Author/s:
Vanderver, A;Simons, C;Helman, G;Crawford, J;Wolf, NI;Bernard, G;Pizzino, A;Schmidt, JL;Takanohashi, A;Miller, D;Khouzam, A;Rajan, V;Ramos, E;Chowdhury, S;Hambuch, T;Ru, K;Baillie, GJ;Grimmond, SM;Caldovic, L;Devaney, J;Bloom, M;Evans, SH;Murphy, JLP;McNeill, N;Fogel, BL;Schiffmann, R;Van Der Knaap, MS;Taft, RJ

Title:
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Date:
2016-06-01

Citation:
Vanderver, A., Simons, C., Helman, G., Crawford, J., Wolf, N. I., Bernard, G., Pizzino, A., Schmidt, J. L., Takanohashi, A., Miller, D., Khouzam, A., Rajan, V., Ramos, E., Chowdhury, S., Hambuch, T., Ru, K., Baillie, G. J., Grimmond, S. M., Caldovic, L. ,... Taft, R. J. (2016). Whole exome sequencing in patients with white matter abnormalities. Annals of Neurology, 79 (6), pp.1031-1037. https://doi.org/10.1002/ana.24650.

Whole exome sequencing in patients with white matter abnormalities

Running Head: Whole exome sequencing in white matter patients

Adeline Vanderver, MD[1,2,3,□,*], Cas Simons, PhD[4,□,*], Guy Helman, BS[1,2], Joanna Crawford, MS[4], Nicole I. Wolf, MD PhD[5], Geneviève Bernard, MD[6], Amy Pizzino, MS GCG[1], Johanna L. Schmidt, MPH MGC[1,2], Asako Takanohashi, DVM PhD[2], David Miller[4,7], Amirah Khouzam MS MA CGC[8], Vani Rajan MS[8], Erica Ramos MS LCGC[8], Shimul Chowdhury, PhD[8], Tina Hambuch PhD[8], Kelin Ru, MS[4], Gregory J. Baillie PhD[4], Sean M. Grimmond PhD[4,7], Ljubica Caldovic, PhD[2], Joseph Devaney, PhD[2], Miriam Bloom, MD[9], Sarah H. Evans, MD[10], Jennifer LP. Murphy, MS CPNP-AC[1], Nathan McNeill, MS[11], Brent L. Fogel, MD PhD[12] The Leukodystrophy Study Group, Raphael Schiffmann, MD[11], Marjo S. van der Knaap, MD PhD[5,13], and Ryan J. Taft, PhD[3,4,8,*]

1. Department of Neurology, Children's National Medical Center, Washington, DC, USA
2. Center for Genetic Medicine Research, Children's National Medical Center, Washington, DC, USA
3. School of Medicine and Health Sciences, George Washington University, Washington DC, USA
4. Institute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland, Australia
5. Department of Child Neurology, VU University Medical Center and Neuroscience Campus Amsterdam, Amsterdam, NL
6. Departments of Pediatrics, Neurology and Neurosurgery, Montreal Children's Hospital, McGill University Health Center, Montreal, Canada
7. The University of Melbourne Centre for Cancer Research, University of Melbourne, Victoria 3010 Australia
8. Illumina, Inc., San Diego, CA, USA
9. Department of Pediatrics, Children's National Medical Center, Washington, DC, USA
10. Department of Physical Medicine and Rehabilitation, Children's National Medical Center, Washington, DC, USA
11. Institute for Metabolic Disease, Baylor Research Institute, Dallas, TX, USA
12. Department of Neurology, Program in Neurogenetics, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA, USA
13. Department of Functional Genomics, VU University, Amsterdam, NL

□ These authors contributed equally to the manuscript

* To whom correspondence should be addressed

Adeline Vanderver: avanderv@childrensnational.org

Cas Simons: c.simons@uq.edu.au

Ryan J Taft: rtaft@illumina.com

Search Terms: Leukodystrophy, Whole Exome Sequencing, MRI Pattern Recognition, Incidental Findings
Word Count: 1,662
Abstract word count: 91
Title Character Count: 66
Running Head Character Count: 47
Tables: 4
Figures: 1

Abstract

Here we report whole exome sequencing (WES) on a cohort of 71 patients with persistently unresolved white matter abnormalities with a suspected diagnosis of leukodystrophy or genetic leukoencephalopathy. WES analyses were performed on trio, or greater, family groups. Diagnostic pathogenic variants were identified in 35% (25/71) of patients. Potentially pathogenic variants were identified in clinically relevant genes in a further 7% (5/71) of cases, giving a total yield of clinical diagnoses in 42% of individuals. These findings provide evidence that WES can substantially decrease the number of unresolved white matter cases.

Introduction

Patients with white matter abnormalities in the central nervous system (CNS) may have one of over a hundred genetic disorders, including the leukodystrophies (Supplemental Table S1).[1] Over the past two decades MRI pattern recognition has transformed the diagnosis of leukodystrophies.[2,3] Despite these advances, the breadth of conditions that present as a possible leukodystrophy continues to challenge even the most astute clinician.[4] Nearly half of these patients will remain unresolved, resulting in a prolonged diagnostic odyssey for affected families.[5-7]

A number of recent reports have provided evidence that whole exome sequencing (WES) can resolve previously intractable genetic disorders, with diagnostic yields ranging from 16% to 53%.[8-19] Given the unmet diagnostic need amongst patients with leukodystrophy, and the potential for agnostic next generation sequencing (NGS) to clarify these cases, we performed whole exome sequencing (WES) on a cohort of 71 patients referred to the Myelin Disorders Bioregistry Project (MDBP) for unresolved leukoencephalopathy of presumed genetic etiology. These patients were collected prospectively from 8.1.2009 to 7.31.2013 in the MDBP or the Amsterdam Database of Unclassified Leukoencephalopathies with approval from the institutional review boards at all collaborating institutions including Children's National Medical Center, the Baylor Neurogenetic Institute, and VU University Medical Center.

