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Setup and Validation of a Targeted Next-Generation Sequencing Approach for the Diagnosis of Lysosomal Storage Disorders

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Lysosomal storage disorders (LSDs) are monogenic diseases, due to accumulation of specific undegraded substrates into lysosomes. LSD diagnosis could take several years because of both poor knowledge of these diseases and shared clinical features. The diagnostic approach includes clinical evaluations, biochemical tests, and genetic analysis of the suspected gene. In this study, we evaluated an LSD targeted sequencing panel as a tool capable to potentially reverse this classic diagnostic route. The panel includes 50 LSD genes and 230 intronic sequences conserved among 33 placental mammals. For the validation phase, 56 positive controls, 13 biochemically diagnosed patients, and nine undiagnosed patients were analyzed. Disease-causing variants were identified in 66% of the positive control alleles and in 62% of the biochemically diagnosed patients. Three undiagnosed patients were diagnosed. Eight patients undiagnosed by the panel were analyzed by whole exome sequencing: for two of them, the disease-causing variants were identified. Five patients, undiagnosed by both panel and exome analyses, were investigated through array comparative genomic hybridization: one of them was diagnosed. Conserved intronic fragment analysis, performed in cases unresolved by the first-level analysis, evidenced no candidate intronic variants. Targeted sequencing has low sequencing costs and short sequencing time. However, a coverage >60× to 80× must be ensured and/or Sanger validation should be performed. Moreover, it must be supported by a thorough clinical phenotyping. *(J Mol Diagn 2020, 22: 488—502; https://doi.org/10.1016/j.jmoldx.2020.01.010)*

Lysosomal storage disorders (LSDs) are a group of >50 inherited rare disorders characterized by the accumulation of specific undegraded metabolites in the lysosomes. This overstorage is commonly caused by a deficient or absent activity of one of the many lysosomal hydrolases or, in a few cases, by the deficit of other non-enzymatic lysosomal proteins. Although singularly considered rare, the combined birth prevalence of LSD is estimated from 7.5 to 23.5 per 100,000 live births.[1] Clinical signs and symptoms may occur from the prenatal period to adulthood, and may develop at different progression rate, according to the pathology, leading to a wide spectrum of disease forms, from mild to extremely severe, that in most cases affect the neurologic compartment.[2]

Generally, the diagnostic approach includes an accurate clinical evaluation, which leads to the formulation of a suspicion for one or more LSDs. This is followed by biochemical tests, aimed to detect the storage products in body fluids, whose results may orient the following enzymatic analyses.[3] Finally, if an enzyme deficit is detected, genetic analysis is performed on the suspected gene. However, this diagnostic route presents several limitations. In

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fact, some LSDs often share clinical signs and symptoms with other LSDs or different disorders; thus, their identification requires deep clinical expertise. Moreover, the above-mentioned biochemical methods are laborious, and they are often subject to high variability. Specifically, the execution of multiple enzyme assays may be expensive, and fluorogenic substrates to perform them may present scarce availability. Finally, not all disorders present with elevated levels of storage products. All this may delay the diagnosis that, in some cases, could be difficult and take several years or could be even unsuccessful.[4,5]

In the past decade, the emergence of next-generation sequencing (NGS) technologies has been proven to be an effective alternative to traditional techniques, in both research and clinical settings, allowing the simultaneous interrogation of several genes in one single reaction, in a short time and at a reduced cost per bp with respect to Sanger sequencing.[6]

Given the limitations of the traditional diagnostic approach and the availability of the NGS technologies, it is conceivable that the previously described diagnostic route for LSD could be potentially reversed.[3] An approach of targeted sequencing could be evaluated as the primary screening tool in the diagnosis of LSD, followed by biochemical and enzymatic tests aimed at confirming the molecular results. This alternative approach would potentially shorten the timing from the onset of first symptoms to the diagnosis, and considerably reduce costs.

In this study, we analyzed the feasibility of such a reversed approach through the evaluation of a targeted panel, including 50 LSD genes, as a potential diagnostic tool. Together with exons, promoters, and untranslated regions (UTRs), the most conserved intronic fragments (CIFs) of the analyzed genes were included in the panel, with the aim to widen variant search to these regions, in case of no appreciable results obtained through the analysis of the canonical regions.

Materials and Methods

Gene Selection and Panel Design

For the selection of the genes to be included in the panel, the Orphanet list of LSD (*https://www.orpha.net/consor/cgi-bin/Disease.php?lng=EN*, last accessed May 24, 2017), the Society for the Study of Inborn Errors of Metabolism LSD list,[7] and the list of genes reported by Fernandez-Marmiesse et al[4] for their panel design were evaluated. Finally, a list of 50 genes was selected (Table 1) by excluding from the whole LSD list both extremely rare disorders and disorders presenting a peculiar phenotype.

The Ion AmpliSeq platform (Thermo Fisher Scientific, Waltham, MA) was used for the design of a panel including the selected genes. The whole target sequence was 202.6 kb; for each gene, the design included the protein-coding transcripts. For each transcript, the exons, a 50-bp flanking sequence on each side, and both UTRs were given to the Ion AmpliSeq Designer software version 4.41 as target sequence. Moreover, the CIFs obtained by identifying highly conserved sequences through the PhastCons tool[8] and merging and

