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THE EFFICACY OF WHOLE-GENOME SEQUENCING IN THE
DIAGNOSIS OF COMPLEX NEUROLOGICAL PHENOTYPES

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of the requirements for the degree of
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Sarah Lawrence College

ABSTRACT

Whole-genome sequencing (WGS) is being increasingly utilized for the diagnosis of neurological disease. The advent of next-generation sequencing (NGS) has replaced Sanger sequencing due to its ability to sequence millions of fragments in parallel, in real-time. It's application in targeted gene panels and whole-exome sequencing (WES) has revolutionized standard investigation practices of neurodevelopmental diseases (NDDs). WGS utilizes NGS technology in order to sequence beyond the exome and into the remaining 98-99% of the genetic code comprising the genome. In addition to increased coverage, WGS allows for the detection of novel gene variants, copy number variants (CNVs) and single nucleotide variants (SNVs) that are not traditionally picked up by WES. Furthermore, RNA sequencing (RNA-Seq) of the blood used in conjunction with WGS may have the ability to validate WGS findings by analyzing gene expression in addition to identifying novel RNA species within the transcriptome. The objective of this retrospective study was to measure the diagnostic yield of trio-based WGS and RNA-Seq against that of negative or inconclusive WES in a patient cohort comprised of complex neurological phenotypes. Whole genome sequencing was performed by Medical Neurogenetics LLC, a CLIA-certified laboratory in Atlanta, Georgia. This laboratory utilized the Illumina NovaSeq 6000 Sequencing System, with a goal of 30x coverage of 99% of mapped genome regions. Alignment and variant interpretation was performed by Dragen v2.2 and CNV analysis by Dragen v2.5. Variants were assessed in accordance with current ACMG criteria. WGS with complementary RNA-Seq resulted in 7 solved patient cases, providing a 31.8% yield. This phenotypically complex cohort was comprised of a spectrum of neurological conditions with suspected underlying genetic mechanisms. The use of WGS in conjunction with RNA-Seq resulted in a markedly increased diagnostic yield over that of preceding WES and conventional

first-tier tests which included chromosomal microarray, targeted gene panels, and metabolic testing. Thus, proving its efficacy in the clinical setting.

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INTRODUCTION

Technological advances in genetic sequencing over the last decade have had considerable impact on genetic testing in both clinical and research settings. Next generation sequencing (NGS) has consequently replaced Sanger sequencing when testing for phenotypically variable syndromes due to its ability to sequence large numbers of genes simultaneously at a reduced cost. This has led to NGS being applied to targeted enrichment methods in addition to whole-exome sequencing (WES) and whole-genome sequencing (WGS) techniques (Petersen et al., 2017). The high resolution of NGS technology utilized in modern-day gene sequencing has allowed for the accelerated discovery of neurodevelopmental disease (NDD) associated genes (Srivastava et al., 2019).

There is a high level of complexity with respect to the evaluation of NDDs and the introduction of WGS to current first-tier standard diagnostic testing technologies. Current workup of NDDs in the clinical setting include chromosomal microarray analysis (CMA), karyotype, MRI and/or ultrasound imaging, Fragile X syndrome (FXS) testing, metabolic testing, and mitochondrial DNA (mtDNA) sequencing (Srivastava et al., 2019). In addition to these first-tier tests, WES has also been applied in clinical settings due to its significantly higher diagnostic yield in comparison to that of CMA.

A molecular diagnosis is important for patients living with NDDs and their families as it may impact clinical management (Srivastava et al., 2019). Introducing trio-based WGS subsequent to current first-tier tests with inconclusive WES, increases diagnostic yield due in part to its increased genomic coverage and ability to capture copy number variants (CNVs) and single nucleotide variants (SNVs). By applying a trio-based approach, novel variants can be ruled in or out during variant analysis and interpretation processes.

Whole-Genome Sequencing vs. Whole-Exome Sequencing

The exome encompasses approximately 95% of the protein-coding regions of the genome and comprises approximately 20,000 genes, while accounting for just 1-2% of the genome itself (Alfares et al., 2018). It is thought that approximately 85% of the pathogenic variants responsible for Mendelian conditions occur in these protein-coding regions of the genetic code or within canonical splice sites (Majewski et al., 2011). Genetic variants associated with complex traits, such as those associated with NDDs, may occur outside of these protein-coding sequences. These genetic variations arise within the remainder of the genome and may affect regulatory elements required for gene expression such as promoter regions, enhancer regions, and silencer sequences (Dinger et al., 2018). Due to WGS' ability to reach beyond the exome and capture the intronic regions not picked up by WES, it is currently considered the most comprehensive genetic test available (Gilissen et al., 2014).

Moreover, it appears that WGS provides better coverage of exons and is less susceptible than WES to distortions and blind spots in addition to being better able to detect copy CNVs and complex genomic rearrangements, such as inversions (Mattick et al., 2018). In addition, WGS is less sensitive to GC content, increasing its likelihood of providing complete coverage within GC-rich regions (Odgerel et al., 2019). WGS has facilitated the discovery of novel pathogenic variants in addition to the CNVs, genomic rearrangements, and SNVs that have been strongly associated with Mendelian conditions that are not picked up by traditional WES (Srivastava et al., 2019).