Methods

Cohort description: A total of 191 persistently unresolved cases were identified during the course of the study. 101 patients were diagnosed using MRI pattern recognition

followed by biochemical or other molecular approaches. These testing approaches included lysosomal enzymes, very long chain fatty acids, specific electron transport chain or mitochondrial enzyme assays, urine organic acids, microarray testing of copy number variations, gross chromosomal abnormality testing by karyotype or microarry, plasma amino acids, cerebrospinal neurotransmitters and alpha interferon, urine mucopolysaccaride or sialic acid testing, and targeted molecular testing, for example for *EIF2B1-5, PLP1* or *GFAP* sequencing based on MRI pattern recognition. Of the 90 persistently unsolved cases, 19 were excluded from WES testing: nine families obtained access to WES at other facilities, and an additional 10 families were excluded because DNA quality for all members of the trio did not meet stringency criteria and attempts to collect additional samples during the course of the study were unsuccessful. Seventy-one families remained for which high quality samples were available for complete trios. These 30 female and 47 male individuals all had abnormal white matter signal on neuroimaging. Individuals ranged in age from 3 years to 26 years at the time of sequencing, but symptom onset ranged from birth to 19 years (see complete Supplemental Case Reports available at http://imb.uq.edu.au/download/Vanderver_AON_2016.case_reports.pdf). Ethnicities were varied and included individuals of mixed and northern European descent, as well as African American, Arab, African, Asian, and Latin American origin. Radiological images and clinical summaries are provided in Supplemental Case Reports.

WES sequencing was performed at the Queensland Centre for Medical Genomics. Exomes were captured using either Illumina Nextera Rapid Capture kit or the SeqCap

EZ Human Exome Library v.3.0. Captured libraries were sequenced on an Illumina HiSeq 2000 (2 x 100nt) or on an Illumina NextSeq 500 (2 x 150nt). WES sequencing was performed such that a minimum of 80% of targeted bases were sequenced to a read depth of 20x or greater (average: 88%). Reads were aligned to the reference human genome (GRCh37) and pedigree informed variant calling was performed using the Real Time Genomics (RTG) integrated analysis tool rtgFamily v3.2.[20] All variants were annotated using SnpEff v3.4[21] and filtered using data from the SnpEff GRCh37.72 database, dbSNP138, and dbNSFP v2.4.

We utilized a custom-built variant annotation and interpretation interface to identify possible causal mutations in each case, incorporating evidence including minor allele frequency, conservation, predicted pathogenicity, disease-association (in public databases or the published literature), established or predicted biological function, confirmation with Sanger sequencing and familial segregation (Supplemental Tables S2-S4).[22] Cases with variants in known disease genes meeting the ACMG criteria for pathogenic or likely pathogenic (see Supplemental Case Reports), and whose clinical features were concordant with the established gene:disease relationship were classified as diagnostically resolved.

Results

In this cohort we were able to unambiguously resolve 25 cases (Table 1, Supplemental Table S2, and Supplemental Case Reports). In three cases we were able to confirm pathogenicity with downstream biochemical testing. For example, we identified a

compound heterozygous mutation in *TERT* (MIM:187270) in a patient that presented with white matter changes, frequent infections, mild developmental delay and hypogammaglobulinemia which was validated by Flow-FISH telomere length analysis and confirmed a diagnosis of atypical Dyskeratosis Congenita with hypomyelination (MIM:613989) (Figure 1, Supplemental Case Reports, LD_0607.0).[23] Likewise, an individual with a compound heterozygous mutation in *GLB1* (MIM:611458) had confirmatory lysosomal enzyme testing (Supplemental Case Reports, LD_0846.0), and an individual with mutations in *ATM* (MIM:607585) had confirmatory elevated alpha-fetoprotein levels (Supplemental Case Reports, LD_0678.0).

Nine of the twenty-five cases had mutations in genes associated with disorders classically defined as leukodystrophies[3] (Table 1 & Supplemental Table S1): two patients were identified with *TUBB4A* (MIM:602662) related hypomyelination (Hypomyelination with atrophy of the basal ganglia and cerebellum [MIM:612438])[24]; two patients were identified with early onset Vanishing White Matter Disease (MIM:603896) (genotype *EIF2B2* [MIM:606454] and *EIF2B5* [MIM:603945])[25, 26]; three families were identified with t-RNA synthetase disorders (*AARS* [MIM:601065] and *DARS* [MIM:603084])[27-29]; and two families identified with a POLR3-related disorder (*POLR3B* [MIM:614366] and *POLR1C* [MIM:610060]) (Table 1, Supplemental Table S2 and Supplemental Case Reports).[30] The remaining individuals had mutations in genes associated with genetic leukoencephalopathies. In these cases expert review confirmed that the clinical presentation and MR imaging was consistent with published phenotypes. These findings are consistent with the estimation that a majority of

disorders associated with abnormal white matter on neuroimaging are not classic leukodystrophies.[1] This suggests that testing of leukodystrophy-associated genes on NGS panels may have limited diagnostic efficacy (predicted to be only 13% in this cohort), which may outweigh the perceived cost benefit and limiting exposure to incidental findings.