Table 1 LSD Genes Included in the Panel and Their Associated Disorder

Gene	Disorder
AGA	Aspartylglicosaminuria
CTNS	Cystinosis
LAMP2	Danon disease
GLA	Fabry disease
ASAH1	Farber disease
FUCA1	Fucosidosis
CTSA	Galactosialidosis
GM2A	Gangliosidosis GM2, activator defect
HEXB	Gangliosidosis GM2, Sandhoff disease
HEXA	Gangliosidosis GM2, Tay Sachs disease
GBA	Gaucher disease
GAA	Glycogenosis type II/Pompe disease
GALC	Krabbe disease
MAN2B1	α-Mannosidosis
MANBA	β-Mannosidosis
ARSA	Metachromatic leucodistrophy
PSAP	Metachromatic leucodistrophy, Krabbe, Gaucher
IDUA	MPS I (Hurler/Scheie syndrome)
IDS	MPS II (Hunter syndrome)
SGSH	MPS III A (Sanfilippo type A)
NAGLU	MPS III B (Sanfilippo type B)
HGSNAT	MPS III C (Sanfilippo type C)
GNS	MPS III D (Sanfilippo type D)
GALNS	MPS IVA (Morquio A)
GLB1	MPS IVB (Morquio B), gangliosidosis GM1
ARSB	MPS VI (Maroteaux Lamy)
GUSB	MPS VII (Sly syndrome)
HYAL1	MPS IX
GNPTAB	Mucolipidosis II α/β, III α/β
GNPTG	Mucolipidosis III γ
MCOLN1	Mucolipidosis IV
SUMF1	Multiple sulfatase deficiency
PPT1	Neuronal ceroid lipofuscinosis 1
CTSD	Neuronal ceroid lipofuscinosis 10
TPP1	Neuronal ceroid lipofuscinosis 2
CLN3	Neuronal ceroid lipofuscinosis 3
DNAJC5	Neuronal ceroid lipofuscinosis 4, Parry type
CLN5	Neuronal ceroid lipofuscinosis 5
CLN6	Neuronal ceroid lipofuscinosis 6
MFSD8	Neuronal ceroid lipofuscinosis 7
CLN8	Neuronal ceroid lipofuscinosis 8
SMPD1	Niemann-Pick A, B
NPC1	Niemann-Pick type I
NPC2	Niemann-Pick type II
CTSK	Pycnodysostosis
NAGA	Schindler disease
SLC17A5	Sialic acid storage disease
NEU1	Sialidosis
GNE	Sialuria
LIPA	Wolman disease

LSD, lysosomal storage disorder; MPS, mucopolysaccharidosis.

filtering identified regions on the basis of length and mutual distance were included. We focused on a multiple alignment among 33 placental mammals and downloaded the corresponding scores from the University of California, Santa Cruz, Genome Browser portal (*http://hgdownload.soe.ucsc.edu/goldenPath/hg19/phastCons46way*, last accessed June 14, 2017). Filtering criteria were 0.85 minimum conservation score, 20-bp minimum length, and 2-bp maximum distance between two fragments, to optimize coverage and number of sequences relatively to library and sequencing costs. For each gene, the 50 CIFs with highest conservation score were included in the panel design. Of importance, exonic and intronic regions were defined by combining Ensembl and RefSeq annotations, to guarantee maximal coverage on functional sequences.

Sample Selection and Ethics Statement

A total of 78 DNA samples were anonymously obtained and processed from the Cell Line and DNA Biobank from Patients Affected by Genetic Diseases, member of the Telethon Network of Genetic Biobanks,[9] and from some clinical and diagnostic centers in Italy and in Croatia. A total of 56 samples were from previously molecularly diagnosed patients [positive controls (PCs)] that were selected to represent most LSDs, and possibly their frequency. Twenty-two samples were evaluated as experimental samples: 13 belonged to patients who were diagnosed only through biochemical analysis and had not received a molecular confirmation of the enzymatic diagnosis [biochemically diagnosed (BD) patients]. Of 22 samples, nine were from patients with moderate to high suspicion of LSD, for whom a diagnosis had not yet been formulated [undiagnosed (UD)]. Informed consent for the targeted sequencing analysis was obtained for all the patients included in the study.

Enrichment, Library Construction, and Sequencing

DNA library preparation was performed according to the Ion AmpliSeq Library Preparation protocol (Thermo Fisher Scientific) in combination with the Ion AmpliSeq Library kit version 2.0. After DNA quantification using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific), the libraries were constructed starting from 10 ng of each DNA sample. The first step of target amplification was performed by using our AmpliSeq LSD-Panel Primer pools. The amplicons were then indexed using the Ion Xpress Barcode Adapters kit and purified using AMPure XP magnetic beads (Beckman Coulter, Inc., Brea, CA). All reactions were performed according to the manufacturer's instructions. Each DNA library was then quantified by real-time quantitative PCR using the KAPA SYBR FAST qPCR kit (Roche, Basel, Switzerland).

Libraries were pooled at 100 pmol/L, amplified by emulsion PCR, and enriched, with the One Touch and the ES machines (Thermo Fisher Scientific). For the Ion Proton sequencing, libraries were loaded into an Ion PI chip and sequenced using the Ion PI HiQ Sequencing 200 kit (all Thermo Fisher Scientific).

Sequencing data were analyzed on a Torrent Server through the Torrent Suite analysis pipeline version 5.0 (Thermo Fisher Scientific).

Runs had the following quality control metrics: 99% enrichment percentage, 22% polyclonal beads, 4% low-quality reads, and 72% usable reads. Each library obtained approximately 300,000 reads with a mean length of 181 bp. Coverage data were obtained using the Coverage Analysis plugin (Thermo Fisher Scientific).

Variant Calling and Prioritization

Alignment and variant calling were performed according to the Torrent Suite 5.0 analysis pipeline. Alignment was performed with tmap version 5.0, with the following parameters: -J 25 –end-repair 15 –do-repeat-clip stage1 map4. Variant calling was performed with the Torrent Variant Caller version 5.0 (Thermo Fisher Scientific), with germline high-stringency parameters, as supplied by the producer.

Variant analysis was performed by using QueryOR,[10] a platform for variant prioritization developed at CRIBI Biotechnology Center of the University of Padova (Padova, Italy). For each sample, variant analysis was split in three distinct queries aiming to prioritize missense, nonsense, and sense variants (query 1), frameshift, in-frame, stop-loss, and stop-gain variants (query 2), and splicing-affecting variants (query 3). In case of no appreciable results obtained with the first three queries, an additional optional fourth query was performed with the aim to prioritize the 5′ UTR, 3′ UTR, and intronic variants (Figure 1). A preliminary analysis of positive controls through these four queries was performed to select the most suitable filters and their cutoff, capable to select the pathogenic variants carried by PC samples. When no results were obtained through the above mentioned four queries, the same were relaunched, removing the coverage filters, to detect uncovered or poor covered variants. In addition, if the output of a single query included more than one variant, during the manual variant evaluation, priority was given to alleles with the lowest frequency, to non-annotated alleles, and to those presenting the highest pathogenic scores.

All identified variants were verified through Integrative Genomics Viewer version 2.3.79[11] for coverage and chromosomal position, and annotated using the Human Genome Variation Society version 15.11[12] nomenclature through Ensembl Variant Effect Predictor (*http://www.ensembl.org/info/docs/tools/vep/index.html*, last accessed December 4, 2018)[13] and through a homemade annotator, developed by the CRIBI laboratory (L.B., unpublished data). In a few specific biochemically diagnosed cases, a manual analysis of the gene of interest was performed through Integrative Genomics Viewer, searching for low covered variants or for homozygous deletions.