The Benefit of RNA-Sequencing Analysis

In addition to WGS, other multi-omic technologies such as epigenomics, lipidomics, metabolomics, and transcriptomics are sometimes being used as diagnostic tools for clinical cases. Evaluation of the transcriptome by RNA-Seq provides the benefit of analyzing the RNA by looking at alternative gene spliced transcripts, post transcriptional modifications, gene fusions, SNVs, changes in gene expression over time and differences in gene expression among different tissues. When used in conjunction with WGS, assays which assess the impact of variants at the mRNA level, such as RNA-Seq, prove beneficial in evaluating the outcomes of variants within deep intronic regions, splice junctions, and untranslated regions (Richards et al., 2015). Identifying genomic variation is important for establishing a relationship between genotype and phenotype and provides further insight into human disease (Piskol, Ramaswami, & Li., 2013).

Whole-Genome Sequencing Limitations

Currently, a key limitation of WGS as a first-tier diagnostic test in the clinical setting is cost. The cost of the sequencing technology in addition to the cost of labour required during the interpretation process and the disclosure of results is a significant obstacle. WGS will likely become more widely used in the future as the cost decreases over time. Furthermore, ACMG guidelines are insufficient for the classification of deep intronic variants that can be detected by WGS (Richards et al., 2015). The current guidelines consist of a 5-tiered system of classification relevant for variants within genes responsible for Mendelian disease, majority of which are exonic.

Study Objectives

This retrospective study reports on the diagnostic yield of trio-based WGS with RNA-Seq in a cohort of patients who had initially been referred for evaluation of a complex neurological phenotype suspicious for an underlying genetic condition. All probands had negative or inconclusive WES results within the antecedent five years. The objective was to assess the diagnostic value of WGS and RNA-Seq after a negative or inconclusive WES result.

MATERIALS & METHODS

Whole Genome Sequencing was performed by CLIA-certified laboratory Medical Neurogenetics, LLC in Atlanta, Georgia (CLIA ID# 11D0703390; George Lab Lic. Code 060-381). Sequencing was performed by Illumina NovaSeq 6000 with a goal of 30x coverage of over 99% of uniquely mappable regions of the genome.. Alignment and variant interpretation (SNV and CNV) was performed by Dragen v2.6. Variants were assessed according to ACMG criteria (Richards et al., 2015). Sequencing of the mitochondrial genome was performed on an Illumina NovaSeq with a goal of >500-fold average coverage. Repeat expansions for *ATNI*, *ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *ATXN8*, *ATXN10*, *C9ORF72*, *TBP*, and *ZNF9* was assessed with ExpansionHunter version v2.5.5 and findings confirmed by repeat-primed polymerase chain reaction (Dolzhenko et al., 2017).

In addition to WGS sequencing, RNA sequencing was also performed by Medical Neurogenetics. Next generation sequencing (NGS) libraries were created using the TruSeq Stranded Total RNA library (Illumina) kit. Sequencing was also performed on an Illumina NovaSeq 6000 instrument with a targeted sequencing yield of 22 giga base pairs (Gbp). RNA secondary analysis was performed following the Nature protocol, “Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown”. Additional RNA-seq analysis was performed using in-house developed analysis for differential expression and small variant calling by GATK HaplotypeCaller v4.1.2.0.

Demographic Data

The patient cohort consisted of 22 patients from 20 families who had initially been referred for evaluation of a complex neurological phenotype suspicious for an underlying genetic condition. The participants in this study were assessed in a pediatric and adult neuromuscular/neurogenetics clinic in Hamilton ON, Canada. The patient cohort was diverse with respect to ethnicity, age at first presentation, and age at which they underwent WGS (current median age: 20 y, current age range: 4 – 49 y). All cases had negative or indeterminate WES results within the antecedent five years. Detailed clinical summaries are available for each patient in the supplemental files.

Solved vs. Unsolved Cases

For the purpose of this study we define a case as “solved” when the variant interpretation and analysis reveals a molecular explanation of the patient phenotype regardless of the WGS outcome. We have provided corresponding numbers from the Online Mendelian Inheritance in Man (OMIM) database for the phenotypes described during case analyses.

RESULTS

In total, each of the 22 patients enrolled in the study had a complete clinical and final result available from WGS. Of these, 3 (13.6%) had positive WGS results, 16 (72.7%) had indeterminate results, and the 3 (13.6%) remaining patients had negative WGS results. When analyzed in conjunction with RNA-Seq results, we received a combined total of 7 (31.8%) solved patient cases (Table 1). WGS results are current as of 2019, reanalysis has not yet been performed. The option of learning ACMG actionable secondary findings was given to each patient/family.

