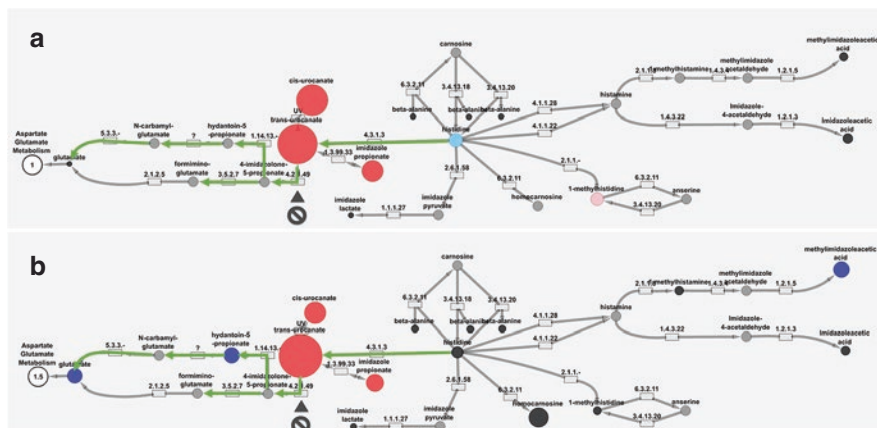


Fig. 2 Metabolic map of the histidine catabolism pathway in a patient with urocanate hydratase (EC 4.2.1.49, urocanase) deficiency in (a) plasma and (b) urine. The affected arm of the pathway, highlighted in green arrows in both plasma and urine, shows the accumulation of analytes immediately upstream to the enzymatic block, trans-urocanate and cis-urocanate. These accumulations then result in the subsequent accumulation of imidazole propionate (in equilibrium with trans-urocanate) and, to a lesser degree, 1-methylhistidine. In urine, the deficiency of the downstream analytes, hydantoin-5-propionate and glutamate, are also shown (blue circles). The color, diameter, and shading of each circle are proportional to the Z-score. Red circles indicate analytes in excess (Z-score $> +2$); blue circles represent deficient analytes (Z-score < -2); pink circles indicate analyte excess with a Z-score $> +1.5 < +2$. Black circles indicate analytes within $-1.5 \leq \text{Z-score} \leq +1.5$. Gray circles indicate analytes that are not measured in this assay. (Adapted from Ginton et al., 2018. Urocanate hydratase (urocanase) is indicated by the black “no entry” sign)

over TM. Diagnoses included disorders of synthesis of neurotransmitters, cholesterol, and peroxisomal biogenesis. γ -aminobutyric acid-(GABA)-transaminase deficiency (MIM 613163). This early infantile epileptic encephalopathy with a movement disorder and hypersomnolence due to GABA catabolism defect, was diagnosed in a 1-year-old patient with hypotonia and movement disorder and compound heterozygous VUS in *ABAT*, which encodes GABA-transaminase. UM showed 2-pyrrolidinone, succinamic acid, and succinimide elevations that were not seen on UOA. These three metabolites are reliably detected in both CSF and plasma, in contrast to CSF GABA alone, the gold standard for diagnosis but prone to false-negative results if not handled appropriately [27]. Specifically, 2-pyrrolidinone is a butyrolactam spontaneously converted from GABA when the latter is not broken down to succinic semialdehyde (SSA) by GABA-transaminase. 2-pyrrolidinone is converted to succinimide and succinamic acid [54], making these three metabolites alternative biomarkers. The same metabolic profile was seen in a 6-year-old with global developmental delay (GDD) and movement disorder who was also homozygous for a VUS and two other patients presenting with encephalopathy, seizures, cortical blindness, motor developmental delay, hypotonia, strabismus, ataxia, and intellectual disability [19, 54].

A diagnosis of Smith-Lemli-Opitz (MIM 270400), a disorder of cholesterol biosynthesis, was made in a typically presenting 10-year-old male with microcephaly, hypotonia, developmental delays, and a congenital cardiac defect. In that patient, 7-dehydrocholesterol was elevated, while cholesterol was decreased. Initially, a diagnosis could be ascertained by comparing this profile to the near identical profile of a molecularly confirmed 3-year-old boy [19] and later confirmed molecularly. In a different study, such a diagnosis was excluded in a 28-year-old patient with developmental delays by demonstrating normal 8-dehydrocholesterol and 7-dehydrocholesterol, downgrading a VUS to likely benign [55].

The latter study presented 16 cases of either homozygous or compound heterozygous VUS in which metabolomics assisted in variant reclassification after ES was nondiagnostic. Noteworthy in this study are (1) a case of a patient presenting with early-onset recurrent nephrolithiasis, urosepsis, and transfusion-dependent anemia, showing an excess of orotic acid in the urine (a metabolite not easily detected in UOA); thus, the two missense VUS were reclassified to pathogenic and likely pathogenic, and treatment with uridine monophosphate was initiated; and (2) a case of psychomotor retardation and retinitis pigmentosa with increased urinary 5-oxoprolin excretion, and despite negative ES, the finding that prompted targeted sequencing of the *GSS* gene, demonstrating homozygosity for a deep intronic variant for this highly heterogeneous condition, 5-oxoprolinase deficiency (MIM 260005).

UM performed on plasma from a 17-year-old with agenesis of the corpus callosum, autism spectrum disorder, lactic acidosis, hyperammonemia, and electrolyte abnormalities revealed reduced pantothenate, carnitine, and carnitine derivatives due to *SLC5A6* deficiency, confirmed by biallelic VUS identified by subsequent exome sequencing [19]. *SLC5A6* is a cellular cotransporter of pantothenate, biotin, and α -lipoic acid in the intestine and blood-brain barrier [56]. *SLC5A6* deficiency leads to early-onset neurodegenerative disorder and also includes failure to thrive, acquired microcephaly, movement disorder, immunodeficiency, gastrointestinal dysfunction, and osteopenia. Early treatment with high-dose pantothenate, biotin, and α -lipoic acid seems to improve outcomes [57, 58]. UM also confirmed the diagnosis of medium-chain acyl-CoA dehydrogenase deficiency, argininemia, and X-linked glycerol kinase deficiency (GKD, MIM 307030), in which molecular diagnoses were inconclusive or missing, among other conditions [19].

To directly analyze the contribution of UM to the interpretation of variants identified in ES, Alaimo et al. examined clinical samples with both ES and UM testing that were sent for diagnostic purposes in a cohort of 170 patients [20]. This cohort was similarly primarily pediatric, with >90% presenting with neurological symptoms. The researchers identified 145 variants in 74 patients in their cohort in 73 genes associated with an IEM. Based on the metabolomic data, the 12.3% diagnostic rate in this cohort facilitated the reclassification of 27 variants (19%). Of the reclassified variants, 24 VUS were reclassified as either likely pathogenic ($n = 15$) or likely benign ($n = 9$), while an additional three variants were upgraded from likely pathogenic to pathogenic. Classifications were done according to the ACMG guidelines [16]. For 17 additional pathogenic variants, the study presented

confirmatory metabolic perturbations. One case upgraded a homozygous VUS to a likely pathogenic variant, making the diagnosis of guanidinoacetate methyltransferase (GAMT) deficiency likely in the patient and allowing the clinician to focus on the treatment for this disorder of cerebral creatine deficiency. Moreover, for autosomal recessive conditions in which sequencing revealed only heterozygous variants, UM ruled out the suspected condition in ~60% of cases by showing a normal metabolite profile for the metabolic pathway in question, thus excluding the possibility of a biochemically symptomatic carrier possessing a second disease-causing allele not detected due to the constraints of ES.

2.7 Automation of Data Interpretation

Similarly to ES diagnostic capabilities, no discussion of UM clinical diagnostic capabilities will be complete without discussing the interpretation of the (untargeted) data. The ability to correctly diagnose a condition based on UM heavily depends on the art of data interpretation. Rather than the convoluted manual analysis of metabolomic data (with or without genomic information), automated bioinformatic tools provide means for pattern recognition and thus hold a great promise of an improved matching between datasets and a particular disease or pathway. One approach applies the clique problem in a metabolomic graph. A clique is defined as an interconnected group of metabolites (nodes), and small highly connected cliques are extracted based on computational analysis bounding cliques' p values [59]. Across 539 plasma samples, this connect-the-dots (CTD) approach reproduced accurate diagnosis of 16 different IEMs [60]. The top-down bioinformatic approach seeks to identify causative genes by providing a likelihood scoring system based on heuristic algorithms predicting the effects they expect to exert on the *-omic* dataset. Several metrics achieved prioritization of candidate genes based on UM. In *cross-omics*, a metabolomic study performed using dried blood spots (DBS) [61], candidate genes were prioritized based on their distance from the perturbed metabolite (where each reaction accounts for 1 step), with a narrowing process of including metabolites that participate in only limited amount of reactions ("uniqueness") and limiting metabolites to only those with a significant Z -score ("significance") to generate gene-specific metabolite sets. Successful prioritization was achieved by considering metabolites up to four reactions away from the primary reaction, uniqueness of up to 15 reactions, and significance of $>+3$ or <-3 . In *metPropagate* [62], each protein is assigned a rank based on its associated metabolite enrichment, and dynamic ranking is propagated with the protein's functional linkage network. This approach successfully prioritized causative genes (within the top 20th centile) in 20% of IEMs in the study group and 82% (9/11) in the test group of neurometabolic disorders. This algorithm outperformed Exomiser [63], a causative variant

