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BRIEF REPORT

Diagnosing, discarding, or de-VUSsing: A practical guide to (un)targeted metabolomics as variant-transcending functional tests

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ABSTRACT

Purpose: For patients with inherited metabolic disorders (IMDs), any diagnostic delay should be avoided because early initiation of personalized treatment could prevent irreversible health damage. To improve diagnostic interpretation of genetic data, gene function tests can be valuable assets. For IMDs, variant-transcending functional tests are readily available through (un)targeted metabolomics assays. To support the application of metabolomics for this purpose, we developed a gene-based guide to select functional tests to either confirm or exclude an IMD diagnosis.

Methods: Using information from a diagnostic IMD exome panel, Kyoto Encyclopedia of Genes and Genomes, and Inborn Errors of Metabolism Knowledgebase, we compiled a guide for metabolomics-based gene function tests. From our practical experience with this guide, we retrospectively selected illustrative cases for whom combined metabolomic/genomic testing improved diagnostic success and evaluated the effect hereof on clinical management.

Results: The guide contains 2047 metabolism-associated genes for which a validated or putative variant-transcending gene function test is available. We present 16 patients for whom metabolomic testing either confirmed or ruled out the presence of a second pathogenic variant, validated or ruled out pathogenicity of variants of uncertain significance, or identified a diagnosis initially missed by genetic analysis.

Conclusion: Metabolomics-based gene function tests provide additional value in the diagnostic trajectory of patients with suspected IMD by enhancing and accelerating diagnostic success.

Introduction

Diagnosing inherited metabolic disorders (IMDs) can be a major challenge because of the phenotypic heterogeneity and lack of experience in the maze of complex diagnostic tests. Diagnostic delay is highly undesirable because an increasing number of IMDs is treatable, and early treatment could potentially prevent permanent damage.[1] Although exome sequencing (ES)/genome sequencing has revolutionized the diagnostic trajectory of IMDs and other rare genetic disorders, many patients are still left without a genetic diagnosis. In these cases, the addition of an extra layer of diagnostic information through testing of gene function can lead the way toward a correct diagnosis. The added value of functional studies or gene function tests is also highlighted in the guidelines of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP).[2]

Richard J. Rodenburg and Karlien L.M. Coene contributed equally.

*Correspondence and requests for materials should be addressed to Richard J. Rodenburg, Department of Pediatrics, 830 Translational Metabolic Laboratory (TML), Radboud University Medical Center, P.O. box 9101, NL-6500HB Nijmegen, the Netherlands. *E-mail address:* Richard.Rodenburg@radboudumc.nl

A full list of authors and affiliations appears at the end of the paper.

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Functional tests are often considered in the context of testing the effect of a specific genetic variant of uncertain significance (VUS); however, it can be time consuming to customize assays for every new variant that is encountered in the genomic data. Gene function tests that cover any variant encountered in a specific gene, for which we would like to introduce the term "variant-transcending gene function test," are more efficient. Especially for IMDs, such tests are readily available in biochemical assays, also known under the term metabolomics, which covers both targeted and untargeted methods. Targeted metabolomics methods specifically analyze a single predefined class of metabolites, often reporting exact concentrations, whereas untargeted metabolomics methods provide an unbiased profile of all metabolites present, not reporting concentrations but relative deviations compared with control profiles.[3-5] The identification of aberrant metabolites can pinpoint a defective pathway or biochemical process, allowing for a targeted evaluation of associated genes in the genomic data. In addition, starting from the identification of a VUS in genes involved in metabolic pathways, metabolomic testing can give insight into putative deleterious functional effects of these variants. Recent studies have shown the feasibility of adding functional information from metabolomic profiling to improve the interpretation of exome data.[6,7] However, the implementation of this combined interpretation in clinical diagnostics could face practical challenges because specific knowledge on metabolomics assays is not always available to the geneticist who evaluates the exome data. To overcome this hurdle, we have developed a practical guide that features both validated and putative variant-transcending gene function tests for >2000 genes associated with human metabolism, which we present in this report. We also showcase the practical application of this guide through actual patients from our clinic, in whom the combination of metabolomics data with the genomic data improved diagnostic interpretation and allowed for optimizing clinical management of patients.