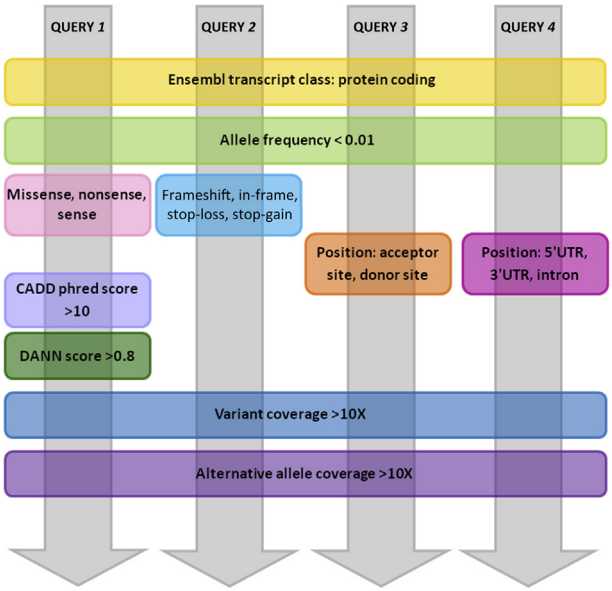

Figure 1 Schematic representation of the four queries and the relative filters used for variant prioritization through QueryOR platform. UTR, untranslated region.

Analysis of CIFs

The intronic variants located in the CIF included in the panel were filtered by QueryOR and analyzed by using different tools. Filtering criteria included frequency <0.01, association to a true major allele in the reference genome, and deleterious substitution types. Splicing-Based Analysis of Variants (SPANR, *http://tools.genes.toronto.edu*, last accessed November 27, 2018)[14] was used to predict both intronic and exonic variants affecting RNA splicing; for each variant, which may be up to 300 nucleotides inside an intron, the tool computes a score for how strongly genetic variant affects RNA splicing. SPANR is based on a bayesian machine learning model trained solely on RNA-sequencing data relative to >10,000 exons with evidence of alternative splicing, thus disregarding disease annotations and population data. Simultaneously, variants falling in regulatory regions and predicted to have a deleterious impact were obtained through Ensembl Variant Effect Predictor.

Variant Validation and Classification

The sequence variants identified in BD and UD patients were checked in Ensembl (*http://grch37.ensembl.org/Homo_sapiens/Info/Ind*, last accessed February 15, 2019), 1000genomes (*http://phase3browser.1000genomes.org/index.html*, last accessed June 19, 2019), and gnomAD version 2.1.1 (*https://gnomad.broadinstitute.org*, last accessed June 19, 2019)[15] for frequency. Moreover, the variants were confirmed by Sanger sequencing, in both directions; duplicate PCR products were obtained through specific set of primers designed through Primer-BLAST (*http://www.ncbi.nlm.nih.gov/tools/primer-blast*, last accessed December 17, 2018).[16] Obtained sequences were compared with the genomic reference sequence of the specific gene analyzed through BLAT (*https://genome.ucsc.edu/cgi-bin/hgBlat*, last accessed January 24, 2019). Finally, variants detected in BD and UD subjects were further classified according to the criteria of the American College of Medical Genetics and Genomics.[17]

Whole Exome Analysis

Library Preparation

Purified genomic DNA (10 ng) extracted from peripheral blood or skin fibroblasts was used for each sample. Genomic DNA quantity and quality were evaluated by Qubit DNA HS assay kit (Thermo Fisher Scientific). DNA libraries were prepared using the Illumina TruSeq Rapid Exome Library Prep Kit (Illumina, San Diego, CA), following the manufacturer's instruction (TruSeq Rapid Exome Reference Guide, number 1000000000751v01).

Exome Enrichment

For TruSeq exome target enrichment, two hybridizations were performed using capture probes, according to manufacturer's protocols. Streptavidin magnetic beads were used to capture the probes hybridized to the targeted regions of interest, and two heated washes were performed to remove non-specific binding from the beads.

Then, the enriched libraries were amplified and cleaned-up products were quantified by using Qubit fluorometer. The size distribution of post-enriched libraries was checked using the Agilent 2100 Bioanalyzer DNA High-Sensitivity Assay (Agilent Technologies, Santa Clara, CA).

Sequencing and Data Analysis

A 75-bp paired-end sequencing of the enriched libraries was performed on Illumina NextSeq500, by using the NextSeq500 High Output Kit version 2. Each sample obtained a mean coverage of 70×. Alignment and variant calling were performed with GATK,[18] according to the GATK Best Practices.[19] The 75-bp paired-end reads were aligned with Burrows-Wheeler Alignment mem version 0.7.12 with default parameters. PCR and optical duplicates were removed using Picard MarkDuplicatesWithMateCigar. Then, to reduce the number of mismatches, reads were realigned with IndelRealigner and base quality scores were recalibrated with the combination of BaseRecalibrator and PrintReads tools of GATK. Variant calling was performed with the HaplotypeCaller module of GATK version 3.4-46 with default parameters. Single VCF files were combined with the GenotypeGVCFs module of GATK to obtain a joint genotype file. VariantRecalibrator tool of GATK was applied to recalculate both single-nucleotide polymorphism and insertion/deletion calling scores. Recalibration, merging, and filtering of both types of variants were finally obtained with ApplyRecalibration tool of GATK.