In a further four cases we identified one or more potentially damaging variants of uncertain significance (VUS) that did not reach the strict burden of proof required to be classified as pathogenic or likely pathogenic. In each of these cases the neuroradiological findings, clinical features and familial segregation of the variants in these individuals were consistent with the published phenotype (Table 2, Supplemental Table S3, and Supplemental Case Reports). We therefore classified these variants as potentially pathogenic and considered the cases clinically resolved by expert assessment. This included cases with variants in *AMPD2*, *FLNA, and NDUFA2* (Supplemental Table S4). This also included one case (LD_0675) where the individual was reported as part of cohort describing a novel disease due to mutations in *AARS2.*[27]

A final case had a *de novo* variant in *FUS* (MIM:205100) classified as pathogenic by ACMG criteria, but because this gene has previously only been associated with only juvenile or adult onset Amyotrophic Lateral Sclerosis (ALS), this was not considered an unambiguous resolution (Table 2, Supplemental Table S3 & Supplemental Case Reports). However, because mutations in other ALS associated genes are associated with early hypomyelination (including *ALS2* [MIM:205100] in this cohort), and because

the *de novo* finding segregated in this family, it was classified as a potentially pathogenic variant and a clinically resolved case.

To investigate the burden of actionable incidental findings that may be identified during trio-based WES investigation of rare genetic disorders, the Illumina Clinical Services Laboratory screened all 56 adult and 49 pediatric ACMG recommended genes for potential incidental variants.[31] This analysis revealed pathogenic or likely pathogenic variants in 3 of the 71 families screened (Table 3). The identified variants were restricted to *KCNQ1* (MIM:607542) associated with long QT syndrome and *SDHB* (MIM:115310) associated with hereditary paragangliomas (Table 2, Supplemental Table S3 and Supplemental Case Reports). Interestingly, mutations in *SDHB* are also now associated with autosomal recessive succinate dehydrogenase deficiency associated leukoencephalopathy, although the lack of a second mutation in LD_0315 precluded definitive association of this genotype with the patient's phenotype.[32] We found less than one known pathogenic or likely pathogenic variant per 46 adult exomes analyzed, suggesting that the impact of incidental findings is likely to be minimal, especially when weighed against the potential benefits of a successful genetic diagnosis in families with severe, life-threatening neurologic illnesses.

Discussion

Using an intention to treat analysis,[33] and if the combined initial cohort of 191 families is considered in which 101 families achieved a diagnosis through standard approaches, the use of WES approach would result in an ~20% diagnostic increase. This yields an

overall rate of diagnosis of ~72% for the combination of standard and WES approaches. Clinical integration of WES (or whole genome sequencing) therefore, may decrease the number of patients with unsolved genetic white matter disorders from 50% to less than 30%. Taking into consideration the clinical and psychosocial costs of prolonged diagnostic odysseys in these families, this is substantial.

Additionally, while the clinical utility of WES as measured by changes to patient care was not addressed in this study, it should be noted that in several cases the results of WES directly influenced clinical care. For example, patients with *ATM* and *TERT* mutations were both referred to specialist clinics where they now undergo oncologic monitoring, and the patient with a *de novo KCNT1* mutation was treated with a potassium channel anticonvulsant to control refractory epilepsy.[34]

The use of WES in large cohorts enables the identification of sequence variants of varying degrees of clinical certainty. ACMG criteria classify the spectrum of identified variants into four tiers; pathogenic, likely pathogenic, variant of unknown significance, or unresolved.[35] However, our study, in which 4 of 31 cases were resolved with MRI pattern recognition[2, 3, 36, 37] and clinical review of the identified variant, suggests that a variant classification system that takes into account clinical context and downstream clinical evaluation and testing (e.g. MRI interpretation) should be considered.

In summary, WES has the potential to decrease the number of unsolved cases of leukodystrophy and genetic leukoencephalopathies. Additional research is needed

establish the potential value of NGS as a first-line diagnostic tool, and to assess the comparative effectiveness of WES, WGS and targeted panels in this disease population.

Acknowledgements

We thank the patients and their families. AV, GH, AP, JLS, AT, and JLPM are supported by the Myelin Disorders Bioregistry Project. GH was supported by the Delman Fund for Pediatric Neurology and Education. This publication was supported by Award Number UL1TR000075 from the NIH National Center for Advancing Translational Sciences. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Center for Advancing Translational Sciences or the National Institutes of Health. We thank the QCMG and IMB sequencing facility teams for their assistance with this project. Computational support was provided by the NeCTAR Genomics Virtual Laboratory and QRIScloud programs. GB has received a Research Scholar Junior 1 award from the Fonds de Recherche du Québec en Santé (FRQS). R.J.T. was supported by an Australian Research Council Discovery Early Career Research Award. This work was supported by National Health and Medical Research Council, Australia Grant (APP1068278) and a University of Queensland Foundation Research Excellence Award. NIW and MSvdK were supported by ZonMw TOP grant number 91211005. BLF reports funding from NIH Grants K08MH086297 (NIMH) and R01NS082094 (NINDS). We also thank, Brian P. Brooks, Sara Zondag, Lisa Green, Sudeshna Mitra, Lucy Civitello, Natasha Shur, Valeria Zincke, Silvia Delgado, Janice E. Brunstrom-Hernandez, Celia Chang, Robert Keating, Jessica Carpenter, Jayne Antony, Shekeeb Mohammad, Marc C. Patterson, Tarannum Lateef, Taeun Chang, James Reese, Shaaron Towns, Diego Preciado, Dewi Depositario-Cabacar, Meganne Leach, Catherine Zorc, Jenny Wilson, Eileen Walters, Steven Leber, Srikanth Muppidi, Kimberly Chapman, Amy Waldman, and Lindsey Scussel for patient referrals to the Myelin Disorders Bioregistry Project.