prioritization tool based on human phenotype ontology data and standard pathogenicity prediction (variant prevalence, conservation, and information from model organisms and inheritance patterns). In another approach, Reaffect [64] assigned a score based on cumulative pathway perturbations, including metabolites with sub-significant *Z*-scores, correctly predicting the causative gene in the top fifth centile in 80% of the 76 patients harboring 36 different IEMs. When combined with the deleteriousness predictive score, Combined Annotation Dependent Depletion (CADD) [65], specificity further increased. Another promising approach is the implementation of a siamese neural network, weighing in the tandem computational metabolic network (for predicting metabolite flux) and machine learning (ML, for matching metabolic networks to diseases) trained by the ML algorithm [66]. The model used a single simulated profile for each disease with real data points from only 2% of the diseases to outperform a generic algorithm that prioritizes causative genes based on distance from real data profiles (using the L1 Manhattan metrics). Limitations to ML include the quality of the training set and the generalizability of metabolic profiles to novel diseases. Nonetheless, implementing predictive ML-based algorithms promises to reduce the number of patient-derived samples required for disease discovery (smaller cohorts), a vital prerequisite in the world of rare diseases such as IEMs [59].

3 Metabolomic Fingerprinting: Identifying Diseases’ Biometrics and Finding New Disease Biomarkers

Biomarkers are crucial for the effective screening, designation, and diagnostic confirmation or exclusion of diseases by UM. Biomarkers are also critical in monitoring an affected patient’s metabolic status and treatment efficacy. Clinical biomarkers for IEMs are abundant [67], although most are derived from TM. Many biomarkers are assessed by routine biochemical tests, such as ammonia, lactate, uric acid, and cholesterol, or from TM, such as UOA (e.g., trimethylamine in fish odor syndrome) or PAA (high phenylalanine in PKU). To increase specificity, either identification of disease-specific biomarkers can assist in the diagnosis (or ruling out) of a disease, such as allo-isoleucine in MSUD or argininosuccinic acid in argininosuccinate lyase (ASL) deficiency, or the identification of several non-pathognomonic metabolites, such as low levels of lysine, ornithine, and arginine in plasma amino acids suggestive of LPI, or the elevation of propionylcarnitine on ACP, methylmalonic acid in UOA, low methionine on PAA, and homocysteine in the blood, indicative of an intracellular cobalamin utilization defect. UM can instigate both strategies. By its untargeted nature, UM has the potential to uncover biomarkers that were not known to be associated with an IEM or not visible by targeted testing.

3.1 Peroxisome Biogenesis

Peroxisome biogenesis disorders in the Zellweger spectrum (PBD-ZSD) are a heterogeneous group of genetic disorders caused by mutations in genes responsible for normal peroxisome assembly and functions [68]. The majority of cases are due to biallelic pathogenic variants in *PEX1* (61%), followed by *PEX6* (15%) and *PEX12* (8%), with additional contribution from at least another 10 *PEX* genes [69], an important group of proteins essential for the assembly of peroxisomes and the recognition and transport of cytoplasmic proteins that contain peroxisomal targeting sequence [70]. The severe end of PBD-ZSD includes neuronal migration defects with leukodystrophy, neonatal onset seizures, hypotonia, failure to thrive, liver dysfunction, bony stippling respiratory insufficiency, cataracts, sensorineural hearing loss, and renal cortical microcysts; the mild end of the spectrum includes developmental delays and intellectual disability that can be mild and slowly progressive retinopathy and sensorineural hearing loss [68, 69, 71]. Severe cases can be screened by elevation of the very-long-chain fatty acid (VLCFA), C26:0-lysophosphatidylcholine (DBS, plasma), phytanic and pristanic acids (plasma); reduction in plasmalogens (plasma, erythrocyte membranes); increase in pipecolic acid (plasma, urine); and increase in the bile acids, dihydroxycholestanoate, and trihydroxycholestanoate (plasma, urine) [69]. Intermediate and mild cases may show only subtle alterations, and in combination with subtle clinical symptoms, screening for PBD-ZSD may not be performed. In a cohort of 19 mild to intermediate PBD-ZSD pediatric patients, Wangler et al. showed a distinct PBD-ZSD plasma metabolome (Fig. 3), with elevated levels of long-chain dicarboxylic acids, pipecolic acid, and the bile acid derivative 7 α -hydroxy-3-oxo-4-cholestenoic acid and with reductions in phosphatidylcholines, phosphatidylethanolamines, and plasmalogens [71]. Pipecolic acid and the lysophospholipid 1-lignoceroyl-GPC (24:0) were most strikingly elevated with a Z-score of +3.7. Less anticipated changes included dicarboxylic acids of 16–22 carbons. UM also proposed novel biomarkers, with a reduction in nine sphingomyelin species. Reduction of several sphingomyelins in the clinical UM database, excluding PBD-ZSD, was observed in only 2% (interestingly, one of which was diagnosed with bifunctional protein deficiency, which can mimic PBD-ZSD). Moreover, the plasma elevations in pipecolic acid and reductions in the sphingomyelins attenuated with age, an expected finding correlating milder phenotypes among older surviving patients.

3.2 Urea Cycle

The urea cycle is the principal mechanism for the clearance of waste nitrogen resulting from protein turnover, the sole source of endogenous production of arginine, ornithine, and citrulline, and a principal component of the nitric oxide (NO) production pathway [72]. The urea cycle is also connected with TCA anaplerosis

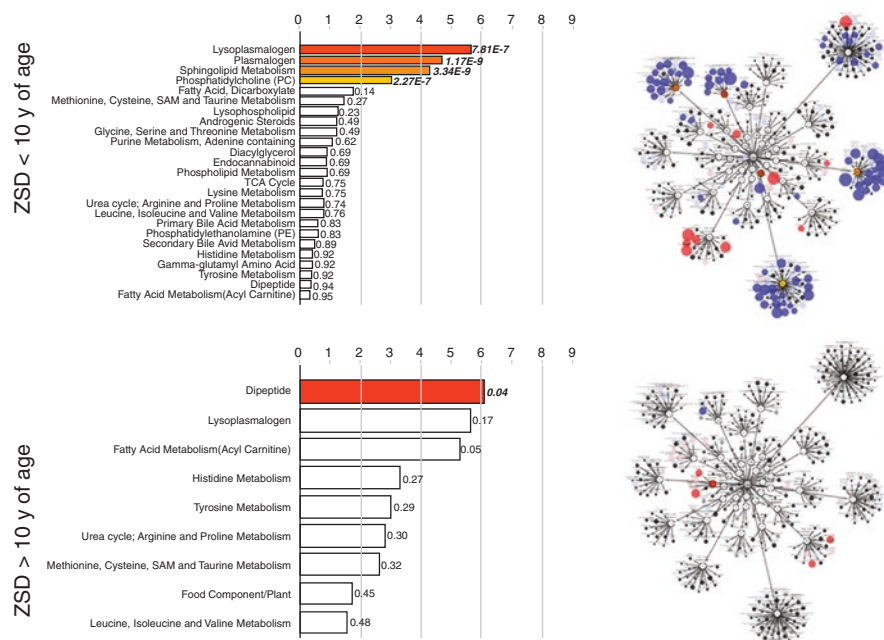


Fig. 3 Plasma metabolomic footprint of peroxisome biogenesis disorder-Zellweger spectrum disorder (PBD-ZSD). **Top: Left panel.** Early diagnosis (age <10 years) of PBD-ZSD shows a more significant metabolic phenotype, with enrichment of lysoplasmalogens, plasmalogens, sphingolipid metabolism, and phosphatidylcholines, reaching statistical significance (colored bars) for patients when compared to control population. **Top: Right panel.** The relative deficiencies of most of these lipids in PBD-ZSD in children under the age of 10 years are represented by the blue circles in the metabolomic tree of lipid analytes (lipidomics). **Bottom: Left panel.** Later diagnosis of PBD-ZSD (age >10 years) exhibits a more subtle metabolic phenotype. **Bottom: Right panel.** The metabolomic fingerprint of PBD-ZSD in individuals >10 years of age showed mild perturbations in which reductions of the plasmalogens and lysoplasmalogens did not reach statistical significance. The lipidomic tree shows considerably fewer perturbations when compared to plasma obtained from a younger patient. Trees were rendered using Cytoscape (<https://www.cytoscape.org>). (Figure adapted from Wangler et al. 2018)