Materials and Methods

Compilation of gene function test guide

Human genes known to be implicated in metabolic disorders were based on an IMD exome panel (version DG 3.2.0) used for diagnostics at Radboudumc Nijmegen (https://www.radboudumc.nl/getmedia/2e0c24d6-c3ef-4d2a-a8e7-8eea13129b74/METABOLICDISORDERS_DG320.aspx). This gene panel was designed by laboratory specialists and clinicians (geneticists, metabolic physicians, neurologists) in our institute and is frequently updated on the basis of genes emerging in the literature. Human genes not yet associated with metabolic disorders, but implicated in metabolic pathways, were extracted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database,[8] for which an academic subscription was purchased in June 2017 for bulk download of database information. Subsequently, from all human genes present in the KEGG database, the genes in the category pathway-metabolism were extracted. From these 2 sources, a list of genes (possibly) amenable for gene function testing was built. For known IMD genes, one or more targeted metabolomics tests were noted as gene function test if available based on cross-linking with Inborn Errors of Metabolism Knowledgebase (IEMbase) (http://www.iembase.org/)[9] and expert knowledge of at least 3 independent laboratory specialists; if no suitable targeted metabolomics test was available, untargeted metabolomics (next-generation metabolic screening [NGMS]) was noted as a putative gene function test. In addition, the gene list was compared with the recent International Classification of Inherited Metabolic Disorders (ICIMD) database.[10] For metabolic genes not yet linked to a human disorder, untargeted metabolomics (NGMS) was listed as a possible gene function test as well. Through this approach, an Excel-based guide was compiled that connects genes to metabolomics-based variant-transcending gene function tests.

Patient samples and data collection

All clinicians and laboratory specialists involved in this work retrospectively collected illustrative patients from real-life clinical practice for whom the sole inclusion criterion was that the combination of metabolomic and genomic testing improved diagnostic interpretation and thereby optimized clinical management. No distinction in ethnicity, age or sex was made for the selection of cases. Written consent was obtained from all patients or their legal guardians for inclusion in this report. Clinical data were extracted from electronic health records.

Targeted metabolomics

Several targeted metabolomics methods were applied as variant-transcending functional tests, including analysis of amino acids,[11] acylcarnitines,[12] bile alcohols, organic acids, homocysteine, sterols, mucopolysaccharides, and purines/pyrimidines.[13] All targeted metabolomics methods were validated for use in clinical diagnostics under ISO15189 accreditation, for which details are available upon request. It goes beyond the scope of this brief report to describe all targeted methods in detail, but some have been previously published or were based on published methods as indicated.

Untargeted metabolomics

Untargeted metabolomics was performed following our previously published NGMS approach.[4] In brief, analyses were performed using a 1290 ultra-high–performance liquid chromatography system (Agilent) coupled to a 6545 QTOF mass spectrometer (Agilent) equipped with a dual electrospray ionization source. Each sample was run in duplicate in both positive and negative

ionization modes. A 2.0-μL aliquot of extracted plasma sample was injected onto an Acquity UHPLC HSS T3 (C18, 2.1 × 100 mm, 1.8 μm) column (Waters) operating at 40 °C. Chromatographic separations were performed by applying a binary mobile phase system. The buffer composition and mass spectrometry settings used were as previously reported by Coene et al.[4] For the analysis of the NGMS data, alignment, annotation, and statistical testing were performed in an integrated manner through an in-house bioinformatic pipeline.[14] Both the analytical and data analysis workflow for NGMS data were validated for clinical diagnostic use according to ISO15189 standards.

ES and variant selection and classification

ES was performed as described before.[15] In brief, exome enrichment was performed using the SureSelect Human All Exon 50 Mb Kit (Agilent). Sequencing was performed on a HiSeq 2000TM sequencer (Illumina). In general, the selection of possibly pathogenic variants was based on the following criteria: >5 variant reads; nonsynonymous coding and splice variants; a frequency of <0.5% in Single Nucleotide Polymorphism Database (v.137), Exome Aggregation Consortium, or Genome Aggregation Database data[16]; and an in-house sequence variant database containing >20,000 exomes. Further variant selection was based on patient-specific criteria, including phenotypic information. Variant classification was performed in line with the guidelines of the ACMG/AMP.[2]

Results

By combining the information from KEGG, IEMbase, and an expert-opinion diagnostic IMD exome panel, we were able to construct a guide that contains variant-transcending gene function tests, either targeted or untargeted metabolomics analysis, for 2047 individual genes (Supplemental Table 1). By using this gene function test guide in clinical practice, we have encountered several patients in whom gene function tests improved diagnostic interpretation of genomic data and thereby contributed to the clinical management of patients. Figure 1 summarizes the different ways in which gene function tests can add crucial information to the diagnostic process of the patients suspected of IMDs, eg, by validating or ruling out the effect and causality of VUS and also identifying a diagnosis missed by genomic testing.