Author Contributions

AV, CS, GH and RJT designed and managed the project, coordinated the manuscript, and performed literature and case review. CS, GH, JC, AK, VR, ER, SC, TH, DM, KR, GJB, SMG, LC, JoDev, NM, AT, and RJT acquired the data and performed analysis of the incidental findings and provided bioinformatics analysis and expertise and performed laboratory studies. AV, CS, GH, JC, AP, NIW, GB, AP, JLS, MB, SHE, JLPM, BLF, RS, MSvdK, and RJT drafted the manuscript and figures.

The Leukodystrophy Study group includes ALG, TS, PLP, EF, SP, BHC, JDR, MRN, CY, YS, MD, EF, LG, CML, CTR, JaDes, HA, KW, VL, MJG, MC, IK, DG, MG, and EDR, who evaluated the patients clinically and referred patients to the Myelin Disorders Bioregistry Project. Their affiliations are included in the supplemental text.

Potential Conflicts of Interest

AV receives funding from Illumina, Inc., Gilead Sciences Inc., Eli Lilly & Co. and Shire Plc. AK, VR, ER, SC, TH, and RJT are employees of Illumina, Inc. The rest of the authors report no conflict of interest.

Supplemental Data

The Supplemental Data for this manuscript includes three tables, and a link to the Supplemental Case Reports including MRI figures from all patients where imaging was available (http://imb.uq.edu.au/download/Vanderver_AON_2016.case_reports.pdf).

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TABLES

Table 1: Cases with pathogenic variants identified by exome sequencing in classical leukodystrophy genes.

Family	Gene	Zygosity	Protein	Classification
LD_0139	TUBB4A	Het, *de novo*	p.Arg391His	Likely pathogenic
LD_0181	DARS	Het	p.Arg494Gly	Likely pathogenic
		Het	p.Arg460His	Likely pathogenic
LD_0604	POLR3B	Het	p.Glu271fs	Pathogenic
		Het	p.Val523Glu	Pathogenic
LD_0672	TUBB4A	Het, *de novo*	p.Val180Gly	Likely pathogenic
LD_0764	EIF2B5	Het	p.Gln562*	Pathogenic
		Het	p.Arg339Trp	Pathogenic
LD_0774	POLR1C	Het	p.Lys295del	Likely pathogenic
		Het	p.Cys146Arg	Likely pathogenic
LD_0869	EIF2B2	Het	p.Gly200Val	Pathogenic
		Het	p.Glu213Gly	Pathogenic

Key: Het –Heterozygous; Hom – Homozygous; rs – Reference Single Nucleotide Polymorphisms

Table 2: Cases with pathogenic variants identified by WES in genes not associated with leukodystrophy.

Family	Gene	Zygosity	Protein	Classification
LD_0106	GRIN1	Het, *de novo*	p.Arg865Cys	Likely pathogenic
LD_0115	AARS	Het	p.Arg751Gly	Pathogenic
		Het	p.Lys81Thr	Pathogenic
LD_0119	KCNT1	Het, *de novo*	p.Phe932Ile	Pathogenic
LD_0157	SZT2	Het, *de novo*	p.Pro1833fs	Pathogenic
		Het	p.Gly2306Arg	Likely pathogenic
LD_0158	CNTNAP1	Hom	p.Arg388Pro	Likely pathogenic
LD_0232	MRPS22	Het	p.Lys248fs	Pathogenic
		Het	p.Arg191Gln	Likely pathogenic
LD_0286	RMND1	Hom	p.Asn238Ser	Likely pathogenic
LD_0333	CNTNAP1	Het	p.Arg107*	Pathogenic
		Het	p.Cys323Arg	Likely Pathogenic
LD_0358	STXBP1	Het, *de novo*	p.Arg367*	Pathogenic
LD_0366	GATAD2B	Het, *de novo*	p.Gln274*	Pathogenic
LD_0463	ALS2	Het	p.Arg1139*	Pathogenic
		Het	p.Gly1083Glu	Pathogenic
LD_0607	TERT	Het, *de novo*	p.Arg819Cys	Pathogenic
		Het	p.Val664Met	Pathogenic
LD_0646[19]	NDUFS7	Hom	p.Arg135Cys	Likely pathogenic
LD_0678	ATM	Het	p.Leu275fs	Pathogenic
		Het	p.Lys2756*	Pathogenic
LD_0755	SDHAF1	Hom	p.Arg55Pro	Pathogenic
LD_0756	SDHB	Hom	p.Asp48Val	Pathogenic
LD_0846	GLB1	Het	p.Arg196Ser	Pathogenic
		Het	p.Tyr240His	Pathogenic
LD_0857	AARS	Hom	p.Arg751Gly	Pathogenic

Key: Het –Heterozygous; Hom – Homozygous;

Table 3. Cases with potentially pathogenic variants identified with whole exome sequencing.