Table 1. Summary of WES, WGS, and RNA-Seq results for all 20 participants

Patient Number	Sex	Clinical Description	WES Results					WGS Results					RNA-Seq Results	Outcome	
			Family	Gene	Nucleotide Change	Amino Acid Change	Origin	Variant Classification	Family	Gene	Nucleotide Change	Amino Acid Change			Origin
1	F	Non-verbal, global developmental delay, seizures, hand flipping/hand obsession, complex I deficiency, slightly underdeveloped corpus callosum and decreased volume of white matter	Trio	Negative				Trio	Negative					Negative	Unsolved
2	F	Neurodegenerative disorder with progressive ataxia, spasticity, seizures, cerebellar atrophy, and retinitis pigmentosa	Duo	Negative				Duo	<i>SHANK3</i> <i>REFLN</i>	c.286G>A c.230C>T	p.Ala953Thr p.Thr77Ile	undetermined mut	VUS VUS	Negative	Unsolved
3	M	Non-verbal, intellectual disability, macrocephaly, periventricular white matter changes, cyanotic seizure-like episodes	Trio	Negative				Trio	Negative					Negative	Unsolved
4	F	Neurodegenerative disorder with regression at 12 months, epilepsy, ataxia, spasticity, non-verbal, brain biopsy suggestive of a necrotizing encephalopathy	Trio	<i>SDH4</i>	c.1532T>C	p.Leu511Pro	pat	VUS	Trio	<i>ENTPD5</i> <i>ENTPD5</i>	c.916G>A c.1229C>T	p.Gln306Lys p.Thr410Met	mut pat	VUS VUS	Negative Unsolved
5	M	Slowly neurodegenerative disorder with cognitive decline, retinitis pigmentosa, scoliosis, cerebellar atrophy, bilateral sensorineural hearing loss, generalized weakness and muscle atrophy, ataxia, and contractures	Trio	<i>MTO1A</i>	c.1165G>A	P_Asp389Asn	pat	VUS	Trio	<i>MTO1A</i>	c.1165G>A	P_Asp389Asn	pat	VUS	Negative Unsolved
6a	M	Dyzygotic twin brothers with epilepsy, Quad severe intellectual disability, choreoathetoid movements, and spasticity	Quad	<i>IDH2</i>	c.439G>C	p.Val147Leu	mut	VUS	Quad	<i>IDH2</i>	c.439G>C	p.Val147Leu	mut	VUS	Negative Unsolved
6b	M			<i>MTH2</i>	c.2963T>C	p.Met688Thr	pat	VUS		<i>MTH2</i>	c.2963T>C	p.Met688Thr	pat	VUS	
				<i>PKX</i>	c.1465G>A	p.Ala489Thr	mut	VUS							

Notes: All variants are heterozygous unless otherwise specified. VUS: variant of uncertain significance, LP: likely pathogenic, P: pathogenic

Table 1 (continued).

Patient Number	Sex	Clinical Description	WES Results					WGS Results								
			Family	Gene	Nucleotide Change	Amino Acid Change	Origin	Variant Classification	Family	Gene	Nucleotide Change	Amino Acid Change	Origin	Variant Classification	RNA-Seq Results	Outcome
7	F	Atypical neurodegeneration with brain iron accumulation (NBIA), progressive cognitive decline, ataxia, rigidity/spasticity, spastic bladder, and retinal detachment with retinitis pigmentosa	Trio	<i>USH2A</i> <i>PANK2</i>	c.2276G>T c.1052C>T	p.Cys799Phe p.Ser351Leu	mat mat	P LP	Trio	<i>ACBD5</i> <i>ACBD5</i>	c.925G>T Exon 4 deletion (Chr10:27,510,501-27,513,251 (GRCh37))	p.Gly309Ter pat	mat pat	LP P	Negative	Solved - <i>ACBD5</i>
8	F	Progressive weakness, ataxia, optic atrophy, ophthalmic neuronal dystrophy, spasticity, peripheral neuropathy, complex I-III deficiency, and 3-methylglutaconic aciduria	Trio	Negative					Trio	Negative					Negative	Unsolved
9	F	Moderate intellectual disability, ataxia, pure motor neuropathy/neuropathically dysarthria, CoQ10 deficiency in muscle	Trio	<i>PLEKHG5</i>	c.2695G>A	p.Gly89Ser	pat	VUS	Trio	<i>AP0A1BP</i> <i>EXOSC8</i>	c.318delG c.98G>A (homo)	p.Ser107AlaLeu*49 p.Arg31Lys	mat mat + pat	LP VUS	Negative	Solved - <i>EXOSC8</i>
10a	F	Intellectual disability, progressive ataxia, scanning speech, vision loss, spasticity, dysarthria, dysphagia, bowed and bladder incontinence, and mild proximal weakness	Duo	Negative					Quad	<i>SLC44I1</i>	Exon 3 deletion (Chr8:108,066,751-108,095,501 (GRCh37)) (homo)	mat + pat	pat	P	Negative	Solved - <i>SLC44A1</i>
10b	M															
11	M	Severe distal axonal peripheral neuropathy, progressive dysphagia, global developmental delay, congenital cataracts, glaucoma, cerebral dysplasia, myositis, and retinal detachment	Duo	<i>POLG</i> <i>DNM2</i> <i>ZNF469</i>	c.2852A>C c.2228C>T c.7469C>A	p.Tyr951Ser p.Pro543Leu p.Pro3490His	undetermined mat undetermined	LP VUS VUS	Duo	<i>POLG</i> <i>DNM2</i> <i>ZNF469</i>	c.2852A>C c.2228C>T c.7469C>A	p.Tyr951Ser p.Pro543Leu p.Pro3490His	undetermined mat undetermined	LP VUS VUS	Negative	Solved - <i>POLG</i>
12	F	Focal epilepsy, global developmental delay, microcephaly, hypotonia, hypermobility, tea-shaped mouth	Trio	<i>TSEN54</i>	c.919G>T	p.Ala307Ser	pat	P	Trio	<i>TSEN54</i> <i>ASS1</i>	c.919G>T c.1186G>A	p.Ala307Ser p.Gly390Arg	pat pat	P P	Negative	Unsolved