via the alternative synthesis of fumarate. UCDs include eight different IEMs resulting from defects in any one of the six enzymes or two transporters involved in the hepatic removal of ammonia as waste nitrogen by its conversion to urea and excretion by the kidneys [73]. Mortality and morbidity primarily contribute to neurological damage resulting from hyperammonemia (HA) and the elevation of other neurotoxic intermediates of metabolism [74]. A typical presentation of a UCD includes neonatal hyperammonemic crisis; however, nontypical presentations of later-onset HA, acute liver dysfunction, intellectual disability, or insidious pyramidal signs of the lower extremities with minimal HA crises (in arginase deficiency) have been described [27]. It has been suggested that arginase deficiency, with its unique presentation compared to other UCDs, exerts a neurotoxic

effect by the guanylation of glycine, with the excess arginine serving as a guanidine donor rather than acute HA crises [75]. In a cohort of 13 patients with argininemia, Burrage et al. demonstrated an increase in additional guanidine compounds, namely, N-acetylarginine, homoarginine, argininate, and 2-oxoarginine, to a greater degree than guanidinoacetate (GA) [76]. Guanidine compound (GC) toxicity is believed to be derived from the observed in vitro neurotoxicity: diminished response to the inhibitory GABA and glycine neurotransmitters [77], promotion of non-apoptotic cell death and axonal hypersprouting [78], and inhibition of Na⁺/K⁺-ATPase activity and glutamate uptake, and decrease in antioxidant defense in the rat brain [79]. Other toxic effects of GC include ethanol-induced liver injury, stimulated osteoclastogenesis, generation of reactive oxygen species (ROS), and modulation of cerebral cortex potentials [79]. In GAMT deficiency, GA is the main GC accumulating and phenotypically exerting a greater degree of intellectual disability, refractory epilepsy, and dystonia. At the same time, pyramidal signs are less dominant as compared to arginase deficiency. While these results may indicate an important role of GA in the developing brain, another important modifier between the two disorders is the creatine level, which is normal in arginase deficiency and low in GAMT deficiency [80]. Peripheral administration of polyethylene glycol amalgamated to (PEGylated) arginase, now in advanced stages of development, will help elucidate further pathomechanistic insights by the peripheral reduction of arginine excess without restoring urea cycle function in the liver.

The same study by Burrage and colleagues also examined a UM profile of OTC deficiency, a severe X-linked disorder bearing high morbidity and mortality among males and affected females. In this UCD, morbidity is attributed to HA crises, and biomarkers are scarce, making this disorder “unscreenable.” In this study, 83% of patients (10/12) with a history of hyperammonemia (excluding females with no such history) showed a significant elevation (*Z*-score >+2) of either orotate or uridine. Other biomarkers of increased pyrimidine metabolism (due to the shunting of the carbamoylphosphate from the dysfunctional urea cycle), β-ureidopropionate, and uracil were not significantly elevated. For screening purposes, the sensitivity of these markers is ~60% (10/17), and the specificity is also low, given shared pathway perturbation with other UCD and pyrimidine metabolism defects; however, these results point to an important consideration for biochemical testing, indicating such abnormalities in cases of uncertain diagnosis given the sensitive nature of this disorder. The authors are aware of a case of a 1.5-year-old female presenting with fulminant liver failure and nonspecific liver biopsy for which only uracil was elevated in urine on traditional metabolic screening. Based on that result, a presumptive diagnosis of OTC deficiency was made, and targeted treatment was promptly provided; molecular testing later confirmed the suspected diagnosis. Both citrullinemia and ASL deficiency did not show unique metabolic fingerprints beyond the accumulation of citrulline and argininosuccinic acid, respectively [76].

3.3 *Pyruvate Kinase*

Pyruvate kinase deficiency (PKD, MIM 266200) is the most common form of inherited anemia due to glycolytic defects. It results in a spectrum of hemolytic anemia that can result in infantile-onset transfusion-dependent anemia or a milder form of compensated anemia [81]. A diagnostic gap exists for PKD due to the unsatisfactory performance of activity assays, a genetic composition complicating molecular diagnosis (variants in regulatory elements or effector genes such as *KLF1*), and the confounding effect of frequent blood transfusion on these methods [82]. In a cohort of 16 patients with PKD (against 32 controls), van Dooijeweert et al. showed three groups of metabolites in DBS that differed between the two cohorts: glycolytic products phosphoenolpyruvate and 2- and 3-phosphoglycerate, as one might expect, along with polyamines, such as spermine, spermidine, N¹-acetylspermidine, and putrescine, which are associated with red blood cell (RBC) membrane integrity, and acylcarnitines such as methylmalonylcarnitine and propionylcarnitine which are involved in turnover and repair of the RBC membrane [83]. Principal core analysis showed a separation of metabolic profiles between the two groups. Interestingly, the mildly affected patients with no history of transfusion dependence or splenectomy more closely resembled the control group, followed by transfusion dependence (severe phenotype), probably due to the frequent retrieval of donor RBC, and the splenectomized patients (moderate phenotype) were furthest away from control. Based on these findings, a machine learning algorithm trained on a subset of the cohort could predict the disease in 94% of cases.

3.4 *Glucose Transporter 1*

UM can also reveal a metabolic fingerprinting of disease-modifying treatments, aiding the monitoring of both therapeutic efficacy and disease progression. The brain glucose transporter GLUT1 facilitates the diffusion of glucose to brain tissue to compensate for insufficient passive diffusion, given the elevated requirement for glucogenic energy production by that tissue. Heterozygous pathogenic variants in *SLC2A1*, which encodes GLUT1, result in a brain energy failure syndrome (GLUT1DS) caused by impaired glucose transport and result in a spectrum of phenotypes including epileptic encephalopathy, intellectual disability, acquired microcephaly, ataxia, action limb dystonia, chorea, and tremor in its severe form and paroxysmal ataxia in its mild end. Diagnosis is made by demonstrating a low CSF concentration of glucose in the presence of plasma normoglycemia and/or identification of a pathogenic variant in *SLC2A1* [84]. Aside from probable energy depletion, it was suggested that depletion of glycolysis intermediates might play a role in the disorder's pathogenesis; however, it is unknown to what extent, as in vitro

models include near-complete abrogation of the transporter [85]. The sole therapeutic option available is a classic ketogenic diet (CKD). By altering the brain energy fuel into mainly ketone bodies, this therapy dramatically affects seizures and can improve cognitive outcomes. However, diet discontinuation was reported in up to 10% of patients due to side effects [86], and low compliance led experts to recommend alternative low carbohydrate diets (such as the modified Atkins diet) for adolescents and adults [87] and even exploring amylopectin-based diet of the low glycemic index [88]. Monitoring CKD is based on serum β -hydroxybutyrate levels, as measuring urine ketones—a convenient and qualitative measure of acetoacetate—has a limited role in diet monitoring [87]. A more comprehensive picture created by UM performed on six treated patients with GLUT1DS [89] showed elevations in β -hydroxybutyrate, β -hydroxybutyrylcarnitine, β -methyladipate, and N-acetylglycine. Other elevated derivatives were α -ketobutyrate, β -hydroxylaurate, 10-nonadecenoate, margarate, 15-methylpalmitate, and α -aminoheptanoate. Furthermore, pathway analysis using Kruskal-Wallis analysis (comparing pathway metabolite perturbations vs. non-pathway metabolite perturbations) showed involvement of long-chain fatty acids, phospholipids, acylcarnitines, and, to a lesser degree, sphingolipids, mono-hydroxy fatty acids, and polyunsaturated fatty acids. In accordance with fatty acid utilization, free carnitine levels were low, while carnitine-bound metabolites were elevated. CSF UM of three patients prior to CKD onset revealed low levels of glycerol 3-phosphate, an intermediary metabolite in lipid metabolism, and an increased level of isocitrate, which can indicate a TCA dysfunction [89]. These results supply a CKD metabolic profile with a more complete ketosis map, which can assist in diet fine-tuning, e.g., increasing the fat-to-carbohydrate ratio to increase overall ketosis or examining the effect of decreasing the ratio in reduced diet tolerability. The results can also guide the need for supplemental carnitine due to increased secondary excretion in ketosis, which, although it is considered a benign supplementary agent, can also exacerbate GI-related symptoms in patients under this GI-unfriendly diet and can also independently elevate plasma trimethylamine N-oxide (TMAO) levels, an atherosclerotic agent [90, 91].