Case series

In Table 1, we present 16 illustrative patients with suspected IMD to further exemplify the different ways in which functional tests can aid the diagnostic process. These cases are divergent in light of genetic and clinical phenotype. In 5 of 16 cases, metabolomic gene function tests excluded the existence of a second pathogenic variant for an autosomal recessive disorder. In 1 case, metabolomic tests pointed to the likely presence of a second pathogenic variant for an autosomal recessive disorder, which was not detected in genomic analyses. In 3 of 16 cases, metabolomics tests rejected a diagnosis suggested by genomic diagnostics, whereas in 4 of 16 cases, metabolomic tests confirmed a diagnosis suggested by ES. Interestingly, in 3 of 16 cases, addition of metabolomics-based functional tests identified an IMD diagnosis not reported in initial genomic analysis. For all patients, functional validation improved clinical management. This is exemplified by patient 7 who had anemia and nephrolithiasis of unknown cause for several years, despite undergoing diagnostic ES. A correct diagnosis of UMPS deficiency was identified through targeted

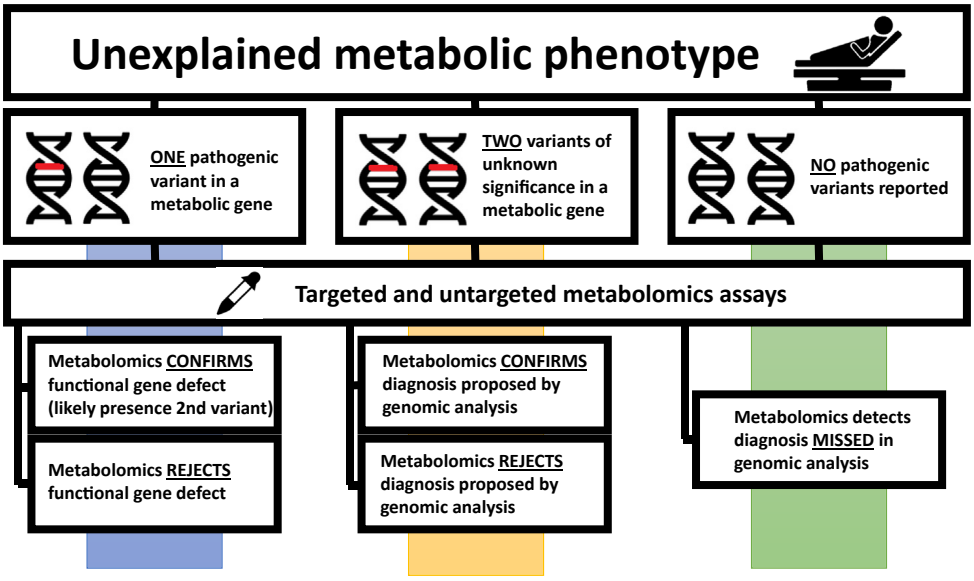

Figure 1 **Flowchart for the application of (un)targeted metabolomics as variant-transcending functional test to improve diagnostic interpretation of genomic variants in patients with suspected inherited metabolic disorder.**