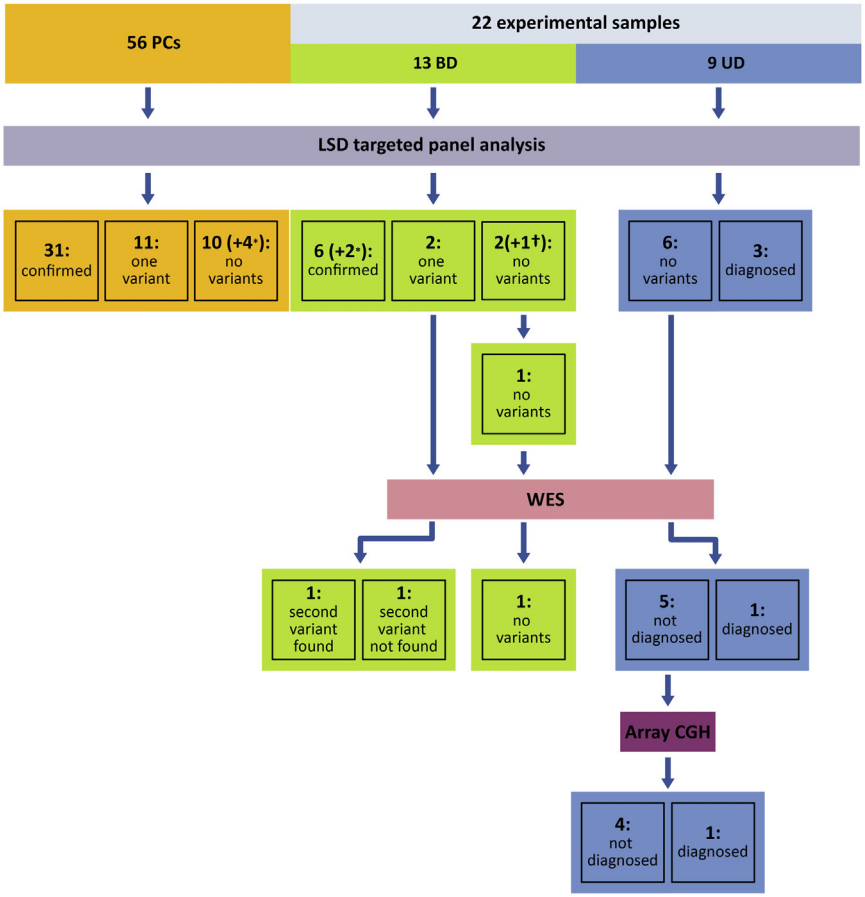

Figure 2 Overview of the number of samples analyzed and the diagnoses confirmed or achieved through the lysosomal storage disorder (LSD) panel and the following analyses applied to specific samples. The **asterisk** indicates patients carrying large deletions in homozygosis or hemizygosis that were not called by the variant caller but were visible through the visualization tool Integrative Genomics Viewer. The **dagger** indicates that for this patient, the results of panel analysis were not consistent with the available biochemical-enzymatic data. BD, biochemically diagnosed; CGH, comparative genomic hybridization; PCs, positive controls; UD, undiagnosed; WES, whole exome sequencing.

Analysis of whole exome sequencing (WES) data was performed using QueryOR[10] platform, following a flowchart similar to that used for the targeted sequencing panel data, setting coverage filters to >50×. In some cases, additional queries were performed setting specific Gene Ontology, Human Phenotype Ontology, and/or DisGeNET terms as filters.

CGH Array Analysis

Five undiagnosed samples (UD2, UD4, UD5, UD7, and UD8) were further analyzed through comparative genomic hybridization (CGH) array. The analysis was conducted using the SurePrint G3 Human CGH Microarray Kit, 8 × 60 K (Agilent Technologies) with a resolution ≥100 kb. Data were analyzed through the software Agilent Genomic Workbench 7.0. In the interpretation of the results, copy number variations (CNVs) identified in loci not disease associated at the time of the analysis, as well as alterations <100 kb, were not considered.

Results

Panel Design and Sample Sequencing

The total target sequence length was 202.6 kb and included 50 LSD genes (Table 1) and 230 CIFs, with an average length of 40 bp. The panel design output was a 187.42-kb sequence covered by 1561 amplicons; the average amplicon length was 240 bp (median, 257 bp) with 93% (median, 95.7%) of the whole target sequence covered. Considering only exons, their flanking sequences, and UTRs, the target sequence coverage does not change significantly, being the uncovered sequences located in the coding sequence. Hence, the least covered genes resulted to be *DNAJC5*, *CLN8*, *IDUA*, *NPC2*, and *HYAL1*, whose sequences (CIFs included) were covered for a percentage between 55% and 80% (Supplemental Table S1). Considering only the coding sequence, the most uncovered gene is *IDUA*, with eight of 14 exons being partially or totally uncovered. Any attempts to increase the coverage of these genes by modifying the setting during the phase of panel design did not result in any improvements. The same problem had been previously encountered by Fernandez-Marmiesse et al.[4]

Sample sequencing was performed through the preparation of four independent libraries, followed by their sequencing in four separate runs. Minimum, maximum, and average values per sample of total aligned bases, target base coverage depth, and percentages of target sequence with coverage at 20× and 100× are reported in Supplemental Table S2. Considering the real gene coverage compared with design coverage, moderate to slight variations were

observed, with the maximum increase with respect to design coverage for *DNAJC55* gene (33%) and the maximum decrease for *ARSA* gene (−21%) (Supplemental Table S1).

Variant Analysis

Figure 2 summarizes the number of samples analyzed and the diagnoses confirmed or achieved through the LSD panel and the following analyses. Pathologies represented in the PC samples reflect the most common LSD in the general population.

Variant analysis was performed through a prioritization process performed by QueryOR platform.[10] The total number of known variants per sample group ranged from 63 to 359, with an average of 254 variants; the average number of novel variants per sample group was 7 (range, 2 to 24). For the three groups of samples analyzed, PCs, BD, and UD, we used the same flowchart for variant prioritization shown in Figure 1, consisting in performing a set of four queries, each capable of detecting a specific type of variant or group of variants.

Positive Control Sample Analysis

An accurate preliminary choice of the most suitable filters capable to select the variants carried by the positive controls was performed: this selection led to the confirmation of the four queries set at the beginning of the study with only a slight modification, a small reduction of the CADD phred score from 15 to 10, to detect specific missense variants known to be pathogenic. The applied filters for each of the four queries are summarized in Figure 1. For positive control samples, all disease-causing variants but few were detected applying the previously adjusted first three queries, being most variants located in the coding regions, in the nearest intron-exon boundaries or in canonical splicing sites. In addition, the *ARSB* intronic variant c.1213+6T>C, resulting in skipping of exon 6 (authors' unpublished data), identified in samples PC2 and PC3, was detected only by applying the set of filters developed for intronic variants (query 4).