3.5 *Serine Metabolism*

Serine biosynthesis defects occur due to deficiency in either of the three enzymes converting 3-phosphoglycerate into serine, phosphoglycerate dehydrogenase (PGDH), phosphoserine aminotransferase (PSAT), and phosphoserine phosphatase (PSP), and result in a severe neurometabolic disorder including severe intellectual disability, ataxia, nystagmus, epilepsy, hypertonia/spasticity, microcephaly, and poor growth. Deficiencies in enzymes of the serine de novo biosynthesis pathway result in low plasma and CSF levels of serine. Low serine impedes the synthesis of sphingolipids (from serine and palmitoyl-CoA), phosphatidylserine, D-serine (an agonist to the ionotropic glutamate receptor N-methyl-D-aspartate (NMDA)), and

5,10-methylenetetrahydrofolate (by converting serine to glycine) [92]. This multi-pathway effect could explain neurodevelopmental abnormalities associated with this disorder; however, a more direct association has not been clinically demonstrated. In four children with two serine biosynthesis defects, PGDH deficiency (three children) and PSAT deficiency (one child), Glinton et al. demonstrated an abnormal phospholipid profile at the time of diagnosis and, upon treatment, normalization thereof. Before initiation of treatment, serine and glycine were both decreased in all patients, and multiple phospholipids were reduced, including species of the mono- and di-unsaturated 18-carbon phosphatidylcholine and phosphatidylethanolamine in three or four of the patients [93]. Multiple sphingolipid species were reduced in two patients, most notably sphingomyelin. Except for phospholipids, no other compounds were found to be altered (either reduced or elevated) consistently in all patients evaluated [93]. The majority of these abnormalities normalized under supplementation. These results emphasize the need for early treatment. De novo synthesis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from choline, ATP, cytidine triphosphate (CTP), and diacylglycerol by choline kinase, phosphocholine cytidyltransferase, and choline transferase (also referred to as the Kennedy pathway) may, in fact, be inadequate at the time of rapid growth or neuronal differentiation in utero and require supplementation of PE and PC from phosphatidylserine pool (by mitochondrial phosphatidylserine decarboxylase). Another possibility is serine palmitoyltransferase (SPT) substrate promiscuity, leading to the condensation of alanine and palmitoyl-CoA into 1-deoxy-sphinganine (instead of 1-dehydro-sphinganine when serine is sufficient). Indeed, decreased sphingomyelin and increased 1-deoxy-sphingomyelin have been reported in targeted metabolomics applied for primary serine biosynthesis defects and secondary serine deficiency due to mitochondrial disorders [94]. Interestingly, a DBS sample from one patient at 38 hours of life showed markedly reduced serine, which could have served as abnormal NBS, as discussed above [93].

3.6 Pentose Phosphate Pathway and Polyol Metabolism

The pentose phosphate pathway is an important cytosolic pathway that converts glucose into ribulose-5-phosphate and produces reduced nicotinamide adenine dinucleotide phosphate (NADPH) for restoration of the antioxidant glutathione. Its oxidation product, ribulose-5-phosphate, can serve as a nucleotide building block or be further converted into phosphorylated mono-sugars that can serve as glycolytic intermediates [95]. The nonoxidative portion includes four enzymes, ribulose 5-phosphate isomerase and reductase, transaldolase (TALDO), and transketolase (TKT). Common features of TALDO deficiency (MIM 606003) are hepatosplenomegaly, anemia, thrombocytopenia, renal tubulopathy, heart abnormalities, and cholestatic liver dysfunction that can develop into cirrhosis [96]. For TKT deficiency (MIM 617044), manifestations include short stature, developmental delays, and congenital heart defects [27]. These manifestations are nonspecific; further,

considering the potential reversibility of these two disorders (despite no currently available treatment), high-yield screening can facilitate diagnosis and improve outcomes. While TALDO deficiency was indicated by UM screening by identification of sedoheptulose, a single polyol that can point out an abnormality within the non-oxidative portion of PPP, Shayota et al. successfully demonstrated high-specificity multiple polyol alterations in plasma and urine metabolomics for the diagnosis of these two enzyme deficiencies (see Table 1) [97]. Two novel biomarkers were shown for both disorders (erythronate and ribonate). Secondary alterations in purine and pyrimidine metabolites, as seen in this work, are most probably due to defect in ribulose-5-phosphate processing.

Table 1 TM versus UM findings in two disorders of the nonoxidative portion of the pentose phosphate pathway [97]

	Metabolic pathway	Targeted metabolomics		Untargeted metabolomics	
		Plasma	Urine	Plasma	Urine
TALDO deficiency	Polyol	Arabitol Erythritol Ribitol Xylitol Sedoheptulose	Arabitol Sedoheptulose	Arabitol/xylitol ^a Ribitol Erythritol Sedoheptulose <u>Erythronate</u> <u>Ribonate</u>	Arabitol/xylitol ^a Ribitol <u>Erythronate</u> <u>Ribonate</u>
	Tryptophan	–	–	Kynurenate Xanthurenate	Quinolinate Xanthurenate
	Purine	–	–		Xanthosine
	Pyrimidine				N-carbamoylaspartate
	TCA	–	–	α-Ketoglutarate	Succinate Fumarate Malate
TKT deficiency	Polyol	Arabitol Erythritol Ribitol Xylitol Sedoheptulose	None	Arabitol/xylitol Ribitol Erythritol Ribose <u>Erythronate</u> <u>Ribonate</u>	Arabitol/xylitol Ribitol Erythritol <u>Erythronate</u> <u>Ribonate</u>
	Tryptophan	–	–	Kynurenine Xanthurenate Quinolinate Indolelactate	Kynurenine 3-hydroxykynurenine
	Purine	–	–	Inosine	Xanthosine Guanosine
	Pyrimidine				N-carbamoylaspartate

Underline—a newly discovered disease biomarker

^aArabitol could not be easily distinguished from xylitol or arabinonate from xylonate by this UM platform, given the similar structure and molecular weight

3.7 *Metabolomics of Muscular Diseases*

The muscular disease has a broad differential diagnosis and multiple pathological mechanisms. An important etiological group is the mitochondrial myopathies, which can have early- or late-onset and acute or subacute course and are progressive in nature [14]. Buzkova et al. compared the metabolomic profiles of mitochondrial myopathies and ataxias (lumping the sporadic inflammatory disorder inclusion body myositis, which also affects mitochondrial functioning) in comparison to non-mitochondrial neuromuscular diseases [98]. This study utilized TM of 94 metabolites but is included herein, given its broad application and interest in disease fingerprinting. The first group demonstrated alterations in the transsulfuration pathway, including elevated cystathionine (1.9–4.1-fold increase) and a less consistent reduction in taurine. In contrast, the second group was characterized by normal levels of cystathionine, depletion of nicotinamide (−1.7-fold change), and increased creatine (2.1-fold change). Alterations were also found in carbohydrate metabolism leading the authors to propose a quad-biomarker set of elevations in sorbitol, alanine, myoinositol, and cystathionine, producing an area under the curve similar to fibroblast growth factor-21 (FGF-21), lactate, and pyruvate, to distinguish the mitochondrial origin of myopathy (with an overall 76% sensitivity, 95% specificity). A particular mitochondrial myopathy, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS, MIM 540000) showed elevations in carbohydrate derivatives (sorbitol, glucuronate, myoinositol, and sucrose), decreased arginine, and an increase in transsulfuration intermediates (cystathionine, γ -glutamyl-cysteine S-adenosylmethionine, and glutamate) with a decrease in adenosine, guanidinoacetate, and betaine. In another UM study of MELAS patients, Sharma et al. demonstrated novel amino acid, acylcarnitine, and fatty acid biomarkers [99]: N-lactoyl attached to the branched-chain amino acids leucine, isoleucine, or valine or to the aromatic amino acids phenylalanine and tyrosine; β -hydroxy acylcarnitines of even-length C10:0 to C16:0 (C10:0, C12:0, C14:0, C16:0); and β -hydroxy fatty acids of even-length C8:0 to C14:0. These biomarkers were associated with the degree of diseased severity (per Karnofsky performance score). Interestingly, β -OH-C16:0 carnitine showed a severity correlation similar to the well-accepted growth/differentiation factor-15 (GDF-15) [100], while β -hydroxy acylcarnitines and β -hydroxy fatty acids correlated with ventricular lactate levels, and the N-lactoyl-amino acids correlated with urine heteroplasmy. Table 2 summarizes selected metabolic pathways in which more than 10% of metabolites are significantly altered in the individual disorders (two-sample *t*-test for significance).

Table 2 Plasma metabolic pathways in which $\geq 10\%$ of metabolites are altered compared to control in selected mitochondrial myopathies and ataxias, compared to non-mitochondrial myopathies [98]

Group	Family	Disorder	OMIM	Gene	Altered metabolic pathways
Mitochondrial	mtDNA depletion	IOSCA	271245	<i>TWNK</i>	Primary bile acids; nitrogen; alanine, aspartate, and glutamine; cysteine and methionine; histidine; butanoate; D-glutamine and D-glutaminate; cyanoamino acids; glutathione
		MIRAS	607459	<i>POLG</i>	Cysteine and methionine; alanine aspartate and glutamine; taurine and hypotaurine; histidine; β -alanine; nitrogen; aminoacyl-tRNA; cyanoamino acids; pantothenate and CoA
	Single mtDNA deletion	PEO	530000 258450 157640 609286	Sporadic mtDNA deletion <i>TWNK</i> <i>POLG</i>	Plasma: Alanine, aspartate, and glutamine; cysteine and methionine; nitrogen; glycine serine and threonine; D-glutamine and D-glutaminate; arginine and proline; aminoacyl-tRNA
		Mitochondrial tRNA defects	MELAS ^a	540000	m.3243A>G
Non-mitochondrial	Immune dysregulation	Inclusion body myositis	147421	Sporadic	D-arginine and D-ornithine; taurine and hypotaurine; cysteine and methionine; alanine, aspartate, and glutamine; arginine and proline; aminoacyl-tRNA; glutathione
	Neuromuscular diseases			AD or AR <i>DMPK</i> <i>ZNF9</i> <i>CAPN3</i> <i>PABPN1</i> <i>SMN1</i> <i>TIA1</i> Or unknown	Nitrogen; glycine serine and threonine; cysteine and methionine; pyrimidine; arginine and proline; glutathione; primary bile acid; histidine
					Arginine and proline; folate (one-carbon pool); glutathione; glycine, serine, and threonine; histidine; butanoate; β -alanine

Pathways are presented in decreasing order of alteration

mtDNA mitochondrial DNA, IOSCA infantile-onset spinocerebellar ataxia, MIRAS mitochondrial recessive ataxia syndrome, MELAS mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes

^aSharma et al.