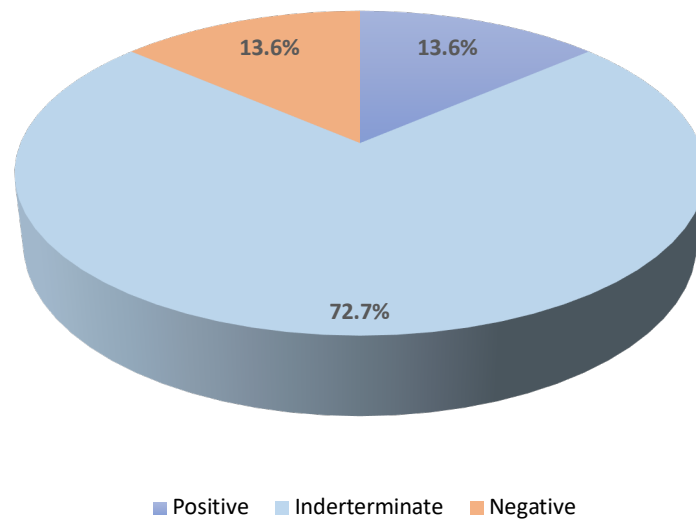
Notes: All variants are heterozygous unless otherwise specified. VUS: variant of uncertain significance, LP: likely pathogenic, P: pathogenic

Table 1 (continued).

WES Results											WGS Results					
Patient Number	Sex	Clinical Description	Family	Gene	Nucleotide Change	Amino Acid Change	Origin	Variant Classification	Family	Gene	Nucleotide Change	Amino Acid Change	Origin	Variant Classification	RNA-Seq Results	Outcome
13	F	Developmental regression, epileptic encephalopathy, infantile spasms/hypsarrhythmia, Lennox-Gaitani syndrome, severe global developmental delay, microcephaly, diffuse cerebellar atrophy	Trio	<i>RAMD1</i>	c.713A>G	p.Asn238Ser	pat	Pathogenic	Trio	<i>RAMD1</i>	c.713A>G	p.Asn238Ser	pat	Pathogenic	Negative	Unolved
				TKT	c.133G>A	p.Ala45Thr	mat	VUS		TKT	c.133G>A	p.Ala45Thr	mat	VUS		
				TKT	c.976C>T	p.Arg236Cys	pat	VUS		TKT	c.976C>T	p.Arg236Cys	pat	P		
14	F	Intellectual disability and movement disorder with myoclonus, dysomia, chorea, supranuclear vertical gaze palsy, and spasticity	Trio	Negative					Duo	<i>SCN3A</i>	c.2204A>G (homo)	p.Asn735Ser	undetermined	VUS	Negative	Unolved
15	M	Congenital hypopneumating neuropathy	Trio	<i>SETX</i>	c.23C>T	p.Thr8Met	de novo	VUS	Trio	<i>SETX</i>	c.23C>T	p.Thr8Met	de novo	LP	Negative	Solved - SETX
				<i>IGHMBP2</i>	c.2176G>A	p.Val720Met	pat	VUS		<i>IGHMBP2</i>	c.2176G>A	p.Val720Met	pat	VUS		
16	F	Moderate intellectual disability, postnatal weakness, spasticity, diffuse cerebellar volume loss in the vermis, poor balance	Trio	Negative					Trio	<i>RAMD1</i>	c.7594pA	p.His254Thrfs*2	de novo	P	Negative	Unolved
										<i>SGT2L6</i>	c.2230C>T	p.Arg744Ter	pat	LP		
17	F	Severe global developmental delay, epilepsy, dysmorphic features, hypertronia, hyperreflexia, cortical visual impairment, cerebellar atrophy and diffuse volume loss on MRI	Trio	Negative					Trio	<i>RAMBP2</i>	c.5579A>C	p.Lys1800Thr	mat	VUS	Negative	Unolved
										<i>CHRNA2</i>	c.859_862delinsGC	p.Phe287_Tyr288del	pat	VUS		
18	F	Spinocerebellar ataxia/heelclary spastic paraplegia overlap, progressive cerebellar atrophy, intellectual disability, myalgias, complex FTH deficiency	Trio	Negative					Trio	<i>KIF1A/656</i>	c.922C>T	p.Gln308Thr	pat	LP	Negative	Unolved
										<i>PNP7T</i>	c.1441+2T>A	splice site variant	mat	LP		
										<i>SGT3A48</i>	c.610C>T	p.Gly206Cys	mat	LP		
19	F	Cognitive decline, dysphasia, neuropsychiatric features (mood and personality changes, anxiety, psychosis, aggression, hallucinations)	Trio						Duo	Negative					Negative	Unolved
				<i>TP53J1</i>	c.588C>T	p.Arg1962Cys	pat	VUS								
				<i>TP53J4</i>	c.869G>T	p.Ile2892Ile	mat	VUS								
20	F	Spinocerebellar ataxia, optic atrophy, intellectual disability, complex I	Singleton	<i>SLC25A10</i>	c.449G>A	p.Arg150H	<i>in trans</i>	VUS	Trio	<i>ACAD9</i>	c.244+1A>G (homo)	splice site variant	mat+pat	LP	Abnormal exon splicing - ACAD9	
				<i>SLC25A10</i>	c.574C>T	p.P92S		VUS								