The analysis led to the identification of pathogenic variants in 66% of the PC alleles, with 31 samples in which both variants were identified and 11 in which only one variant was identified. In 14 PC samples, no variants were detected; among them, five present with gross deletions or complex rearrangements: this kind of genetic modification could not be detected by our panel. If we exclude the PC samples carrying large deletions and rearrangements, the percentage of identified alleles increases to 70%. Also, variants covered by low-quality reads [eg, variants falling in specific regions of the genes harboring a pseudogene (ie, *IDS* and *GBA*)] were not retrieved by our variant search. Supplemental Table S3 reports the list of genotypes of positive control samples included in the present study.[20-72]

The presence of large deletions encompassing one or more exons was confirmed by checking the degree of coverage of the affected exon(s) through the visualization tool Integrative Genomics Viewer. This approach allowed the identification of large deletions only if present in homozygosis, differently from what was reported by Fernandez-Marmiesse et al.[4] However, an attempt to apply the same approach for the detection of gross insertions/deletions was made, with no statistically significant results, given the inter-run and intra-run coverage variability between samples observed in our study.

Confirmation of Biochemical Diagnosis

Panel analysis led to the confirmation of previous enzymatic diagnoses for 8 cases of 13, with the molecular characterization of 62% of the biochemically diagnosed patients. In two cases, no variants were identified; in two other cases, only one variant was found. In one subject, panel analysis results were not consistent with the biochemical data available. Table 2 reports the identified genotypes of the biochemically diagnosed subjects. Five patients carried homozygous variants, two were compound heterozygous and one was hemizygous. Most variants were missense, two were gross deletions, one was a small deletion, and one a nonsense variant. The identification of gross deletions was possible by checking the read coverage through Integrative Genomics Viewer, as both deletions were carried in homozygosis status.

Four new variants, one missense in the *GALNS* gene, one nonsense in the *SGSH* gene, one gross deletion in the *HEXB* gene, and one small deletion in the *IDUA* gene, were identified and are described below.

A fifth potentially novel missense variant in the *NPC1* gene was not further confirmed by Sanger sequencing.

Variant c.860C>G [p.(Ser287Trp)] in the *GALNS* gene, found in heterozygosis in patient BD3, is not present in gnomAD. It is located in the same chromosomal position of p.Ser287Leu, a pathogenic variant previously described in homozygosis by Bunge et al[73] in a severe mucopolysaccharidosis (MPS) IVA patient. In our opinion, c.860C>G could be reasonably considered pathogenic, given the results of the tools, implemented in QueryOR, used to predict its deleterious effect on the coded N-acetylgalactosamine-6-sulfatase enzyme (CADD phred = 27.1; DANN = 0.991). Of interest, patient BD3 carries both c.860C>G [p.(Ser287Trp)] and c.860C>T [p.(Ser287Leu)] variants, allowing us to conclude that these variants are necessarily located on opposite alleles.

Variant c.1486C>T [p.(Gln496*)] in the *SGSH* gene was found in homozygosis in patient BD13; it is not present in gnomAD, and it is not described in literature. Being a nonsense variant, it should be reasonably considered the disease-causing variant for this patient.

The gross deletion detected in homozygosis in subject BD7 involves exon 7 of the *HEXB* gene; no deletions involving this exon have been previously described in literature.

In patient BD9, a novel 16-bp deletion (c.793-10_798del) encompassing the boundary intron 6−exon 7 was detected

Table 2 Results Obtained for the BD Samples Included in the LSD Panel

Patient code (sex)	Ethnogeographical origin	Enzymatic diagnosis	Gene	Nucleotide change
BD1 (M)	Turkey	MPS type VI	*ARSB*	ND
BD2 (F)	NA	Krabbe	*GALC*	ND
BD3 (F)	Croatia	MPS type IVA	*GALNS*	c.860C>T
				c.860C>G
BD4 (F)	Croatia	MPS type IVA	*GALNS*	c.860C>T
BD5 (F)*	Croatia	Galactosialidosis	*CTSA*	ND
BD6 (F)	Croatia	Gangliosidosis GM2, Sandhoff	*HEXB*	Exon 7 deletion
BD7 (M)	NA	Gangliosidosis GM2, Sandhoff	*HEXB*	Exon 1-5 deletion
BD8 (M)	Croatia	MPS type II	*IDS*	c.262C>T
BD9 (F)	Germany	MPS type I	*IDUA*	c.793-10_798del
				ND
BD10 (F)	Croatia	Niemann-Pick type I	*NPC1*	c.2764C>T
				c.3467A>G
BD11 (M)	Bosnia and Herzegovina	MPS type IIIA	*SGSH*	c.220C>T
				c.734G>A
BD12 (F)	Croatia	MPS type IIIA	*SGSH*	c.1167C>A
BD13 (F)	Croatia	MPS type IIIA	*SGSH*	c.1486C>T

(*table continues*)

*In patient BD5, no variants were detected in the *CTSA* gene; instead, two variants were revealed in the *GNPTAB* gene (see *Results*).
†This variant was identified by WES. Allele frequencies refer to European non-Finnish population frequencies obtained from gnomAD version 2.1.1 (*https://gnomad.broadinstitute.org*); the source (exome or genome data) is reported after the allele frequency in the seventh column.
F, female; M, male; BD, biochemically diagnosed; HE, hemizygous; HO, homozygous; HT, heterozygous; LSD, lysosomal storage disorder; MPS, mucopolysaccharidosis; N, no; NA, data not available; ND, not detected; WES, whole exome sequencing; Y, yes.

in heterozygosis in the *IDUA* gene; this deletion disrupts the splicing acceptor site of intron 6, presumably leading to the skipping of exon 7. Further confirmation of exon skipping on cDNA was not possible given the unavailability of RNA sample.

In one case, the panel result was not consistent with the biochemical analysis previously performed. Indeed, in a subject biochemically reported as affected by galactosialidosis (patient BD5) whose causative gene is *CTSA*, no variants in the *CTSA* gene were identified; instead, we found two variants in the *GNPTAB* gene, which is associated with mucolipidosis II α/β, III α/β (ML II α/β, III α/β). One of the variants had been previously reported in the literature as pathogenic (c.3503_3504del; p.Lys1168GlnfsX5),[80] whereas the second one (c.571+2T>C) is a novel variant affecting the splicing donor site of intron 6. This last variant was not present in either gnomAD or 1000 Genomes Database. In addition, both tools used to predict its effect on splicing (Human Splicing Finder and SPANR) strongly suggested it as a dangerous variant, disrupting the splicing donor site and potentially causing the skipping of exon 5. Unfortunately, as patient's cells were not available, the cDNA analysis could not be performed. Moreover, Sanger sequencing validation was not feasible, given the inability to amplify by PCR the patient's DNA, likely because of its degradation. Finally, a deeper clinical reevaluation of the patient was not possible because of the poor collaboration of the family.