4 Future Perspectives

We shall end our discussion of bedside UM with a futuristic perspective of UM in the service of autism spectrum disorder (ASD). The autism spectrum is a range of neurodevelopmental conditions exhibiting persistent deficits in reciprocal social interaction and restricted, repetitive patterns of behavior, interests, or activities. Historically, ASD gained an independent psychiatric status from schizophrenia only in the second half of the twentieth century (the word autism was initially coined to describe severe schizophrenia) [101] and became a spectrum only at the end of that century. We now appreciate a 60–80% heritability of ASD [102, 103], with an estimated genetic or genomic etiology in 30–40% of cases [104]. Within the group of ASD, due to a known genetic variant, an estimated 3–5% are due to an IEM [105]. Examples include [106] amino acidopathies (PKU, homocystinuria, S-adenosylhomocysteine hydrolase deficiency, MSUD, UCD), organic acidurias (PA, L-2-hydroxyglutaric aciduria), cholesterol biosynthesis defects (Smith-Lemli-Opitz syndrome), disorders of neurotransmitter synthesis or degradation (SSA deficiency; SSADH deficiency), disorders of purine metabolism (ADSLD, Lesch-Nyhan syndrome), cerebral creatine deficiency syndromes (GAMT deficiency, creatine transporter defect), disorders of folate transport and metabolism (cerebral folate deficiency, MTHFR deficiency), lysosomal storage disorders (mucopolysaccharidosis type III, neuronal ceroid lipofuscinoses (NCL), Niemann-Pick disease type C), CTX, MELAS, Wilson disease, and several types of neurodegeneration with brain iron accumulation, among others. Moreover, multiple lines of evidence have suggested biochemical alterations in children with ASD compared to peers. Alterations can derive from the large intestine microbiome, showing differential fecal content for isopropanol, p-cresol, and short-chain fatty acids. Mitochondrial dysfunction can result in complex I, IV, or V deficiencies in children with ASD (up to 7%). Alterations were also demonstrated in some cases of glutathione reduction in the CNS [107]. While attempts to find unifying markers of autism demonstrated interesting candidates, they failed to show consistency across multiple UM platforms and ASD cohorts. Glutaric acid, arginine, histidine (and its catabolites), taurine, β -alanine, and succinic acid were most consistently elevated, along with a reduction of creatine and creatinine [107]. In a cohort of 52 pregnant women whose children developed ASD, compared to 62 control pregnant women in an NMR-based UM, differences were found in glycosphingolipid metabolism, N-glycan and pyrimidine metabolism, bile acid pathways, and C21-steroid hormone biosynthesis but with only a mild perturbation in each metabolite [108]. The latter two candidates are of interest due to the involvement of cholesterol metabolism, as ASD is highly prevalent among patients with Smith-Lemli-Opitz syndrome, a disorder of cholesterol synthesis. Abnormal bile acid synthesis can cause perturbations in taurine, and pyrimidine synthesis defects can alter β -alanine. However, a lesson learned from these works is that ASD may represent shared neurodevelopmental outcomes of quite different neurometabolic processes, complicating the search for biomarkers, as the case separation from control may be shadowed by intragroup variability

[109]. Instead, a machine learning approach to recognize a pattern of altered seemingly unrelated metabolites in a study group could allow the discovery of heterogeneous biomarkers, indicating a high-risk status for ASD, Alzheimer's disease, Parkinson's disease, or other conditions, and, once defined, may also be then targeted for treatment and/or monitoring. Such a learning process was performed on UM data from a cohort of 500 children with ASD (against 200 controls) [110]. The group was halved into study and discovery groups. The study group yielded 34 groups sharing metabolic phenotypes with a specificity of $\geq 95\%$. Those groups were then clustered into six metabolic groups, each with an abnormal ratio of either α -ketoglutarate, 4-hydroxyproline, glycine, lactate/pyruvate, ornithine, or succinic acid compared to other metabolites. These clusters were then validated in the discovery group. When screening for the clusters together, this assay had 53% sensitivity and 91% specificity for indicating "high risk" for ASD. These outcomes demonstrate the potential for a higher level of complexity in data interpretation. Identification of such abnormalities by the physician ordering an "ASD risk stratification" test may be a difficult task. Still, computer-assisted data analysis could flag such abnormal results and promote a revolution in the field of developmental neuroscience.

While UM offers an opportunity for discovery both inside and outside clinical settings, its use in the clinical lab supports broad screening for inborn errors of metabolism well beyond the newborn screen, supports the development of metabolic profiles for a disease that may be monitored during treatment, and, when integrated with genomics, provides a precision medicine approach to the diagnosis of rare disease.

Glossary

Dried blood spot (DBS) A method of whole blood sample collection in which a small amount of fresh blood is blotted onto an absorbent filter paper, followed by drying. This method provides a convenient storage and shipment platform and is widely used for newborn screening. Typically, a small punch from the DBS paper is eluted with phosphate-buffered saline, availing the sample for testing.

Elevation/reduction (of a metabolite) In the context of this chapter, a metabolite is considered *reduced* (insufficient) or *elevated* (in excess) when UM reveals a Z-score $\geq +2$ or ≤ -2 . The Z-score is the number of standard deviations that a data point differs from the population means, representing the relative level of a given metabolite. Raw values for individual metabolites are \log_2 -transformed, and the relative Z-score is calculated compared to a lab-specific reference population [91, 111–113].

Inborn error of metabolism (IEM) A heterogeneous group of mostly inherited disorders involving a failure of the certain metabolic pathway(s) to break down or store biomolecules (typically carbohydrates, lipids, or amino acids) in the cell.

Although any given inborn error of metabolism is rare, taken as a group, inborn errors of metabolism occur in 1 in 2000 births [114].

Molecular confirmation A suspected diagnosis, as suggested by biochemical testing such as UM, is said to be molecularly confirmed when genomic sequencing reveals pathogenic variants, either monoallelic for autosomal dominant or X-linked disorders or biallelic for recessive disorders in the gene associated with the metabolic abnormality. Sequencing can be targeted for the specific gene(s) or untargeted as exome or genome sequencing. In the latter case, further confirmation by Sanger sequencing may be performed to validate the variants identified.

Reference population A lab-specific reference population created by performing UM on samples received in the clinical laboratory, with careful inclusion and exclusion of clinical samples to ensure pathways and analytes are covered for comprehensive clinical assessment. Raw data for each metabolite are median scaled, \log_2 transformed, extreme outliers removed, and Z-scores generated based on the mean and standard deviation in this reference population [91, 111–113].

Traditional screening methods Screening tools for certain common abnormalities indicative of diseases. While there is no definition per se for traditional screening methods, they usually include organic acids measured in urine (urine organic acids, UOA); measurements of standard amino acids in plasma (PAA); and carnitine conjugates of fatty acids (acylcarnitine profile, ACP). ACP and PAA are examples of TM where predefined biochemicals are quantitatively measured compared to known standards. UOA is a semiquantitative, untargeted analysis in which analytes are qualitatively compared against a few laboratory-specific internal standards. UOA and PAA are typically performed by liquid chromatography (LC) and/or gas chromatography (GC) coupled with mass spectrometry (MS). In contrast, ACP is performed by tandem MS/MS, where indicators are aimed at detecting carnitine daughter ions.

References

1. Zhang A, Sun H, Yan G, Wang P, Wang X. Metabolomics for biomarker discovery: moving to the clinic. *Biomed Res Int*. 2015;2015:354671.
2. Clish CB. Metabolomics: an emerging but powerful tool for precision medicine. *Cold Spring Harb Mol Case Stud*. 2015;1(1):a000588.
3. Ashrafian H, Sounderajah V, Glen R, Ebbels T, Blaise BJ, Kalra D, et al. Metabolomics: the stethoscope for the twenty-first century. *Med Princ Pract*. 2021;30(4):301–10.
4. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, et al. Metabolite profiles and the risk of developing diabetes. *Nat Med*. 2011;17(4):448–53.
5. Kalhan SC, Guo L, Edmison J, Dasarathy S, McCullough AJ, Hanson RW, et al. Plasma metabolomic profile in nonalcoholic fatty liver disease. *Metabolism*. 2011;60(3):404–13.
6. Mimmi MC, Finato N, Pizzolato G, Beltrami CA, Fogolari F, Corazza A, et al. Absolute quantification of choline-related biomarkers in breast cancer biopsies by liquid chromatography electrospray ionization mass spectrometry. *Anal Cell Pathol (Amst)*. 2013;36(3–4):71–83.
7. Glunde K, Jacobs MA, Bhujwalla ZM. Choline metabolism in cancer: implications for diagnosis and therapy. *Expert Rev Mol Diagn*. 2006;6(6):821–9.