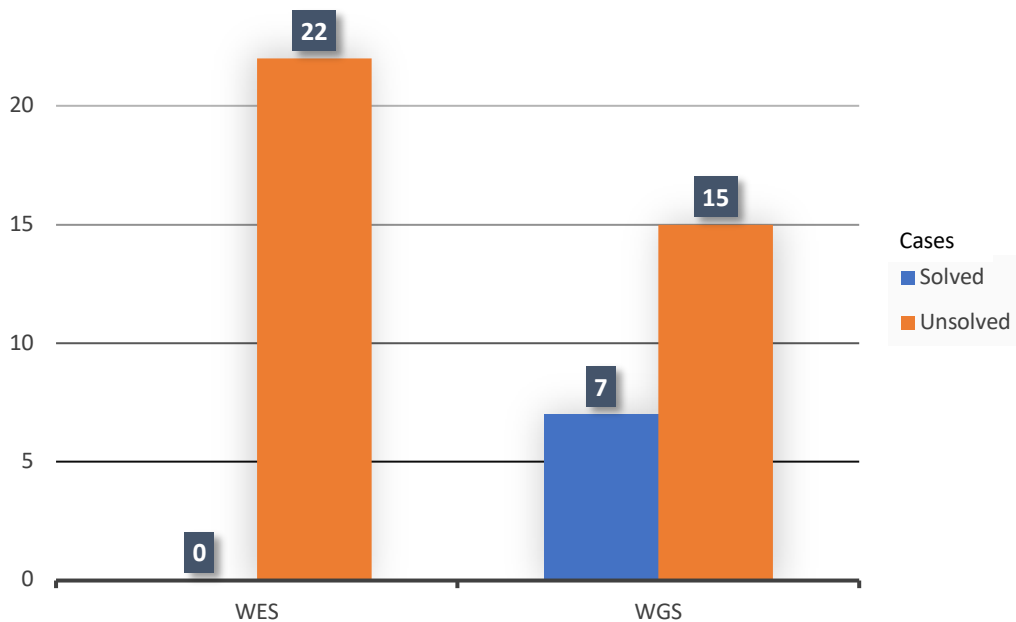
All variants are heterozygous unless otherwise specified. VUS: variant of uncertain significance, LP: likely pathogenic, P: pathogenic

Figure 1. WGS Results



Notes: Chart of WGS results showing the proportion of patient cases that yielded a negative, positive, or indeterminate result.

Figure 2. Diagnostic yield of WGS vs WES



Notes: When analyzed in conjunction with RNA-Seq results, we received a combined total of 7 (31.8%) solved patient cases (Table 1). WGS results are current as of 2019, reanalysis has not yet been performed. The option of learning ACMG actionable secondary findings was given to each patient/family.

DISCUSSION

In this study, WGS and RNA-Seq was performed for 22 patients with complex neurological phenotypes and previous negative or indeterminate WES results. The goal was to assess the diagnostic yield of WGS and RNA-Seq in patients with nondiagnostic WES results. Many studies have been conducted to report the diagnostic yield of WGS as a first-line test among a variety of conditions. In these study populations, the patients have either had no prior genetic testing or have undergone an array of genetic testing including targeted gene sequencing, sequencing panels, CMA, or WES. Our study differs as we are evaluating the diagnostic yield of WGS in a population who homogeneously had inconclusive or negative WES completed prior to.

At typical sequencing depth, WGS offers improved uniformity of exonic coverage compared to that employed by WES, in addition to increased coverage and longer read lengths. (Wilfert et al., 2017). Demonstrated in Supplemental Case 9, WGS (trio) identified homozygous *EXCOSC8* variants inherited maternally and paternally while WES (trio) in 2016 was negative. The lower coverage exhibited by WES in comparison to WGS in that region resulted in the variants being missed. The *EXCOSC8* variants at c.98G>A (p.Arg33Lys) has been classified as likely pathogenic, accurately representing the patients unexplained phenotype rendering the case solved.

WGS has the ability to sequence beyond the protein-coding regions of the genome and detect genomic variation outside of these regions, which play a role in the development of complex traits and diseases (Alfares et al., 2018). WGS offers improved uniformity of exonic coverage compared to that employed by WES when restricted to regions covered by both platforms. For example, in gnomAD, 89.4% of the exome was covered by WES at 20x coverage while WGS covered 97.1% at this same threshold.

It is known that WGS allows for superior detection of copy number variations in known disease genes over both WES and CMA. This includes *de novo* mutation (DNM) identification in genes which were previously restricted to large CNVs. The identification of small deletions in known/candidate disease genes led to a diagnosis in Case 7 and sibling Cases 10a/10b.

WES for siblings 10a and 10b (duo) was completed in 2015 and was negative. WGS (quad) of the sibling pair in addition to each parent identified a homozygous deletion of exon 3 within *SLC44A1* in both siblings. This deletion was predicted to result in a frameshift at amino acid position 90 and the subsequent termination of 18 codons (Chapelle et al., 2019). *SLC44A1* encodes for choline transporter-like protein 1, which has recently been associated with a childhood-onset neurodegenerative condition with progressive ataxia, dysphagia, tremors, cognitive decline, dysarthria, and optic atrophy (Fagerberg et al., 2020).