Table 1 Case series

Case	Ethnicity and Age at Clinical Presentation	Clinical Symptoms	Gene	Genetic Variant(s)	Zygosity	Variant Class (Adjusted Class)	Metabolic Gene Function Test Results	Medical Impact
				Metabolomic gene function tests excluded the existence of a second pathogenic gene variant for an autosomal recessive disorder				
1	Dutch, 2 y	Growth delay, mild psychomotor delay, regression, encephalopathy, ataxia, progressive epilepsy	*HLCS*	Chr21(GRCh37): g.38308860del; NM_001352514.1:c.1326del; p.(Val443Serfs*20)	Heterozygous	5	Carnitine profile normal[a]; organic acids normal[b]	o Exclusion of HLCS deficiency (OMIM 253270) o Biotin supplementation trial was discontinued o Diagnostic trajectory diverted
2	North-African Dutch, 9 y	Developmental disorder, intellectual disability, urolithiasis, hyperlaxity, ocular problems, fatigue	*CBS*	Chr21(GRCh37):g.44482453C>T; NM_000071.3:c.1007G>A; p.(Arg336His)	Heterozygous	5	Homocysteine and amino acids (including methionine) normal[a]	o Exclusion of CBS deficiency/homocystinuria (OMIM 236200) o Diagnostic trajectory diverted
3	Dutch, 12 y	Convulsions, gray matter MRI abnormalities	*GLDC*	Chr9(GRCh37):g.6644626del; NM_000170.2:c.322del; p.(Glu108Lysfs*123)	Heterozygous	5	CSF/plasma glycine ratio normal[a]	o Exclusion of nonketotic hyperglycinemia (OMIM 605899) o Diagnostic trajectory diverted
4	Dutch, 1 y	Prematurity (27 wk), nephrocalcinosis and hypercalciuria	*SLC7A9*	Chr19(GRCh37):g.33355167C>T; NM_014270.4:c.313G>A; p.(Gly105Arg)	Heterozygous	5	Cystine↑[b], lysine↑[b], arginine↑ (slightly)[b], ornithine↑ (slightly)[b]	o Exclusion of cystinuria (OMIM 220100) o Confirmation of pathogenic SLC7A9 variant o Diagnostic trajectory diverted o Prevention of unnecessary PEG placement for cystinuria
5	Dutch, 1.5 y	Focal seizures with an altered level of consciousness, generalized seizures in the form of absences	*GPHN*	Chr14(GRCh37): g.67647581C>T; NM_020806.4:c.2237C>T; p.(Pro746Leu) (de novo)	Heterozygous	3	Taurine[a]; taurine[a, b], uric acid[b], xanthine[b], hypoxanthine[b] normal, no S-sulfocysteine[b]	o Exclusion of molybdenum cofactor C deficiency (OMIM 615501) o Diagnostic trajectory diverted

(continued)

Table 1 Continued

Case	Ethnicity and Age at Clinical Presentation	Clinical Symptoms	Gene	Genetic Variant(s)	Zygosity	Variant Class (Adjusted Class)	Metabolic Gene Function Test Results	Medical Impact
Metabolomic gene function tests indicated a second pathogenic gene variant for an autosomal recessive disorder								
6	Dutch, 3 mo	Neonatal screening positive for phenylketonuria	*PAH*	Chr12(GRCh37): g.103246714G>A; NM_000277.3: c.721C>T; p.(Arg241Cys)	Heterozygous	5	Phenylalanine↑[a], sapropterin dihydrochloride response test positive (Phenylalanine decrease of 55%)[a]	o Confirmation of phenylketonuria/ hyperphenylalaninemia (OMIM 261600) o Sapropterin supplementation initiated o Second pathogenic genetic variant very likely
Metabolomic gene function tests led to a diagnosis missed by exome sequencing								
7[19]	Dutch, 1 d	Multiple episodes of nephrolithiasis and urosepsis, transfusion-dependent anemia	*UMPS*	Chr3(GRCh37):g. 124456970A>G; NM_000373.:c.866A>G; p.(Asp289Gly) Chr3(GRCh37):g. 124458952A>G NM_000373.4: c.1064A>C; p.(Gln355Pro) (both variants not reported in the diagnostic result)	Heterozygous	2 (↑4) 3 (↑5)	Orotic acid↑[b]	o Confirmation of UMPS deficiency (OMIM 258900) o Variants were upgraded to 4/5 o Uridine monophosphate treatment initiated
8	Turkish Dutch, 1 d	Psychomotor retardation, adipositas, retinitis pigmentosa	*GSS*	No *GSS* variants detected in exome sequencing and targeted sequencing of *GSS* exons	–	–	5-Oxoproline↑[b]	o Confirmation of GSS deficiency (OMIM 266130) o Follow-up sequencing identified GSS homozygotic variant c.129+1663A>G p.(?) (class 5)[20]

(continued)