8. Lionel AC, Costain G, Monfared N, Walker S, Reuter MS, Hosseini SM, et al. Improved diagnostic yield compared with targeted gene sequencing panels suggests a role for whole-genome sequencing as a first-tier genetic test. *Genet Med*. 2018;20(4):435–43.
9. Srivastava S, Love-Nichols JA, Dies KA, Ledbetter DH, Martin CL, Chung WK, et al. Meta-analysis and multidisciplinary consensus statement: exome sequencing is a first-tier clinical diagnostic test for individuals with neurodevelopmental disorders. *Genet Med*. 2019;21(11):2413–21.
10. Manickam K, McClain MR, Demmer LA, Biswas S, Kearney HM, Malinowski J, et al. Exome and genome sequencing for pediatric patients with congenital anomalies or intellectual disability: an evidence-based clinical guideline of the American College of Medical Genetics and Genomics (ACMG). *Genet Med*. 2021;23:2029.
11. Baxter SK, King MC. A time to sequence. *JAMA Pediatr*. 2017;171(12):e173435.
12. Theunissen TEJ, Nguyen M, Kamps R, Hendrickx AT, Sallevelt S, Gottschalk RWH, et al. Whole exome sequencing is the preferred strategy to identify the genetic defect in patients with a probable or possible mitochondrial cause. *Front Genet*. 2018;9:400.
13. Lehtonen JM, Auranen M, Darin N, Sofou K, Bindoff L, Hikmat O, et al. Diagnostic value of serum biomarkers FGF21 and GDF15 compared to muscle sample in mitochondrial disease. *J Inherit Metab Dis*. 2021;44(2):469–80.
14. Mancuso M, Klopstock T. *Diagnosis and management of mitochondrial disorders*. Berlin: Springer International; 2019.
15. Friedman JM, Jones KL, Carey JC. *Exome sequencing and clinical diagnosis*, vol. 324. JAMA; 2020. p. 627.
16. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17(5):405–24.
17. Cooper GM. Parlez-vous VUS? *Genome Res*. 2015;25(10):1423–6.
18. Bertier G, Héту M, Joly Y. Unsolved challenges of clinical whole-exome sequencing: a systematic literature review of end-users' views. *BMC Med Genet*. 2016;9(1):52.
19. Liu N, Xiao J, Gijvanekar C, Pappan KL, Glington KE, Shayota BJ, et al. Comparison of untargeted metabolomic profiling vs traditional metabolic screening to identify inborn errors of metabolism. *JAMA Netw Open*. 2021;4(7):e2114155.
20. Alaimo JT, Glington KE, Liu N, Xiao J, Yang Y, Reid Sutton V, et al. Integrated analysis of metabolomic profiling and exome data supplements sequence variant interpretation, classification, and diagnosis. *Genet Med*. 2020;22(9):1560–6.
21. Amendola LM, Jarvik GP, Leo MC, McLaughlin HM, Akkari Y, Amaral MD, et al. Performance of ACMG-AMP variant-interpretation guidelines among nine Laboratories in the Clinical Sequencing Exploratory Research Consortium. *Am J Hum Genet*. 2016;98(6):1067–76.
22. Trujillano D, Bertoli-Avella AM, Kumar Kandaswamy K, Weiss ME, Köster J, Marais A, et al. Clinical exome sequencing: results from 2819 samples reflecting 1000 families. *Eur J Hum Genet*. 2017;25(2):176–82.
23. Shashi V, McConkie-Rosell A, Schoch K, Kasturi V, Rehder C, Jiang YH, et al. Practical considerations in the clinical application of whole-exome sequencing. *Clin Genet*. 2016;89(2):173–81.
24. Park KJ, Park S, Lee E, Park JH, Park JH, Park HD, et al. A population-based genomic study of inherited metabolic diseases detected through newborn screening. *Ann Lab Med*. 2016;36(6):561–72.
25. Atwal PS, Donti TR, Cardon AL, Bacino CA, Sun Q, Emrick L, et al. Aromatic L-amino acid decarboxylase deficiency diagnosed by clinical metabolomic profiling of plasma. *Mol Genet Metab*. 2015;115(2–3):91–4.
26. Wassenberg T, Molero-Luis M, Jeltsch K, Hoffmann GF, Assmann B, Blau N, et al. Consensus guideline for the diagnosis and treatment of aromatic l-amino acid decarboxylase (AADC) deficiency. *Orphanet J Rare Dis*. 2017;12(1):12.

27. Saudubray JM, Baumgartner MR, Walter JH. Inborn metabolic diseases: diagnosis and treatment. Berlin, Heidelberg: Springer; 2016.
28. Pearson TS, Gilbert L, Opladen T, Garcia-Cazorla A, Mastrangelo M, Leuzzi V, et al. AADC deficiency from infancy to adulthood: symptoms and developmental outcome in an international cohort of 63 patients. *J Inherit Metab Dis.* 2020;43(5):1121–30.
29. Fusco C, Leuzzi V, Striano P, Battini R, Burlina A, Spagnoli C. Aromatic L-amino acid decarboxylase (AADC) deficiency: results from an Italian modified Delphi consensus. *Ital J Pediatr.* 2021;47(1):13.
30. Luc QN, Querubin J. Clinical management of dystonia in childhood. *Paediatr Drugs.* 2017;19(5):447–61.
31. Kim R, Jeon B, Lee WW. A systematic review of treatment outcome in patients with Dopa-responsive dystonia (DRD) and DRD-plus. *Mov Disord Clin Pract.* 2016;3(5):435–42.
32. Pappan KL, Kennedy AD, Magoulas PL, Hanchard NA, Sun Q, Elsea SH. Clinical metabolomics to segregate aromatic amino acid decarboxylase deficiency from drug-induced metabolite elevations. *Pediatr Neurol.* 2017;75:66–72.
33. Almontashiri NAM, Zha L, Young K, Law T, Kellogg MD, Bodamer OA, et al. Clinical validation of targeted and untargeted metabolomics testing for genetic disorders: a 3 year comparative study. *Sci Rep.* 2020;10(1):9382.
34. Rodan LH, Anyane-Yeboah K, Chong K, Klein Wassink-Ruiter JS, Wilson A, Smith L, et al. Gain-of-function variants in the ODC1 gene cause a syndromic neurodevelopmental disorder associated with macrocephaly, alopecia, dysmorphic features, and neuroimaging abnormalities. *Am J Med Genet A.* 2018;176(12):2554–60.
35. Bupp CP, Schultz CR, Uhl KL, Rajasekaran S, Bachmann AS. Novel de novo pathogenic variant in the ODC1 gene in a girl with developmental delay, alopecia, and dysmorphic features. *Am J Med Genet A.* 2018;176(12):2548–53.
36. Marbaix AY, Noël G, Detroux AM, Vertommen D, Van Schaftingen E, Linster CL. Extremely conserved ATP- or ADP-dependent enzymatic system for nicotinamide nucleotide repair. *J Biol Chem.* 2011;286(48):41246–52.
37. Marbaix AY, Tyteca D, Niehaus TD, Hanson AD, Linster CL, Van Schaftingen E. Occurrence and subcellular distribution of the NADPHX repair system in mammals. *Biochem J.* 2014;460(1):49–58.
38. Van Bergen NJ, Guo Y, Rankin J, Paczia N, Becker-Kettern J, Kremer LS, et al. NAD(P)HX dehydratase (NAXD) deficiency: a novel neurodegenerative disorder exacerbated by febrile illnesses. *Brain.* 2019;142(1):50–8.
39. Kremer LS, Danhauser K, Herebian D, Petkovic Ramadža D, Piekutowska-Abramczuk D, Seibt A, et al. NAXE mutations disrupt the cellular NAD(P)HX repair system and cause a lethal Neurometabolic disorder of early childhood. *Am J Hum Genet.* 2016;99(4):894–902.
40. Van Bergen NJ, Walvekar AS, Patraskaki M, Sikora T, Linster CL, Christodoulou J. Clinical and biochemical distinctions for a metabolite repair disorder caused by NAXD or NAXE deficiency. *J Inherit Metab Dis.* 2022;45(6):1028–38.
41. Manor J, Calame DG, Gijavanekar C, Tran A, Fatih JM, Lalani SR, et al. Niacin therapy improves outcome and normalizes metabolic abnormalities in an NAXD-deficient patient. *Brain.* 2022;145(5):e36–40.
42. Pillai NR, Amin H, Gijavanekar C, Liu N, Issaq N, Broniowska KA, et al. Hematologic presentation and the role of untargeted metabolomics analysis in monitoring treatment for riboflavin transporter deficiency. *Am J Med Genet A.* 2020;182(11):2781–7.
43. Amir F, Atzinger C, Massey K, Greinwald J, Hunter LL, Ulm E, et al. The clinical journey of patients with riboflavin transporter deficiency type 2. *J Child Neurol.* 2020;35(4):283–90.
44. Liu Z, Peng Q, Li J, Rao C, Lu X. BVVLS2 overlooked for 3 years in a pediatric patient caused by novel compound heterozygous mutations in *SLC52A2* gene. *Clin Chim Acta.* 2021;523:402–6.
45. Kalafatic Z, Lipovac K, Jezerinac Z, Juretic D, Dumic M, Zurga B, et al. A liver urocanase deficiency. *Metabolism.* 1980;29(11):1013–9.