Family	Gene	Zygosity	Protein	Classification
LD_0493	FLNA	Hem, *de novo*	p.Leu2271Arg	VUS
LD_0664	FUS	Het	p.Gly500fs	Pathogenic
LD_0673	AMPD2	Hom	p.Arg843His	VUS
LD_0675	AARS2	Het	p.Gly965Arg	Likely pathogenic
		Het	p.Glu405Lys	VUS
LD_0821	NDUFA2	Het	p.Lys45Thr	VUS
		Het	p.Thr75fs	VUS

* Key: Het –Heterozygous; Hom – Homozygous;

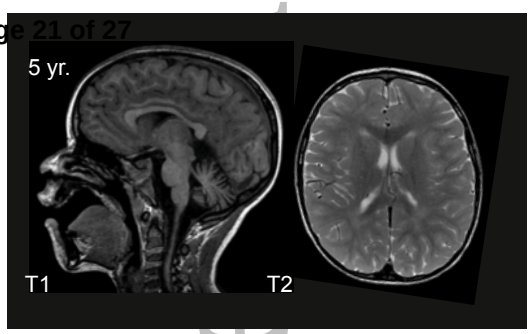
Table 4. Incidental Findings Identified in Cohort

	Number of individuals screened	Number of individuals with incidental findings	Reported Incidental Finding
Unaffected Adults	142	3 (2.1%)	*KCNQ1* (NM_000218.2) c.514G>A *KCNQ1* (NM_000218.2) c.1189C>T *SDHB* (NM_003000.2) c.541-2A>G
Affected Children	79	3 (3.7%)	As above
Unaffected Siblings	10	0	NA

FIGURES

Figure 1. MRI and pathogenic variants for three cases. A) MRI of individual LD_0607.0 – a male of mixed European descent with a multisystem disorder characterized by elevated creatine kinase, recurrent infection with hypogammaglobulinemia, dyskeratosis congenita, and mild transaminase abnormalities. MRI revealed moderate cerebellar atrophy and diffuse multifocal white matter changes. Follow-up MRI one year later showed unchanged T2 hyperintensities. The variants found in TERT in this patient were classified as pathogenic per the ACMG guidelines and the diagnosis was supported by telomere length analysis (data not shown). **B)** Schematic of the TERT protein showing heterozygous variants identified in LD_0607.0. Predicted domains: GQ, GQ motif; QFP, QFP motif; RT, Reverse transcriptase domain. **C)** Clustalo alignment of vertebrate homologs of TERT showing conservation of mutated residues. **D)** MRI of individual LD_0756.0 – male of Turkish descent with motor delays were noted at birth who abruptly decompensated at 7 months of age, and a history of ataxia, hypotonia, and spasticity. MRI at 3 years and 6 months of age was significant for signal abnormality of the supratentorial white matter with sparing of the U fibers, a swollen appearance to the corpus callosum, involvement of the cerebellar white matter, and the brain stem. This pattern has been seen in previously published cases and supports the SDHB variant categorization as potentially pathogenic. **E)** Schematic of the SDHB protein showing a homozygous variant identified in LD_0756.0. Predicted domains: MTS, mitochondrial targeting signal; SDH, Succinate dehydrogenase domain. **F)** Clustalo alignment of vertebrate homologs of SDHB showing conservation of mutated residues. **G)** MRI of individual LD_0286.0B – male of mixed-European descent

with leukoencephalopathy and a history of sensorineural hearing loss, developmental delay and febrile seizures. MRI is significant for bilateral temporal lobe cysts, small corpus callosum, and periatrial white matter abnormalities. Hearing loss and other clinical manifestations were consistent with the phenotype reported for mutations in RMND1, and the variant was classified as likely pathogenic based on supporting evidence. **H)** Schematic of the RMND1 protein showing heterozygous variants identified in LD_0286. Predicted domains: MLS, mitochondrial localization signal; DUF155, domain of unknown function 155; CC, coiled-coil domain; and TM, trans-membrane domain. **I)** Clustalo alignment of vertebrate homologs of RMND1 showing conservation of mutated residues.



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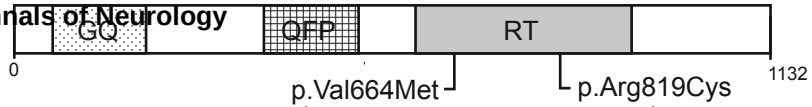

C.

```
H. sapiens      RLTSRVKALFSVLNYERARRPGL VFLRFMCHHAVRIRGKSYVQC--
M. musculus     CFTQSLKTLFSVLNYERTKHPNL FFLRFVRHSVVKIDGRFYVQC--
R. norvegicus   HFTQRLKTLFSMLNYERTKHPHL FFLHFLRHSVVKIGDRCYTQC--
B. taurus       HLSSRVKTLFAVLNYERARRPGL LFLHLVRSHVIRIGGRSYIQC--
X. tropicalis   YFQSCVRVLQNVLSVCVREAPGP VFQQMIRSHILRIEDRIYSCKLA
D. rerio        HFTSRVRNLFSVLNYEWNRNCSL FFQKMLSSYVIHYDQQMFRQV--
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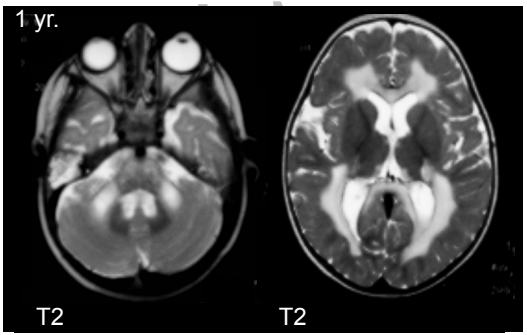
D.



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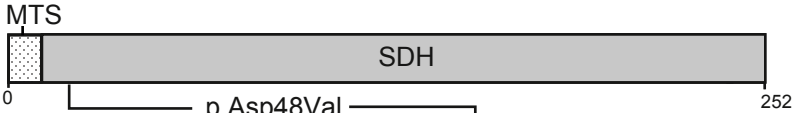
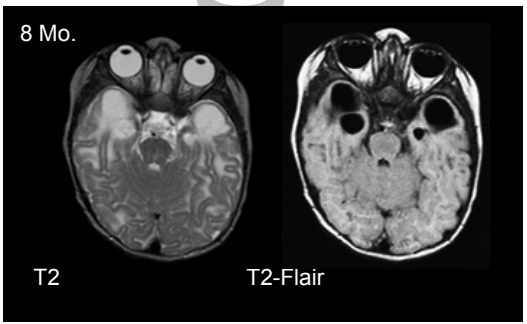


F.