For the other two subjects, an MPS VI and a Krabbe patient, no variants were detected through the panel analysis.

New Diagnoses Achieved

The targeted panel was also tested on nine UD patients who were referred to us with an LSD suspicion. For seven of them, a specific or quite specific suspicion toward a particular LSD or class of LSDs was previously formulated. Moreover, for one of them (patient UD2), sequencing of the *ARSB* gene (MPS VI) and real-time quantitative PCR analysis for detection of exonic deletions had been previously performed, with no results.

The LSD panel analysis led to the achievement of a diagnosis for three of nine patients. Table 3 reports the main clinical data available for these patients and the related genotype, where detected.

In a child suspected of MPS I/MPS II (patient UD1), a novel single-nucleotide insertion, c.1390_1391insA [p.(Ser464Lysfs*15)], was found in hemizygosis in the *IDS* gene; the same variant was found in heterozygosis in the mother. The variant is not reported in gnomAD and likely causes the alteration of the coding sequence from codon 464, with the insertion of a stop codon at position 478.

A diagnosis of ML II α/β, III α/β was achieved for a patient (patient UD3) whose clinical signs and symptoms were suggestive of mucolipidosis, but whose enzymatic data were ambiguous; thus, a deeper investigation, including molecular analysis, had not been conducted. Our

Table 2 (*continued*)

Amino acid change	Accession number (allele frequency; source)	WES analysis performed	Zigosity	Reference
		Y		
		Y		
p.(Ser287Leu)	*rs770053354* (0.00001865; exomes)	N	HT	73
p.(Ser287Trp)	NA			This study
p.(Ser287Leu)	*rs770053354* (0.00001865; exomes)	N	HO	73
ND		N		
		N	HO	This study
	NA	N	HO	74
p.(Arg88Cys)	*rs398123249* (no frequency data available)	N	HE	75
		N		This study
p.(Gln922*)†	*rs786204641* (no frequency data available)	Y	HT	76
p.(Asn1156Ser)	*rs28942105* (0.00003556; exomes)			77
p.(Arg74Cys)	*rs104894636* (0.0003423; genomes)	N	HT	78
p.(Arg245His)	*rs104894635* (0.0006478; genomes)			79
p.(Asn389Lys)	*rs764057581* (0.00004409; exomes)	N	HO	41
p.(Gln496*)	*rs1232231848* (no frequency data available)	N	HO	This study

analysis showed that the patient carried two previously described pathogenic variants in the *GNPTAB* gene: the missense variant c.1514G>A [p.(Cys505Tyr)] and the deletion c.3503_3504del [p.(Leu1168Glnfs*5)].[80,81] Further analyses found each parent a carrier of one of these mutations.

Finally, in a child (patient UD9) suspected of GM1 gangliosidosis, two known pathogenic variants were found in the *GLB1* gene: c.176G>A [p.(Arg59His)] and c.808T>G [p.(Tyr270Asp)].[55,56] In this case, the parents' DNA was not analyzed because of sample unavailability.

Variant Validation

Sanger validation, performed on the resolved BD and UD samples, confirmed panel results with exception of one case in which a poorly covered missense mutation was not confirmed. For the gross deletions in the *HEXB* gene, validations were performed through PCR amplification by using the primers reported by Neote et al,[74] for the deletion of exons 1 to 5 in patient BD6, and the primers for genomic DNA of exon 7 published by Zampieri et al,[84] for the exon 7 deletion in patient BD7.

Analysis of CIFs

CIFs were analyzed to identify potentially dangerous variants located in intronic regions, and the analysis was focused on samples from UD or BD patients in whom no variants had been found through the previous analysis. A total of 345 intronic variants with frequency <0.01 or with no frequency (not annotated variants) filtered by QueryOR were uploaded in SPANR and in Ensembl Variant Effect Predictor. Nine variants were selected by SPANR as potentially deleterious, but unfortunately none of them was carried by the mentioned UD or BD patients. The same intronic variants analyzed by Variant Effect Predictor gave 61 candidate variants mapping in regulatory regions, two of which were carried by UD2 and BD1 samples in regions predicted as promoter and enhancer for *LIPA* and *PSAP* genes, respectively. However, their frequency, although <1%, was relatively high (0.8% and 0.56%), likely excluding their involvement as disease-causing variants in these two patients.

WES of Undiagnosed Patients

Six undiagnosed and two biochemically diagnosed patients, for whom the LSD panel could not identify pathogenic variants, and one BD patient, for whom only one pathogenic variant had been identified, were further analyzed by WES, to widen the analysis to the remaining coding sequences (Figure 2). Statistical data on coverage of WES runs are reported in Supplemental Table S4. WES analysis was resolving for two patients: for subject BD10, we detected the second variant in the *NPC1* gene; in patient UD6, for whom a Morquio-like disease was suspected, we detected in the transient receptor potential cation channel subfamily V member 4 (*TRPV4*) gene the variant

Table 3 Suspected Disorder, Main Signs and Symptoms, and Biochemical Data for the UD Patients Analyzed through the LSD Panel

Patient code (sex)	Ethnogeographical origin	Main clinical features	Diagnosis achieved	Gene
UD1 (M)	Croatia	Suspected MPS type I, type II	MPS II	*IDS*
UD2 (F)	Italy	Suspected MPS type VI		
UD3 (F)	Italy	Suspected mucolipidosis	Mucolipidosis II α/β, III α/β	*GNPTAB*
UD4 (M)	Hungary	Suspected MPS type III. Developmental delay, coarse facial features. Elevated excretion of glycosaminoglycans in urine with the main fraction heparane sulfate. Normal activity of lysosomal enzymes for MPS III A, B, C, and D.		
UD5 (M)	Italy/Tunisia	Gastric distress, cognitive and motor delay, hepatomegaly, corneal opacity, absence of skeletal involvement. Elevated urinary glycosaminoglycans. LSD panel analysis was performed before enzymatic analyses.	Williams-Beuren syndrome (MIM number 194050)*	
UD6 (M)	Romania	Low stature, pectus carinatum, skeletal involvement with pain, normal at cognitive level. Borderline quantitative urinary glycosaminoglycans. Suspected Morquio disease. Enzymatic activities of MPS IV and other enzymes were negative.	Spondylometaphyseal dysplasia, Kozlowski type (MIM number 184252)	*TRPV4*
UD7 (F)	Italy	Facies sui generis, hepatosplenomegaly, short neck, fused cervical vertebrae. Normal at cognitive level. Negative enzymatic analyses.		
UD8 (F)	Bosnia and Herzegovina	Suspected NCL. Epilepsy, retinal detachment, GRODS. Normal enzyme analysis of PPT1 and TPP1 enzymes.		
UD9 (F)	Bosnia and Herzegovina	Suspected GM1 gangliosidosis. Two relatives died from the same disorder.	GM1 gangliosidosis	*GLB1*