For Case 7, WGS was able to detect a deletion in exon 4 of *ACBD5* which was not identified by CMA or WES. The patient, a 29-year-old female, was known to have one maternally inherited heterozygous variant of uncertain clinical significance in *ACBD5* (c.925G>T, p.Gly309Ter). At the time WES was performed in 2015 *ACBD5* was listed as a candidate gene not yet associated with a human phenotype. WGS identified the maternally inherited *ACBD5* variant in addition to the novel discovery of the exon 4 deletion, which was of paternal origin. RNA-Seq detected the deletion in *ACBD5* in approximately 48% of reads covering its position at c.935G>T (Table 1).

Additionally, RNA-Seq was proved an added benefit to WGS in this cohort and provided a diagnosis for Case 20. WES for Case 20 was performed in 2017 and identified several variants of interest, including a homozygous splice site variant of uncertain significance in *ACAD9* (c.244+3A>G) and compound heterozygous variants (*in trans*) in candidate gene *SLC25A10*

(c.G449A, p.R150H and c.C574T, p.P192S). The *ACAD9* homozygous variants were not felt to be diagnostic at the time because there was not a match with the described phenotype. The classic phenotype of *ACAD9*-related mitochondrial complex I deficiency is muscle weakness, hypotonia, cardiomyopathy, liver failure, encephalopathy, and Reye-like episodes in infancy/childhood. The *ACAD9* homozygous splice site variant was again identified by WGS and RNA-Seq in blood confirmed that exon splicing was affected by this variant. Ataxia is still a rarely reported phenotype of *ACAD9*-related mitochondrial complex I deficiency and it is likely that this patient represents a milder phenotype of this condition.

In Cases 11 and 15, while unable to identify new variants or provide multi-omic support to the pathogenicity of variants, WGS and RNA-Seq led to a final diagnosis by acting as a reanalysis due to updated medical knowledge. Case 15 is a 9 year old male with congenital hypomyelination syndrome. WES in 2014 identified a heterozygous *de novo* variant of uncertain significance in *SETX* (c.23C>T, p.Thr8Met). Some heterozygous variants in *SETX* have been associated with juvenile ALS type 4 (OMIM: 602433) while some biallelic variants have been associated with ataxia-oculomotor apraxia type 2 (OMIM: 606002). The *SETX* (c.23C>T, p.Thr8Met) variant has since been reclassified as “likely pathogenic” as we have been made aware of a few other cases of children with the same *de novo* variant in *SETX* and identical phenotype. Further work is being done to better characterize and publish this possible expansion in phenotype.

Case 11 was known to have a likely *de novo*, likely pathogenic variant in *POLG* which was felt to explain part of his phenotype. A recent publication by Castiglioni et al. in 2018 describes an expansion of the *POLG* phenotype which matches most of Case 11’s clinical symptoms. WGS also identified biallelic variants of uncertain significance in *ZNF469* potentially

associated with Brittle cornea syndrome 1 (OMIM: 229200) and a heterozygous variant in *EPHA2* of unknown parental origin possibly associated with autosomal dominant cataracts type 6, multiple types (OMIM: 116600). It is presently unclear if these variants are playing any role in the complex eye phenotype of this patient.

CONCLUSION

In summary, WGS is revolutionizing the ability to diagnose complex neurological phenotypes associated with NDDs in addition to acute monogenic disorders. With the ability of WGS to poll more of the exome and reach the remaining 98-99% of the genetic code, WGS has the ability to produce a higher diagnostic return. In our study, a 31.8% diagnostic yield is reported by WGS subsequent to inconclusive or negative WES offered homogenously to this patient cohort.

Other studies have reported the diagnostic yield of WGS to be as high as 21-34% in individuals with broad spectrum disorders or congenital malformations and neurodevelopmental disorders. These results parallel the results of our study. For example, Lionel et al. reported the diagnostic yield of WGS to be as high as 41%, which was higher than that of WES by approximately 26% (Lionel et al., 2018). It is worth mentioning that studies to support this claim are few, and the patient population in this study did not homogenously undergo WES prior to WGS. This emphasizes the need for additional studies directly comparing the diagnostic yield of WGS when performed subsequent to indeterminate or negative WES among the same patient population. In contrast to ours, a study by Alfares et al. found that prior to reanalysis, WGS yielded a positive result in only 7% of their patient population subsequent to previously negative WES results (Alfares et al., 2018).

In addition to being less susceptible to distortions, rearrangements, and blind spots, WGS is also less sensitive to GC-rich content. With its additional ability to detect CNVs and SNVs, WGS identifies novel variants that are not identified by conventional or WES. Ultimately, WGS provides the complete dataset of an individual's genetic composition.

Current ACMG classification guidelines do not directly apply to variants identified within deep intronic regions. This may be a contributing factor to the many variants resulting in negative or indeterminate findings within our study. Providing additional sequencing of the RNA transcriptome is becoming increasingly beneficial in establishing the significance of an increasing amount of VUS results being yielded by WES and WGS. RNA sequencing has proven useful in the identification of functional consequences of VUS results by providing examination at the mRNA level. It has improved our ability to understand unknown variants by establishing their effects within both regulatory regions and coding regions of the genome. It can be integrated with WGS in order to interpret exonic and intronic SNVs and structural variants to increase the diagnostic rate. To our knowledge, our study is one of few to integrate RNA-Seq of the blood transcriptome into WGS in hopes of establishing increased diagnostic yield of complex neurological phenotypes.

While our study focused on the benefits of NGS and diagnostic yield of WGS compared to WES, there are some limitations worth noting. Phenotypes presented in our study consisted only of complex neurological phenotypes, therefore, the results of our study may not be directly applicable across specialties. There is a need for more research on the efficacy of WGS outside of the neurologic setting.