```
H. sapiens      TAAATA------PRIKKFAIYRWDPDKAGDKPHMQTYEVDLNKCGP
M. musculus     TAAAAA------PRIKKFAIYRWDPDKTGDKPRMQTYEVDLNKCGP
R. norvegicus   TAAAAA------PRIKTFAIYRWDPDKAGDKPRMQTYKVDLNKCGP
B. taurus       TAAAAA------PRIKKFAIYRWDPDKTGDKPHMQTYEIDLNNCGP
X. tropicalis   TAAAAAPASQAEARIKKFAIYRWDPDKPGDKPRMQTYEVDLNECGS
D. rerio        -FAQTAAAPAAQPRIKKFQIYRWDPDTVGDKPRMQTYEIDLNTCGP
```

G.



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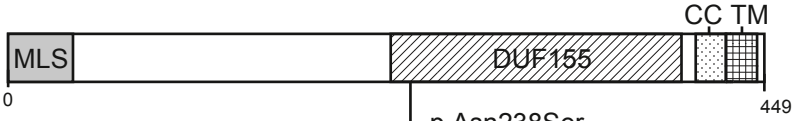

I.

```
H. sapiens      ENSAKEGDPGTIFFFREGAAVFWNVKDKTMKHVMKVLEKHEIQPYEI
M. musculus     ESSAKEGDAGTIFLFREGAAVFWNVKDKTMKHVMQVLERHETQPYEV
R. norvegicus   ESSAKEGDPGTIFLFREGAAVFWNVKEKTMKHVMQVLERHETQPYEV
B. taurus       ENSAKEGDPGTIFFFREGAAVFWNVKDQTMKHVMQVLEKHEIQPYEI
X. tropicalis   DNGAKDTDSGTVFFFREGAVVFWNVEERIMKHTLQILERHEIQPYEV
D. rerio        DNSAKPYDSGTIFFFREGSVVFWNVEDKTMKTTMKILEQHEIQPYEV
```

Supplemental note:
Case summaries including a description of the radiologic findings of affected individuals, the phenotypic presentation of affected individuals and their families, and the details of each candidate pathogenic variant identified are available to download at the following URL: http://imb.uq.edu.au/download/Vanderver_AON_2016.case_reports.pdf

S1: Leukodystrophies and their causative genes.

Leukodystrophy	Gene/s
Pol-III related disorders (4H syndrome (hypomyelination, hypodontia and hypogonadotropic hypogonadism))	POLR3A, POLR3B, POLR1C
18q minus syndrome	18q-
X linked Adrenoleukodystrophy (X-ALD)	ABCD1
Adult onset leukodystrophy with neuroaxonal spheroids and pigmented glia (including hereditary diffuse leukoencephalopathy with spheroids, HDLS, and pigmentary type of orthochromatic leukodystrophy with pigmented glia, POLD)	CSF1R
Aicardi–Goutières Syndrome (AGS)	TREX1, RNASEH2A, RNASEH2B, ADAR1, IFIH1, SAMHD1
Alexander disease (AxD)	GFAP
Autosomal Dominant Leukodystrophy with Autonomic disease (ADLD)	LMNB1
Canavan disease	ASPA
Cerebrotendinous Xanthomatosis (CTX)	CYP27A1
Chloride Ion Channel 2 (ClC-2) related leukoencephalopathy with intramyelinic oedema	CLC2
eIF2B related disorder (Vanishing White Matter Disease or Childhood ataxia with central nervous system hypomyelination (CACH)	EIFB2B1, EIF2B2, EIF2B3, EIF2B4, EIF2B5
Fucosidosis	FUCA1
Globoid cell Leukodystrophy (Krabbe)	GALC
Hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC)	TUBB4A
Hypomyelination with brainstem and spinal cord involvement and leg spasticity (HBSL)	DARS
Hypomyelination with congenital cataract (HCC)	FAM126A
Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL)	DARS2
Leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL)	EARS2
Megalencephalic leukoencephalopathy with subcortical cysts (MLC)	MLC1, HEPACAM
Metachromatic leukodystrophy (MLD) and its biochemical variants	ARSA
Oculodentodigital dysplasia	GJA1
Pelizaeus-Merzbacher disease (PMD)	PLP1
Pelizaeus-Merzbacher like-disease (PMLD)	GJC2
Peroxisomal Biogenesis disorders (including Zelleweger, neonatal Adrenoleukodystrophy and Infantile Refsum)	PEX genes
Polyglucosan Body Disease (PGBD)	GBE1
RNAse T2 deficient leukoencephalopathy	RNASET2
Sialic acid storage disorders (Salla disease, Infantile Sialic Acid Storage Disease and Intermediate form)	SLC175A
Single enzyme deficiencies of peroxisomal fatty acid beta oxidation (including D-Bifunctional Protein Deficiency; SCPx deficiency ; Peroxisomal acyl-CoA-Oxidase Deficiency	Numerous genes
Sjögren–Larsson syndrome	ALDH3A2
SOX10-associated PCWH: peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome, and Hirschsprung disease	SOX10

* Leukodystrophy associated genes reviewed in Vanderver et al. (2015)

S2: Cases with pathogenic variants identified by exome sequencing in classical leukodystrophy genes.

Family	Gene	Zygosity	Transcript	CDS	Protein	rsID	Max AF*	Classification
LD_0139	TUBB4A	Het, *de novo*	ENST00000264071	c.1172G>A	p.Arg391His	-	-	Likely pathogenic