(*table continues*)

Allele frequencies refer to European non-Finnish population frequencies obtained from gnomAD version 2.1.1 (*https://gnomad.broadinstitute.org*); the source (exome or genome data) is reported after allele frequency.

*Deletion revealed by comparative genomic hybridization array.

†Mutation detected by WES.

F, female; M, male; GRODS, granular osmiophilic dense deposits; LSD, lysosomal storage disorder; MIM, Mendelian Inheritance in Man; MPS, mucopolysaccharidosis; N, no; NA, data not available; NCL, neuronal ceroid lipofuscinosis; PPT, palmitoyl-protein thioesterase; TPP, tripeptidyl peptidase; UD, undiagnosed; WES, whole exome sequencing; Y, yes.

c.1781G>A [p.(Arg594His)], previously described in the literature.[83] Mutations in this gene are associated with spondylometaphyseal dysplasia, Kozlowski type (Mendelian Inheritance in Man number 184252), a pathology with dominant inheritance. *TRPV4* gene codes for a calcium permeable nonselective cation channel of 871 amino acids, which plays a key role in ion homeostasis and as important integrator of sensory information required for taste, vision, nociception, and the detection of temperature and mechanical forces. Moreover, it has an important role in differentiation of chondrocytes and terminal differentiation of osteoclasts via calcium influx.[85] Spondylometaphyseal dysplasia, Kozlowski type is a well-defined autosomal-dominant spondylometaphyseal dysplasia, characterized by significant scoliosis and mild metaphyseal abnormalities in the pelvis; the vertebrae exhibit platy spondyly and overfaced pedicles. Arginine 594 is highly conserved among the TRPV channels and is critical for the detection and transduction of chemical stimuli. Hence, HEK293 cells expressing this variant show increased constitutive and agonist-responsive TRPV4 activity.[77] Our patient presented with a series of clinical features partly overlapping those described in literature. As expected, the variant carried by patient UD6 is a *de novo* variant, with the parents totally asymptomatic and the analysis of their carrier status negative.

Table 3 (*continued*)

Nucleotide change	Amino acid change	Accession number (European non-Finnish allele frequency; source)	WES analysis performed	Reference
c.1390_1391insA	p.(Ser464Lysfs*15)	NA	N	This study
			Y	
c.1514G>A	p.(Cys505Tyr)	*rs281864980* (0.00002638; exomes)	N	81
c.3503_3504del	p.(Leu1168Glnfs*5)	*rs34002892* (0.0006430; genomes)		80
			Y	
Microdeletion of 1.4 Mb at q11.23 of chromosome 7*			Y	82
c.1781G>A	p.(Arg594His)†	NA	Y	83
			Y	
			Y	
c.176G>A	p.(Arg59His)	*rs72555392* (0.00003534; exomes)	N	56
c.808T>G	p.(Tyr270Asp)	*rs376663785* (0.00004664; genomes)	N	55

Array CGH Analysis

Samples UD2, UD4, UD5, UD7, and UD8, for which neither LSD panel nor WES analyses evidenced potential disease-causing variants, were further analyzed by array CGH, searching for pathogenic CNV. Analysis of the obtained results evidenced for patient UD5 a microdeletion of 1.4 Mb at q11.23 of chromosome 7: arr(GRCh37) 7q11.23(72,365,957x2,72,726,578_74,139,390x1,74,338,985x2).

This alteration causes a developmental disorder called Williams-Beuren syndrome (Mendelian Inheritance in Man number 194050), a multisystemic pathology characterized by intellectual disability, peculiar facial features, and cardiovascular problems.[82] For the other subjects analyzed, the array CGH analysis did not identify any pathogenic CNV.

American College of Medical Genetics and Genomics Classification of Variants

The variants detected in BD and UD patients were further analyzed using the criteria suggested by the American College of Medical Genetics and Genomics[17]: results of this classification are reported in Supplemental Table S5. As for the novel variants detected, three of them resulted to be

likely pathogenic (*IDS*:c.1390_1391insA; *IDUA*:c.793-10_798del; and *SGSH*: c.1486C>T), whereas one resulted to be of uncertain significance (*GALNS*:c.860C>G).

Discussion

The application of an NGS approach, like our LSD targeted panel, to the diagnosis of LSD could have several advantages with respect to the classic diagnostic approach. It potentially reduces the timing of diagnosis that, in addition, could be achieved at lower costs, with respect to traditional approaches (enzymatic assays, followed by specific Sanger sequencing analyses), considering that in some cases several enzymatic and/or genetic analyses are needed to achieve a defined diagnosis.

In this respect, our panel analysis revealed a previous misdiagnosis obtained by a biochemical approach: patient BD5 was suspected of galactosialidosis; however, he carried two mutations in the *GNPTAB* gene, which is associated with ML II α/β, III α/β. A similar case was recently reported by Gheldof et al,[5] who described a patient with a suspicion of galactosialidosis, later found to carry two known pathogenic variants in the *GNPTAB* gene. This is likely because of the peculiarity of mucolipidosis, which affects the activities of different enzymes tagged with mannose-6-phosphate molecule; and it is confirmed by Leroy et al,[86] who reported also infantile galactosialidosis among the disorders to consider in the differential diagnoses of ML II α/β, III α/β. Also, our undiagnosed patient UD3, finally defined as affected by ML II α/β, III α/β through the panel herein proposed, had ambiguous enzymatic data, which did not fully suggest a mucolipidosis. This further stresses the advantages of an approach like a targeted panel, which could be really useful in the differential diagnosis of LSD with overlapping clinical and biochemical phenotypes.