A limitation to both WGS and WES is that a disease-causing variant may occasionally be overlooked if it has not yet been identified as such. If a variant is not yet identified as causative of a specific disease phenotype or the variant in question is within a gene of unknown function a diagnosis may be missed. This addresses the need for increased variant reporting especially in the case of extremely rare and phenotypically complex neurological diseases.

In addition, our study employed a trio-based approach to facilitate comprehensive interpretation of results, however, we did not address additional factors that may have influenced the diagnostic yield of WGS. For example, it was not clearly stated for each patient the degree of clinical involvement of a genetics team prior to their involvement with the neuromuscular clinic. This includes unknown frequency of consults with a geneticist and whether or not they had routine follow-ups with the genetics specialty prior to their involvement with our clinic. As a result, this may have directly impacted the amount of time elapsed before further comprehensive examination, testing and perhaps a diagnosis.

Analysis and discussion of secondary findings was beyond the scope of our study; however, participants were informed of the possibility of secondary findings as per ACMG. The option to report actionable secondary findings was provided to each patient case.

Conflicts of Interest

Author Bianca Blake declares that she has no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). A waiver of consent was obtained according to IRB protocol for the purpose of this case review.

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APPENDIX

Appendix I: Clinical Case Summaries

Case 1

An 11-y-old girl first presented at 16 mo of age with global developmental delay, hypotonia, bilateral ptosis, joint hypermobility, feeding difficulties, and failure to thrive (FTT). She was born weighing 5 lbs 8 oz at 36 wk by C-section after an induced labour due to placental abruption. At 20 mo of age she was not able to sit up on her own but could sit independently if placed in that position. She was 3 y old when she had her first febrile seizure and 5 y old when she had her first unprovoked generalized tonic-clonic seizure with loss of consciousness. At last examination, she has profound intellectual disability, continues to have seizures, and is nonverbal.

Karyotype analysis, array based comparative genomic hybridization (aCGH), *CDKL5*, Angelman and Rett syndrome testing were negative. Lactate levels ranged from 1.1 to 2.3 nmol/L (*N*: 0.5 – 2.2 nmol/L) and she had a normal CK. Muscle ultrastructure pathology showed a minimal amount of mitochondrial pleomorphism. Enzyme testing of skeletal muscle revealed a mild complex I + III deficiency. NGS of mtDNA from skeletal muscle was negative for deletions but revealed a homoplasmic variant in the *MT-ND4* subunit (m.11240C>T; p.Leu161Phe). This variant was also homoplasmic in her asymptomatic mother and was thus considered to likely be benign.

WES (trio) performed in 2014 was negative. In 2019, the result of WGS (trio) was negative. RNA-Seq of the entire fibroblast transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patients.

Case 2

This 33-y-old woman was born by uncomplicated delivery at term. She weighed 6 lb. 6 oz and her Apgar scores were within normal limits. She met all developmental milestones and there were no concerns until around 10 y of age when it was noticed that she had reduced vision. By 16 y of age she had been diagnosed with progressive visual loss due to retinitis pigmentosa. She could no longer perceive light by 32 y of age.

At 17 y of age she started to show symptoms of progressive ataxia, also affecting speech. She became pregnant at 23 y of age and gave birth to a male child. Her condition worsened after birth with the commencement of seizures and severe and sharp headaches. By 30 y of age it became clear that she had a progressive neurodegenerative disorder. She was displaying abnormal behaviors and significant emotional outbursts with possible hallucinations and decreasing cognition. Physical examination found progressive spasticity and ataxia. She passed away at 33 y of age.

Initial investigations included a muscle biopsy which showed normal pathology and muscle mitochondrial enzyme activities for complex I + III, IV and citrate synthase did not show a significant deficiency. Multiple brain MRIs showed significant atrophy of the cerebellum and vermis.

WES (trio) was performed in 2014 and was negative. WES re-analysis in 2016 was also negative. WGS (duo) in 2018 identified two variants of uncertain significance. The two heterozygous variants of uncertain significance were identified, one in each of the *SHANK3* and *RELN* genes. The *SHANK3* (c.2863G>A; p.Ala955Thr) was of unknown parental origin and was not identified in large databases. The *RELN* variant (c.230C>T; p.Thr77Ile) was inherited from a clinically unaffected mother. RNA-Seq of the entire blood transcriptome did not identify any

significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Case 3

This 16-y-old boy who presented at 22 mo of age with global developmental delay, macrocephaly (54 cm, >98th percentile), and periventricular white matter changes. He experienced a seizure-like episodes once to twice a month with lip smacking, cyanosis of the lips, and breath holding. These episodes were usually followed by vomiting. Evaluation by a geneticist suggested a clinical diagnosis of MOMO Syndrome (Macrosomia, Obesity, Macrocephaly, and Ocular Abnormalities – OMIM# 157980). He remains largely non-verbal.