LD_0181	DARS	Het	ENST00000264161	c.1480C>G	p.Arg494Gly	rs147077598	0.00012	Likely pathogenic
		Het	ENST00000264161	c.1379G>A	p.Arg460His	-	0.00006	Likely pathogenic
LD_0604	POLR3B	Het	ENST00000228347	c.813_819del	p.Glu271fs	-	-	Pathogenic
		Het	ENST00000228347	c.1568T>A	p.Val523Glu	rs138249161	0.00049	Pathogenic
LD_0672	TUBB4A	Het, *de novo*	ENST00000264071	c.539T>G	p.Val180Gly	-	-	Likely pathogenic
LD_0764	EIF2B5	Het	ENST00000273783	c.1864C>T	p.Gln562*	-	-	Pathogenic
		Het	ENST00000273783	c.1015C>T	p.Arg339Trp	rs113994068	0.00007	Pathogenic
LD_0774	POLR1C	Het	ENST00000372389	c.883-885del	p.Lys295del	-	-	Likely pathogenic
		Het	ENST00000372389	c.436T>C	p.Cys146Arg	-	-	Likely pathogenic
LD_0869	EIF2B2	Het	ENST00000266126	c.599G>T	p.Gly200Val	rs113994012	0.00058	Pathogenic
		Het	ENST00000266126	c.638A>G	p.Glu213Gly	rs104894425	0.00006	Pathogenic

* Maximum reported population allele frequency for any single sub-population represented in ExAC, 1000 Genomes or Exome Variant Server datasets. "-" indicates allele not present in these databases.

Key: AF – Allele Frequency; CDS – Coding DNA Sequence; ENST – Ensembl Gene Spliced Transcript; Het –Heterozygous; Hom – Homozygous; rs – Reference Single Nucleotide Polymorphisms

S3: Cases with pathogenic variants identified by WES in genes not associated with leukodystrophy.

Family	Gene	Zygosity	Transcript	CDS	Protein	rsID	max AF*	Classification
LD_0106	GRIN1	Het, *de novo*	ENST00000371546	c.2593C>T	p.Arg865Cys	-	-	Likely pathogenic
LD_0115	AARS	Het	ENST00000261772	c.2251A>G	p.Arg751Gly	rs143370729	0.00012	Pathogenic
		Het	ENST00000261772	c.242A>C	p.Lys81Thr	-	-	Pathogenic
LD_0119	KCNT1	Het, *de novo*	ENST00000298480	c.2794T>A	p.Phe932Ile	-	-	Pathogenic
LD_0157	SZT2	Het, *de novo*	ENST00000562955	c.5499del	p.Pro1833fs	-	-	Pathogenic
		Het	ENST00000562955	c.6916G>A	p.Gly2306Arg	-	-	Likely pathogenic
LD_0158	CNTNAP1	Hom	ENST00000264638	c.1163G>C	p.Arg388Pro	-	0.00006	Likely pathogenic
LD_0232	MRPS22	Het	ENST00000310776	c.741dup	p.Lys248fs	-	-	Pathogenic
		Het	ENST00000310776	c.572G>A	p.Arg191Gln	-	0.00010	Likely pathogenic
LD_0286	RMND1	Hom	ENST00000367303	c.713A>G	p.Asn238Ser	rs144972972	0.00047	Likely pathogenic
LD_0333	CNTNAP1	Het	ENST00000264638	c.319C>T	p.Arg107*	-	-	Pathogenic
		Het	ENST00000264638	c.967T>C	p.Cys323Arg	-	0.00003	Likely Pathogenic
LD_0358	STXBP1	Het, *de novo*	ENST00000373302	c.1099C>T	p.Arg367*	-	-	Pathogenic
LD_0366	GATAD2B	Het, *de novo*	ENST00000368655	c.820C>T	p.Gln274*	-	-	Pathogenic
LD_0463	ALS2	Het	ENST00000264276	c.3415C>T	p.Arg1139*	-	0.00003	Pathogenic
		Het	ENST00000264276	c.3248G>A	p.Gly1083Glu	-	-	Pathogenic
LD_0607	TERT	Het, *de novo*	ENST00000310581	c.2455C>T	p.Arg819Cys	-	-	Pathogenic
		Het	ENST00000310581	c.1990G>A	p.Val664Met	-	-	Pathogenic
LD_0646[19]	NDUFS7	Hom	ENST00000414651	c.403C>T	p.Arg135Cys	-	-	Likely pathogenic
LD_0678	ATM	Het	ENST00000278616	c.824del	p.Leu275fs	-	-	Pathogenic
		Het	ENST00000278616	c.8266A>T	p.Lys2756*	rs371638537	0.00012	Pathogenic
LD_0755	SDHAF1	Hom	ENST00000378887	c.164G>C	p.Arg55Pro	rs137853193	-	Pathogenic
LD_0756	SDHB	Hom	ENST00000375499	c.143A>T	p.Asp48Val	rs202101384	0.00036	Pathogenic

LD_0846	GLB1	Het	ENST00000445488	c.586C>A	p.Arg196Ser	rs192732174	0.00132	Pathogenic
		Het	ENST00000445488	c.718T>C	p.Tyr240His	-	-	Pathogenic
LD_0857	AARS	Hom	ENST00000261772	c.2251A>G	p.Arg751Gly	rs143370729	0.00012	Pathogenic

* Maximum reported population allele frequency for any single sub-population represented in ExAC, 1000 Genomes or Exome Variant Server datasets. "-" indicates allele not present in these databases.

Key: AF – Allele Frequency; CDS – Coding DNA Sequence; ENST – Ensembl Gene Spliced Transcript; Het –Heterozygous; Hom – Homozygous; rs – Reference Single Nucleotide Polymorphisms

Table S4 Cases with potentially pathogenic variants identified with whole exome sequencing.