46. Espinós C, Pineda M, Martínez-Rubio D, Lupo V, Ormazabal A, Vilaseca MA, et al. Mutations in the urocanase gene UROC1 are associated with urocanic aciduria. *J Med Genet.* 2009;46(6):407–11.
47. Camp BW, Broman SH, Nichols PL, Leff M. Maternal and neonatal risk factors for mental retardation: defining the ‘at-risk’ child. *Early Hum Dev.* 1998;50(2):159–73.
48. Boyle CA, Yeargin-Allsopp M, Doernberg NS, Holmgreen P, Murphy CC, Schendel DE. Prevalence of selected developmental disabilities in children 3-10 years of age: the metropolitan Atlanta developmental disabilities surveillance program, 1991. *MMWR CDC Surveill Summ.* 1996;45(2):1–14.
49. Keyfi F, Nasser M, Nayerabadi S, Alaei A, Mokhtariye A, Varasteh A. Frequency of inborn errors of metabolism in a northeastern Iranian sample with high consanguinity rates. *Hum Hered.* 2018;83(2):71–8.
50. Afzal RM, Lund AM, Skovby F. The impact of consanguinity on the frequency of inborn errors of metabolism. *Mol Genet Metab Rep.* 2018;15:6–10.
51. Saad HA, Elbedour S, Hallaq E, Merrick J, Tenenbaum A. Consanguineous marriage and intellectual and developmental disabilities among Arab Bedouins children of the Negev region in southern Israel: a pilot study. *Front Public Health.* 2014;2:3.
52. Jamra R. Genetics of autosomal recessive intellectual disability. *Med Genet.* 2018;30(3):323–7.
53. Glinton KE, Levy HL, Kennedy AD, Pappan KL, Elsea SH. Untargeted metabolomics identifies unique though benign biochemical changes in patients with pathogenic variants in UROC1. *Mol Genet Metab Rep.* 2019;18:14–8.
54. Kennedy AD, Pappan KL, Donti T, Delgado MR, Shinawi M, Pearson TS, et al. 2-Pyrrolidinone and Succinimide as clinical screening biomarkers for GABA-transaminase deficiency: anti-seizure medications impact accurate diagnosis. *Front Neurosci.* 2019;13:394.
55. Ferreira EA, Veenliet ARJ, Engelke UFH, Kluijtmans LAJ, Huigen M, Hoegen B, et al. Diagnosing, discarding, or de-VUSSing: a practical guide to (un)targeted metabolomics as variant-transcending functional tests. *Genet Med.* 2023;25(1):125–34.
56. Subramanian VS, Constantinescu AR, Benke PJ, Said HM. Mutations in SLC5A6 associated with brain, immune, bone, and intestinal dysfunction in a young child. *Hum Genet.* 2017;136(2):253–61.
57. Byrne AB, Arts P, Polyak SW, Feng J, Schreiber AW, Kassahn KS, et al. Identification and targeted management of a neurodegenerative disorder caused by biallelic mutations in SLC5A6. *NPJ Genom Med.* 2019;4:28.
58. Holling T, Nampoothiri S, Tarhan B, Schneeberger PE, Vinayan KP, Yesodharan D, et al. Novel biallelic variants expand the SLC5A6-related phenotypic spectrum. *Eur J Hum Genet.* 2022;1-11:439.
59. Thistlethwaite LR, Petrosyan V, Li X, Miller MJ, Elsea SH, Milosavljevic A. CTD: an information-theoretic algorithm to interpret sets of metabolomic and transcriptomic perturbations in the context of graphical models. *PLoS Comput Biol.* 2021;17(1):e1008550.
60. Thistlethwaite LR, Li X, Burrage LC, Riehle K, Hacia JG, Braverman N, et al. Clinical diagnosis of metabolic disorders using untargeted metabolomic profiling and disease-specific networks learned from profiling data. *Sci Rep.* 2022;12(1):6556.
61. Kerkhofs M, Haijes HA, Willemsen AM, van Gassen KLI, van der Ham M, Gerrits J, et al. Cross-omics: integrating genomics with metabolomics in clinical diagnostics. *Metabolites.* 2020;10(5):206.
62. Graham Linck EJ, Richmond PA, Tarailo-Graovac M, Engelke U, Kluijtmans LAJ, Coene KLM, et al. metPropagate: network-guided propagation of metabolomic information for prioritization of metabolic disease genes. *NPJ Genom Med.* 2020;5:25.
63. Smedley D, Jacobsen JO, Jäger M, Köhler S, Holtgrewe M, Schubach M, et al. Next-generation diagnostics and disease-gene discovery with the exomiser. *Nat Protoc.* 2015;10(12):2004–15.
64. Bongaerts M, Bonte R, Demirdas S, Huidekoper HH, Langendonk J, Wilke M, et al. Integration of metabolomics with genomics: metabolic gene prioritization using metabolomics data and genomic variant (CADD) scores. *Mol Genet Metab.* 2022;136(3):199–218.

65. Rentzsch P, Schubach M, Shendure J, Kircher M. CADD-splice-improving genome-wide variant effect prediction using deep learning-derived splice scores. *Genome Med.* 2021;13(1):31.
66. Messa GM, Napolitano F, Elsea SH, di Bernardo D, Gao X. A Siamese neural network model for the prioritization of metabolic disorders by integrating real and simulated data. *Bioinformatics.* 2020;36(Suppl_2):i787–i94.
67. Garg U, Smith LD. Biomarkers in inborn errors of metabolism: clinical aspects and laboratory determination. Amsterdam: Elsevier Science; 2017.
68. Braverman NE, Raymond GV, Rizzo WB, Moser AB, Wilkinson ME, Stone EM, et al. Peroxisome biogenesis disorders in the Zellweger spectrum: an overview of current diagnosis, clinical manifestations, and treatment guidelines. *Mol Genet Metab.* 2016;117(3):313–21.
69. Steinberg SJ, Raymond GV, Braverman NE, Moser AB. Zellweger spectrum disorder. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Gripp KW, et al., editors. *GeneReviews*(®). Seattle, WA: University of Washington; 1993.
70. Kim PK, Hettema EH. Multiple pathways for protein transport to peroxisomes. *J Mol Biol.* 2015;427(6 Pt A):1176–90.
71. Wangler MF, Hubert L, Donti TR, Ventura MJ, Miller MJ, Braverman N, et al. A metabolomic map of Zellweger spectrum disorders reveals novel disease biomarkers. *Genet Med.* 2018;20(10):1274–83.
72. Ah Mew N, Simpson KL, Gropman AL, Lanpher BC, Chapman KA, Summar ML. Urea cycle disorders overview. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Gripp KW, et al., editors. *GeneReviews*(®). Seattle, WA: University of Washington; 1993.
73. Stone WL, Basit H, Jaishankar GB. Urea cycle disorders. In: *StatPearls.* Treasure Island, FL: StatPearls; 2022.
74. Sen K, Whitehead M, Castillo Pinto C, Caldovic L, Gropman A. Fifteen years of urea cycle disorders brain research: looking back, looking forward. *Anal Biochem.* 2022;636:114343.
75. Amayreh W, Meyer U, Das AM. Treatment of arginase deficiency revisited: guanidinoacetate as a therapeutic target and biomarker for therapeutic monitoring. *Dev Med Child Neurol.* 2014;56(10):1021–4.
76. Burrage LC, Thistlethwaite L, Stroup BM, Sun Q, Miller MJ, Nagamani SCS, et al. Untargeted metabolomic profiling reveals multiple pathway perturbations and new clinical biomarkers in urea cycle disorders. *Genet Med.* 2019;21(9):1977–86.
77. De Deyn PP, Marescau B, Macdonald RL. Guanidino compounds that are increased in hyperargininemia inhibit GABA and glycine responses on mouse neurons in cell culture. *Epilepsy Res.* 1991;8(2):134–41.
78. Hanna-El-Daher L, Béard E, Henry H, Tenenbaum L, Braissant O. Mild guanidinoacetate increase under partial guanidinoacetate methyltransferase deficiency strongly affects brain cell development. *Neurobiol Dis.* 2015;79:14–27.
79. Ostojic SM. Safety of dietary Guanidinoacetic acid: a villain of a good guy? *Nutrients.* 2021;14(1):75.
80. Ingoglia F, Chong JL, Pasquali M, Longo N. Creatine metabolism in patients with urea cycle disorders. *Mol Genet Metab Rep.* 2021;29:100791.
81. Grace RF, Bianchi P, van Beers EJ, Eber SW, Glader B, Yaish HM, et al. Clinical spectrum of pyruvate kinase deficiency: data from the pyruvate kinase deficiency natural history study. *Blood.* 2018;131(20):2183–92.
82. Bianchi P, Fermo E, Glader B, Kanno H, Agarwal A, Barcellini W, et al. Addressing the diagnostic gaps in pyruvate kinase deficiency: consensus recommendations on the diagnosis of pyruvate kinase deficiency. *Am J Hematol.* 2019;94(1):149–61.
83. Van Dooijeweert B, Broeks MH, Verhoeven-Duif NM, Van Beers EJ, Nieuwenhuis EES, Van Solinge WW, et al. Untargeted metabolic profiling in dried blood spots identifies disease fingerprint for pyruvate kinase deficiency. *Haematologica.* 2021;106(10):2720–5.
84. Wang D, Pascual JM, De Vivo D. Glucose transporter type 1 deficiency syndrome. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Gripp KW, et al., editors. *GeneReviews*(®). Seattle, WA: University of Washington; 1993.