Table 1 Continued

Case	Ethnicity and Age at Clinical Presentation	Clinical Symptoms	Gene	Genetic Variant(s)	Zygosity	Variant Class (Adjusted Class)	Metabolic Gene Function Test Results	Medical Impact
Metabolomic gene function tests rejected a diagnosis proposed by exome sequencing								
9	Dutch, 1 d	Severe global developmental delay, feeding difficulties, hypothyroidy, vesicourethral reflux, hearing and visual impairment, sleeping disorder, short stature, abnormal MRI (focal lesions, myelination delay)	*GLDC*	Chr9(GChr37): g.6558558-?_6645693+?del; p.0?) Chr9(GRCh37): g.6556208A>T; NM_000170.2: c.2147T>A; p.(Leu716His)	Heterozygous	5 4 (↓2)	CSF/plasma glycine ratio normal[a]	o Exclusion of nonketotic hyperglycinemia (OMIM 605899) o Diagnostic trajectory diverted o Variant downgraded to class 2
10	Moluccan Dutch, 28 y	Global developmental delay	*DHCR7*	Chr11(GRCh37): g.71146886C>G; NM_001360.3: c.964-1G>C; (p.?) Chr11(GRCh37): g.71146696C>T; NM_001360.2: c.1153G>A; p.(Ala385Thr)	Heterozygous	4 3 (↓2)	8-Dehydrocholesterol, 7-dehydrocholesterol normal[a]	o Exclusion of Smith-Lemli-Opitz syndrome (OMIM 270400) o Diagnostic trajectory diverted o Variant downgraded to class 2
11	Turkish Dutch, 3 y	Global developmental delay, intellectual disability, seizures, hypotonia, exercise intolerance, torticollis, tingling paresthesia, strabismus, depression, anxiety	*ALDH4A1*	Chr1(GRCh37):g.19203910G>A; NM_170726.2: c.1137C>T; (p.=)	Homozygous	3 (↓2)	Proline normal[a]; no detectable pyrrole-2-carboxylic acid[b]	o Exclusion of hyperprolinemia (OMIM 239510) o Diagnostic trajectory o Variant downgraded to class 2 o Diagnostic trajectory diverted

(continued)

Table 1 Continued

Case	Ethnicity and Age at Clinical Presentation	Clinical Symptoms	Gene	Genetic Variant(s)	Zygosity	Variant Class (Adjusted Class)	Metabolic Gene Function Test Results	Medical Impact
				Metabolomic gene function tests confirmed a diagnosis proposed by exome sequencing				
12	Dutch, 18 y	Short stature, relatively late puberty with little growth spurt	*ARSB*	Chr5(GRCh37): g.78260300T>C; NM_000046.4: c.629A>G; p.(Tyr210Cys) Chr5(GRCh37): g.78280934G>T; NM_000046.4:c.138C>A; p.(His46Gln)	Heterozygous	5 3 (↑4)	Arylsulfatase B activity in leukocytes↓, dermatan sulfate↑[b]; Mucopolysaccharides↑[b]	o Confirmation of mucopolysaccharidosis type VI (Maroteaux-Lamy, OMIM 253200) o Clinical suspicion Noonan syndrome discarded o Variant upgraded to class 4
13	Turkish Dutch, 1 d	Convulsions after stem cell transplantation for MKL1 deficiency (OMIM 618847)	*MOCOS*	Chr18(GRCh37): g.33846769C>T; NM_017947.3: c.2467C>T; p.(Arg823*)	Homozygous	3 (↑4)	Xanthine↑[b], hypoxanthine↑[b], uric acid↓[b]	o Confirmation of xanthinuria type II (OMIM 603592) o Variant upgraded to class 4 o Initiation of purine-restricted diet
14	Turkish Dutch, 5 y	Developmental delay, behavioral problems, protein losing enteropathy	*ASL*	Chr7(GRCh37): g.65546907G>A; NM_000048.3: c.130G>A; p.(Ala44Thr)	Homozygous	3 (↑4)	Argininosuccinic acid↑[b]; argininosuccinic acid↑ (slight)[a], argininosuccinate lyase enzyme activity in erythrocytes↓[a]	o Confirmation of ASL deficiency (OMIM 207900) o Variant was upgraded to class 4 o Initiation of protein-restricted diet and prevention of long periods of fasting
15	Turkish Dutch, n.r.	Psychomotor developmental delay	*TMLHE*	Intragenic gain (arr[GRCh37] Xq28(154741706_154762853)x2)	Hemizygous	3 (↑4)	Trimethyllysine↑[a], gamma-butyrobetaine↓[a,b], gamma-butyrobetaine/trimethyllysine ratio↓[a]	o Confirmation of TMLHE deficiency (OMIM 300872) o Variant was upgraded to class 4 o Carnitine supplementation considered