Family	Gene	Zygosity	Transcript	CDS	Protein	rsID	Max AF*	Classification
LD_0493	FLNA	Hem, *de novo*	ENST00000369850	c.6812T>G	p.Leu2271Arg	-	-	VUS
LD_0664	FUS	Het	ENST00000568685	c.1500_1507	p.Gly500fs	-	-	Pathogenic
LD_0673	AMPD2	Hom	ENST00000256578	c.2528G>A	p.Arg843His	-	0.00006	VUS
LD_0675	AARS2	Het	ENST00000244571	c.2893G>A	p.Gly965Arg	rs543267101	0.00035	Likely pathogenic
		Het	ENST00000244571	c.1213G>A	p.Glu405Lys	-	0.00009	VUS
LD_0821	NDUFA2	Het	ENST00000252102	c.134A>C	p.Lys45Thr	-	0.00008	VUS
		Het	ENST00000252102	c.225del	p.Thr75fs	-	-	VUS

* Maximum reported population allele frequency for any single sub-population represented in ExAC, 1000 Genomes or Exome Variant Server datasets. "-" indicates allele not present in these databases.

Key: AF – Allele Frequency; CDS – Coding DNA Sequence; ENST – Ensembl Gene Spliced Transcript; Het –Heterozygous; Hom – Homozygous; rs – Reference Single Nucleotide Polymorphisms

Leukodystrophy Study Group Authors and Affiliations:

Andrea L. Gropman, MD[1], Tena Rosser, MD[2], Phillip L. Pearl, MD[3], Eva Fung, MBChB[4], Sumit Parikh, MD[5], Bruce H. Cohen, MD[6], James D. Reggin, MD[7], Cengiz Yalcinkaya, MD[8], Yuval Shafrir, MD[9], Marc DiFazio, MD[1] Emily Freilich, MD[1], Charles M. Lourenco, MD[10], Carolina Tesi-Rocha, MD[11], Jay Desai, MD[12], Hernan Amartino, MD[13], K. Nicole Weaver, MD[14], Valynne Long MS CGC[15], Michael J. Gambello, MD PhD[15], Melissa L. Cirillo, MD[16], Ilana Kahn, MD[1], Deepak Gill, BsC FRACP[17], Maria Gieron, MD[18], Emily de Los Reyes, MD[19], Bennett Lavenstein, MD[1], Brendan C. Lanpher, MD[20], Gerhard Kurleman, MD[21]

1. Department of Neurology, Children's National Medical Center, Washington, DC, USA
2. Division of Neurology, Children's Hospital Los Angeles, California, Keck School of Medicine of University of Southern California, Los Angeles, California
3. Division of Epilepsy and Clinical Neurophysiology, Boston Children's Hospital, Boston, MA USA
4. Department of Paediatrics, The Chinese University of Hong Kong
5. Department of Neurology, Center for Child Neurology, Cleveland Clinic Children's Hospital, Cleveland, Ohio, USA
6. Department of Pediatrics, NeuroDevelopmental Science Center, Children's Hospital Medical Center of Akron, Akron, Ohio, USA
7. Providence Sacred Heart Medical Center and Children's Hospital, Spokane, WA, USA
8. Division of Pediatric Neurology, Department of Neurology, Cerrahpasa School of Medicine, Istanbul University, Istanbul, Turkey
9. Department of Pediatrics, Sinai Hospital, Baltimore, MD, USA
10. Neurogenetics Unit, Department of Medical Genetics, School of Medicine, University of Sao Paulo, Ribeirao Preto, Brazil
11. Department of Neurology and Neurological Sciences, Stanford University Medical Center, Palo Alto, CA, USA
12. Division of Neurology, Children's Hospital Los Angeles, California, Keck School of Medicine of University of Southern California, Los Angeles, California
13. Hospital Universitario Austral, Buenos Aires, Argentina
14. Division of Human Genetics, Department of Pediatrics, Cincinnati Children's Hospital Medical Center and University of Cincinnati, Cincinnati, OH, USA
15. Department of Human Genetics, Division of Medical Genetics, Emory University School of Medicine, Atlanta, GA, USA
16. Division of Neurology, Ann & Robert H. Lurie Children's Hospital of Chicago, Department of Pediatrics, Northwestern University Feinberg School of Medicine, Chicago, IL, USA
17. Department of Neurology, The Children's Hospital at Westmead, Sydney, Australia
18. Departments of Pediatrics and Neurology, Morsani College of Medicine, Tampa, FL, USA
19. Division of Child Neurology, Ohio State University, Columbus, OH, USA
20. Department of Genetics, Children's National Medical Center, Washington, DC, USA
21. Department of General Pediatrics, Neuropediatrics, University Children's Hospital Muenster, Münster, Germany