85. Tang M, Monani UR. Glut1 deficiency syndrome: new and emerging insights into a prototypical brain energy failure disorder. *Neurosci Insights*. 2021;16:26331055211011507.
86. Wibisono C, Rowe N, Beavis E, Kepreotes H, Mackie FE, Lawson JA, et al. Ten-year single-center experience of the ketogenic diet: factors influencing efficacy, tolerability, and compliance. *J Pediatr*. 2015;166(4):1030–6.e1.
87. Klepper J, Akman C, Armeno M, Auvin S, Cervenka M, Cross HJ, et al. Glut1 deficiency syndrome (Glut1DS): state of the art in 2020 and recommendations of the international Glut1DS study group. *Epilepsia Open*. 2020;5(3):354–65.
88. Almuqbil M, Go C, Nagy LL, Pai N, Mamak E, Mercimek-Mahmutoglu S. New paradigm for the treatment of glucose transporter 1 deficiency syndrome: low glycemic index diet and modified high amylopectin cornstarch. *Pediatr Neurol*. 2015;53(3):243–6.
89. Cappuccio G, Pinelli M, Alagia M, Donti T, Day-Salvatore DL, Veggiotti P, et al. Biochemical phenotyping unravels novel metabolic abnormalities and potential biomarkers associated with treatment of GLUT1 deficiency with ketogenic diet. *PLoS One*. 2017;12(9):e0184022.
90. Miller MJ, Bostwick BL, Kennedy AD, Donti TR, Sun Q, Sutton VR, Elsea SH. Chronic Oral L-Carnitine Supplementation Drives Marked Plasma TMAO Elevations in Patients with Organic Acidemias Despite Dietary Meat Restrictions. *JIMD Rep*. 2016;30:39–44. https://doi.org/10.1007/8904_2016_539. Epub 2016 Mar 3. PMID: 26936850; PMCID: PMC5110437.
91. Kennedy AD, Miller MJ, Beebe K, Wulff JE, Evans AM, Miller LA, et al. Metabolomic profiling of human urine as a screen for multiple inborn errors of metabolism. *Genet Test Mol Biomarkers*. 2016;20(9):485–95.
92. El-Hattab AW. Serine biosynthesis and transport defects. *Mol Genet Metab*. 2016;118(3):153–9.
93. Glington KE, Benke PJ, Lines MA, Geraghty MT, Chakraborty P, Al-Dirbashi OY, et al. Disturbed phospholipid metabolism in serine biosynthesis defects revealed by metabolomic profiling. *Mol Genet Metab*. 2018;123(3):309–16.
94. Ferreira CR, Goorden SMI, Soldatos A, Byers HM, Ghauharali-van der Vlugt JMM, Beers-Stet FS, et al. Deoxysphingolipid precursors indicate abnormal sphingolipid metabolism in individuals with primary and secondary disturbances of serine availability. *Mol Genet Metab*. 2018;124(3):204–9.
95. Litwack G. Chapter 7 - Glycogen and Glycogenolysis. In: Litwack G, editor. *Human biochemistry*. Boston: Academic Press; 2018. p. 161–81.
96. Banne E, Meiner V, Shaag A, Katz-Brull R, Gamliel A, Korman S, et al. Transaldolase deficiency: a new case expands the phenotypic Spectrum. *JIMD Rep*. 2016;26:31–6.
97. Shayota BJ, Donti TR, Xiao J, Gijavanekar C, Kennedy AD, Hubert L, et al. Untargeted metabolomics as an unbiased approach to the diagnosis of inborn errors of metabolism of the non-oxidative branch of the pentose phosphate pathway. *Mol Genet Metab*. 2020;131(1–2):147–54.
98. Buzkova J, Nikkanen J, Ahola S, Hakonen AH, Sevastianova K, Hovinen T, et al. Metabolomes of mitochondrial diseases and inclusion body myositis patients: treatment targets and biomarkers. *EMBO Mol Med*. 2018;10(12):e9091.
99. Sharma R, Reinstadler B, Engelstad K, Skinner OS, Stackowitz E, Haller RG, et al. Circulating markers of NADH-reductive stress correlate with mitochondrial disease severity. *J Clin Invest*. 2021;131(2):e136055.
100. Yatsuga S, Fujita Y, Ishii A, Fukumoto Y, Arahata H, Kakuma T, et al. Growth differentiation factor 15 as a useful biomarker for mitochondrial disorders. *Ann Neurol*. 2015;78(5):814–23.
101. Evans B. How autism became autism: the radical transformation of a central concept of child development in Britain. *Hist Human Sci*. 2013;26(3):3–31.
102. Sandin S, Lichtenstein P, Kuja-Halkola R, Hultman C, Larsson H, Reichenberg A. The heritability of autism Spectrum disorder. *JAMA*. 2017;318(12):1182–4.
103. Colvert E, Tick B, McEwen F, Stewart C, Curran SR, Woodhouse E, et al. Heritability of autism Spectrum disorder in a UK population-based twin sample. *JAMA Psychiatry*. 2015;72(5):415–23.

104. Schaefer GB, Mendelsohn NJ. Clinical genetics evaluation in identifying the etiology of autism spectrum disorders: 2013 guideline revisions. *Genet Med.* 2013;15(5):399–407.
105. Ghaziuddin M, Al-Owain M. Autism spectrum disorders and inborn errors of metabolism: an update. *Pediatr Neurol.* 2013;49(4):232–6.
106. Žigman T, Petković Ramadža D, Šimić G, Barić I. Inborn errors of metabolism associated with autism Spectrum disorders: approaches to intervention. *Front Neurosci.* 2021;15:673600.
107. Glington KE, Elsea SH. Untargeted metabolomics for autism spectrum disorders: current status and future directions. *Front Psych.* 2019;10:647.
108. Ritz B, Yan Q, Uppal K, Liew Z, Cui X, Ling C, et al. Untargeted metabolomics screen of mid-pregnancy maternal serum and autism in offspring. *Autism Res.* 2020;13(8):1258–69.
109. Courraud J, Ernst M, Svane Laursen S, Hougaard DM, Cohen AS. Studying autism using untargeted metabolomics in newborn screening samples. *J Mol Neurosci.* 2021;71(7):1378–93.
110. Smith AM, Natowicz MR, Braas D, Ludwig MA, Ney DM, Donley ELR, et al. A metabolomics approach to screening for autism risk in the Children’s autism metabolome project. *Autism Res.* 2020;13(8):1270–85.
111. Ford L, Kennedy AD, Goodman KD, Pappan KL, Evans AM, Miller LAD, et al. Precision of a clinical metabolomics profiling platform for use in the identification of inborn errors of metabolism. *J Appl Lab Med.* 2020;5(2):342–56.
112. Kennedy AD, Pappan KL, Donti TR, Evans AM, Wulff JE, Miller LAD, et al. Elucidation of the complex metabolic profile of cerebrospinal fluid using an untargeted biochemical profiling assay. *Mol Genet Metab.* 2017;121(2):83–90.
113. Kennedy AD, Wittmann BM, Evans AM, Miller LAD, Toal DR, Lonergan S, et al. Metabolomics in the clinic: a review of the shared and unique features of untargeted metabolomics for clinical research and clinical testing. *J Mass Spectrom.* 2018;53(11):1143–54.
114. Waters D, Adeloye D, Woolham D, Wastnedge E, Patel S, Rudan I. Global birth prevalence and mortality from inborn errors of metabolism: a systematic analysis of the evidence. *J Glob Health.* 2018;8(2):021102.