However, the results of our analysis showed a detection rate of 70% of the alleles for positive control samples, if we do not consider large deletions and rearrangements, and 62% of the samples for biochemically diagnosed subjects, highlighting also the limitations of this diagnostic approach. The diagnostic yield for our PC samples with respect to the genetic classified patients of Fernandez-Marmiesse et al[4] is lower. This is because of the higher number of probands (approximately three times as much) analyzed by us and the types of LSD tested; moreover, some of our subjects carried complex rearrangements or large deletions, not detectable by our platform of variant analysis. One of the technical limitations confirmed by this study is the poor or absent amplification of some specific regions, thus resulting as low-covered or uncovered regions, like several *IDUA* exons. In addition, some regions are covered by poor-quality reads: this is the case of repeated regions because of gene-pseudogene sequences located one after the other, as in *IDS* and *GBA* genes. These two limitations could be overcome, in presence of a strong LSD suspicion, by filling the gaps of not fully covered genes by using classic PCR amplification, followed by Sanger sequencing.

A further limitation is the poor ability, peculiar of the chosen NGS approach, to detect specific genetic alterations (complex rearrangements and CNVs). As for large deletions, this kind of alteration could be detected by checking manually the coverage of the suspected gene: the degree of coverage of the examined region with respect to the same region in other samples of the same run could suggest the presence of a deletion in heterozygosis or could reveal one in homozygosis; however, in both cases, different molecular techniques should be used to confirm the suspected deletion(s), as well as to exclude potential allelic dropout events.

Given the LSD panel results, we decided to examine in-depth some unresolved cases, analyzing nine patients through WES: this analysis led to resolution in two cases. The diagnostic yield of our WES analysis is similar to that reported in the literature (25% to 30%) in large-scale studies, in children with broad clinical presentations, in which WES was applied after multiple genetic and clinical investigations.[87] Also, it is comparable to that reported by Wang et al,[88] who performed WES on 14 patients with suspicion of a lysosomal disorder, and found disease-causing or candidate disease-causing variants in lysosomal genes in four cases and in non-lysosomal genes in two cases. Indeed, most of our patients analyzed by WES had been previously biochemically tested for at least one or sometimes more lysosomal enzymes and in most cases with negative or ambiguous results; moreover, one of them had been genetically analyzed for MPS VI (*ARSB* gene).

This confirms that the classic diagnostic route could become a long or even endless odyssey for the patients and their relatives, and that several lysosomal disorders could remain undiagnosed after extensive genetic and biochemical investigations.[4,89]

To increase the low detection rate, a deeper clinical characterization of LSD patients performed by a specialist is essential. In fact, these patients may show unspecific symptoms overlapping with other nonlysosomal disorders, as other neurometabolic or musculoskeletal disorders, that are sometimes not recognized by the specialists. In addition, the counseling of a geneticist would be useful to target the patients to the most appropriate genetic test. Once the test is performed, a close collaboration of the laboratory with the clinics would be important to help address the variant filtration process toward the right direction, hopefully leading to the identification of the disease-causing variants. Moreover, when parents' DNA is available, analysis of the trio would be highly preferable to singleton analysis, because this considerably increases the detection rate, as previously reported.[90,91] Also, trio analysis would be necessary to discriminate real homozygosis from apparent homozygosis resulting from a deletion.

Our inclusion of the CIFs was aimed at identifying possible intronic variants that presumably could be the cause of the disease, being located in highly conserved genomic regions. In fact, our results, as well as others reported in the literature,[87,88] show that numerous disease-causing variants escape the proposed NGS exomic approach of analysis. However, unfortunately, it is still difficult to demonstrate the pathogenicity of the intronic variants, excluding the splicing ones; at the moment, only *in silico* predictions and evaluations based on allele frequency can be performed, or mRNA analyses when RNA samples are available. As a proof of this, recently Caciotti et al[92] identified a disease-causing deep intronic variant in *GALNS* gene in a Morquio A patient who had only one pathogenic allele characterized. Our CIF analysis could not detect candidate pathogenic intronic variants; however, we believe that the inclusion of these sequences in the panels may be useful in the future, representing a first step toward the analyses of these regions, whose function is still unknown. The choice of including only highly conserved intronic regions provides the advantage of limiting the analysis to the intronic regions likely important for the gene function, thus maintaining low costs of the analysis.

Finally, an array CGH analysis was performed on five undiagnosed samples for which both panel and WES analyses resulted inconclusive. In one patient (UD5), array CHG revealed a deletion at q11.23 of chromosome 7: this alteration causes a developmental disorder called Williams syndrome.[82] Instead, for the other four patients, the results excluded the presence of potentially pathogenic CNV in the subjects analyzed, further highlighting the complexity of the diagnosis for some LSD-suspected cases.

Conclusions

Targeted sequencing is an appealing approach to implement routine diagnostic strategy, given its low sequencing costs and short sequencing time. However, a good coverage must be ensured and, when this is not reached, validation by Sanger sequencing needs to be performed on the proband and on the parents as final step, also to exclude the presence of deletions in cases of homozygous variant finding. Moreover, the possibility to fill the gaps in the panel design must be guaranteed, especially in case of strong suspicion for a specific disease. Indeed, Sanger sequencing still remains a reliable sequencing technique, and it should be considered an important support to NGS approaches, especially for confirmation of variants with a coverage below the good coverage threshold. Therefore, each laboratory should have a diagnostic flowchart, providing appropriate molecular genetic tools to address the clinical suspicion.

We believe that the application of the panel or, in a near future, of a WES or WGS analysis as first or one of the first steps in diagnostic route, supported by a thorough phenotyping of the patients, and a tight collaboration between clinics and laboratory could increase the yield of the diagnostic process of LSD, paving the way to a new reversed approach.

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Author Contributions

A.Z. designed the panel, collected the samples, analyzed next-generation sequencing (NGS) data, and wrote the manuscript; F.D., L.B., and G.Z. designed the panel, selected the conserved intronic fragments, and analyzed NGS data; E.F. and F.D.P. performed the panel and exome sequencing; A.R. collected and analyzed clinical data; M.F. performed array comparative genomic hybridization analysis and critically revised the manuscript; G.V. and R.T. gave support in NGS data analysis and critically revised the manuscript; all authors read and approved the final manuscript; R.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Supplemental Data

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