(continued)

Table 1 Continued

Case	Ethnicity and Age at Clinical Presentation	Clinical Symptoms	Gene	Genetic Variant(s)	Zygosity	Variant Class (Adjusted Class)	Metabolic Gene Function Test Results	Medical Impact
16[21]	Dutch, 10 d	Poor sucking, respiratory distress, generalized convulsions and Reye-like syndrome, mild hepatomegaly, failure to thrive, hypertonia, developmental delay	*DPYD*	Chr1(GRCh37): g.98165074_98165082del; NM_000110.4: c.505_513del; p.(Pro169_Ile171del)	Homozygous	3 (↑4)	Uracil↑[a], thymine↑[a], 5-hydroxymethyluracil↑[a] DPD activity in leukocytes↓	o Confirmation of DPD deficiency (OMIM 274270) o Variant was upgraded to class 4 o Initiation of appropriate anticonvulsant therapy

↑, increased levels; ↓, decreased levels; *CSF*, cerebrospinal fluid; *MRI*, magnetic resonance imaging; *n.r.*, not reported; *PEG*, percutaneous endoscopic gastrostomy.
[a]Plasma.
[b]Urine.

metabolomics, which enabled personalized therapy through supplementation with uridine monophosphate, resolving his disabling symptoms.

Discussion

In this brief report, we aim to showcase the added value of (un) targeted metabolomics as functional test in parallel to genomic analysis to either confirm or discard an IMD diagnosis, illustrated by 16 anecdotical cases encountered in clinical practice. A cross-omics diagnostic approach facilitates the process of de-VUSsing: changing from VUSs to functional evidence–supported known significance. We present a flowchart for the application of metabolomics alongside genomics in the diagnostic process for patients with suspected IMD and provide the diagnostic community with a practical guide to facilitate the selection of available metabolomic variant-transcending gene function tests for genes implicated in human metabolism. By sharing this guide, we hope to reduce the gap between genomic diagnostics and (un)targeted metabolomics analyses as variant-transcending functional tests and improve and accelerate the diagnostic yield for patients. The 16 cases we highlighted show that apart from supporting the pathogenicity of variants detected in sequencing data, excluding pathogenicity or even detecting an otherwise missed diagnosis is also possible through functional testing. In addition, upon the detection of a single pathogenic variant in a recessive disease gene, gene function tests can either prove or exclude the existence of a second genetic variant that may have been missed in the genetic evaluation, thereby avoiding unnecessary diagnostic delay and additional testing.

Our guide contains both known IMD-related genes and metabolically implicated genes not yet linked to human disease with variants that could be encountered in open exome or genome analysis. We realize that for genes not yet related to human disease, untargeted metabolomics has yet to prove itself as a suitable functional test. In these cases, clinical validation of untargeted metabolomics can only be performed if patients with VUS in these genes are indeed referred for metabolomics testing. Based on the untargeted nature of the assay, we hypothesize that genetic disruptions in any metabolic process could be detected if they result in significantly altered metabolites in patient's body fluids. The first example of a new IMD, NANS-congenital disorder of glycosylation, discovered through such a combined untargeted genomic and metabolomic diagnostic strategy has already been described.[17] We hope that through the guide we present in this article, more patients with VUS in putative IMD genes will be offered variant-transcending gene function testing through untargeted metabolomics methods. The identification of these cases and linking them to metabolomic testing will provide the necessary validation in light of suitability of untargeted metabolomics as a functional test for a specific gene. Based on these validation cases, the gene function test guide can be further fine-tuned. In addition, we envision that upon increased use and

knowledge of gene function tests, a scoring matrix regarding their diagnostic value should also be constructed, in parallel to the diagnostic classification system for genetic variants.

We realize that the gene function test guide we present in this article is static and contains information from KEGG, ICIMD, and IEMbase as was available at the specific time of compilation. As our knowledge on human metabolism and genetic regulation thereof steadily increases, we aim to establish a real-time digital connection between KEGG, ICIMD, IEMbase, and even other databases such as Reactome[18] with up-to-date information. However, the format of a gene function test guide as we now present in a simple Excel file offers the possibility for any genetic laboratory to directly start using this source of information and find the matching gene function test for their potential disease gene of interest. This guide could likely be easily coupled to genomic interpretation tools/diagnostic interfaces used by genetic laboratories. We believe that the time is right to share this knowledge as more and more metabolic laboratories are now offering untargeted metabolomics techniques apart from targeted metabolomics assays in their diagnostic toolbox. We hope our work will stimulate the efficient application of these novel methods as variant-transcending gene function tests, thereby improving diagnostic success for patients with IMD.

Data Availability

De-identified data are available upon request by contacting the corresponding author.

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Author Information

Conceptualization: C.D.M.v.K., R.J.R., K.L.M.C.; Investigation: E.A.F., A.R.J.V., U.F.H.E., L.A.J.K., M.C.D.G.H., B.H., L.d.B., M.C.d.V., B.W.v.B., E.L., E.A.M.C., C.A.H., J.H.S., M.E.R.-G., I.M.L.W.K.-K., L.M.M., S.D., J.M., A.J.M.R.; Writing-original draft: E.A.F., K.L.M.C.; Writing-review and editing: E.A.F., C.D.M.v.K., R.J.R., K.L.M.C.

Ethics Declaration

Written consent was obtained from all patients or their legal guardians for inclusion in this report. Formal research ethics approval at each of the institutions was not required because the cases initially were evaluated independently, and then, for the purpose of this study, de-identified data were retrospectively compiled.

Conflict of Interest

The authors declare no conflicts of interest.

Additional Information

The online version of this article (https://doi.org/10.1016/j.gim.2022.10.002) contains supplementary material, which is available to authorized users.

Authors

Elise A. Ferreira[1,2], Annemarijne R.J. Veenvliet[3], Udo F.H. Engelke[4], Leo A.J. Kluijtmans[4], Marleen C.D.G. Huigen[4], Brechtje Hoegen[5], Lonneke de Boer[3], Maaike C. de Vries[3], Bregje W. van Bon[5], Erika Leenders[5], Elisabeth A.M. Cornelissen[6], Charlotte A. Haaxma[7], Jolanda H. Schieving[7], M. Estela Rubio-Gozalbo[2,8,9,10], Irene M.L.W. Körver-Keularts[2,9], Lara M. Marten[11], Susann Diegmann[11], Jeroen Mourmans[12], Alexander J.M. Rennings[13], Clara D.M. van Karnebeek[1,2,14], Richard J. Rodenburg[3,4,*], Karlien L.M. Coene[4,15]

Affiliations

[1]Department of Pediatrics, Emma Children's Hospital, Amsterdam Gastroenterology Endocrinology Metabolism, Amsterdam University Medical Centers, Amsterdam, The Netherlands; [2]United for Metabolic Diseases, Amsterdam, The Netherlands; [3]Department of Pediatrics, Amalia Children's Hospital, Radboud University Medical Center, Nijmegen, The Netherlands; [4]Department of Laboratory Medicine, Translational Metabolic Laboratory (TML), Radboud University Medical Center, Nijmegen, The Netherlands; [5]Department of Human Genetics, Radboud University Medical Center, Nijmegen, the Netherlands; [6]Department of Pediatric Nephrology, Amalia Children's Hospital, Radboud University Medical Center, Nijmegen, The Netherlands; [7]Department of Pediatric Neurology, Amalia Children's Hospital, Radboud University Medical Center, Nijmegen, The Netherlands; [8]Department of Pediatrics, Maastricht University Medical Centre, Maastricht University, Maastricht, The Netherlands; [9]Department of Clinical Genetics, Maastricht University Medical Center,

Maastricht University, Maastricht, The Netherlands; [10]GROW-School for Oncology and Developmental Biology, Faculty of Health, Medicine and Life Sciences, Maastricht University, Maastricht, The Netherlands; [11]Division of Pediatric Neurology, Department of Pediatrics and Adolescent Medicine, University Medical Centre Göttingen, University of Göttingen, Göttingen, Germany; [12]Department of Pediatrics, Deventer Ziekenhuis, Deventer, The Netherlands; [13]Department of Internal Medicine, Radboud University Medical Center, Nijmegen, The Netherlands; [14]Department of Human Genetics, Amsterdam Reproduction and Development, Amsterdam University Medical Centers, Amsterdam, The Netherlands; [15]Department of Clinical Chemistry and Hematology, Elisabeth-TweeSteden Hospital, Tilburg, The Netherlands

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