Initial workup included mtDNA sequencing of blood and muscle tissue, genetic testing for Prader-Willi syndrome, a genetic epilepsy panel, transferrin isoelectric focusing, microarray, and metabolic screen. WES (trio) was performed in 2014 and was negative. WES re-analysis in 2016 was also negative. WGS (trio) was negative. RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Case 4

This 11-y-old female had a neurodegenerative phenotype with epilepsy, global developmental delays, and spasticity. Her development proceeded normally until ten mo of age when a stagnation in development and a slightly broad-based gait with stiff legs was noted. Regressions in her speech and motor skills became apparent at one year of age. Brain biopsy at three y of age was suggestive of a necrotizing encephalopathy. Seizures began around four y of age with a frequency of approximately five to eight per night. At five y of age she experienced an additional regression of cognitive and motor skills. She became fully non-verbal and experienced difficulties using her communication board. Due to increasing spasticity in her lower limbs she required assistance with walking (braces and orthotics). By the age of seven y her seizures occurred in clusters with a maximum of 25 within a timespan of 20 min each night. Each cluster involved multiple episodes of posturing and stiffened leg or arm stretches followed by post-ictal agitation. At age nine y of age she began having episodes of tonic stiffening during the day. Brain MRI at three and a half years of age showed multifocal areas of acute ischemia in the frontal lobes (bilateral), basal ganglia, and right cerebral peduncle. MR spectroscopy showed a decrease of NAA in relation to creatine and a lactate peak. Serial MRIs over the next several years showed continued slow progression.

Initial investigations included enzymatic testing of hexosaminidase, *B*-galactosidase, and arylsulfatase. Genetic testing for Rett and Angelman syndrome, microarray, mtDNA sequencing and mtDNA deletion/depletion analysis in muscle tissue, a leukodystrophy panel, and an epilepsy panel were negative. Transferrin isoelectric focusing and immunoperoxidase for parvoviruses were both negative. Muscle biopsy of the right vastus lateralis at three y of age showed unremarkable pathology. Mitochondrial work up on fibroblasts, including analysis for complex V deficiency and lactate to pyruvate ratio were negative.

WES was done in 2014 and identified one paternally inherited heterozygous likely pathogenic variant in *SDHA* (c.1532T>C; p.Leu511Pro). Biallelic pathogenic variants in *SDHA* have been associated with autosomal recessive Leigh syndrome (OMIM: 600857).

Deletion/duplication analysis of the *SDHA* gene was normal and no second variant was identified.

WGS (trio) was completed and identified compound heterozygous variants of uncertain significance (*in trans*) in *ENTPD5* (c.916G>A, p.Glu306Ly + c.1229C>T, p.Thr410Met). The *ENTPD5* gene encodes for an ectonucleoside triphosphate diphosphohydrolase but is currently a candidate gene and does not have any definitive disease associations in humans. No gene or phenotype matches were made through GeneMatcher (Sobreira et al., 2015). Transferrin isoelectric focusing was unremarkable. RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Case 5

A 25-y-old man was born at 42 week gestation with Apgar scores of 2 @ 1 min and 7 @ 10 min and was diagnosed with cerebral palsy in and this diagnosis persisted for the first 17 y of his life. He was first seen at 18 y of age with a complex phenotype of retinitis pigmentosa, scoliosis, cerebellar atrophy/hypoplasia, bilateral sensorineural hearing loss (SNHL), progressive generalized weakness, non-progressive ataxia, and significant contractures. During his most recent evaluation he reported a decline in strength and overall function with progressive cognitive decline and muscle atrophy.

Initial work-up included a normal; aCGH, skeletal muscle mtDNA sequencing, and genetic testing for; Friedreich ataxia, *POLG*, *FCMD*, *FKRP*, *POMgNT1*, *POMT1*, and *POMT2*. His CK was elevated at 901 iU/L (*N*: < 225 iU/L). Muscle biopsy of the right vastus lateralis revealed extensive skeletal muscle atrophy with degeneration/necrosis, increased adipose tissue, and nonspecific mitochondrial changes.

In 2014, WES (trio) identified one paternally inherited variant of uncertain significance in *MYO1A* (c.1165G>A; p.Asp389Asn). Pathogenic variants in *MYO1A* can be associated with an autosomal dominant nonsyndromic hearing loss (DFNA48) (OMIM: 607841) and the proband's father felt that he had some non-specific hearing loss, although this was not clinically tested. In 2018, reanalysis of the WES results revealed no new findings. WGS (trio) identified the same *MYO1A* VUS that was identified on WES, but no other findings. RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Cases 6a and 6b

Case 6a is the 20-year-old male dizygotic twin of case 6b. He initially presented at 18 mo of age with global developmental delay and a history of seizures. His first febrile seizure was described as tonic-clonic and lasting approximately 30 seconds. Developmentally, he was able to sit independently at 18 mo and walk with support. Neurological examination at 18 mo found lax ankle joints, brisk symmetric reflexes, and macrocephaly (head circumference = 50.5 cm, >97th percentile).

By three y of age he was experiencing myoclonic, convulsive, and focal seizures with left sided stiffening and postictal shivering. He could crawl, walk with support, and feed himself with his fingers. He had six to eight words in his vocabulary. At ~ 5 y of age he had a regression while the frequency of seizures increased. At 16 y of age he displayed choreoathetosis and quadriplegic cerebral palsy-like features along with profound intellectual disability. By 20 y old he was having four to five generalized seizures per month, with a maximum seizure-free period was one week.

Case 6b is the 20-year-old male dizygotic twin of case 6a. He presented with the same phenotype of global developmental delay, hypotonia, and seizures occurring approximately 17 times a day. His head circumference was below the 90th percentile (49 cm). By 16 y he also had choreoathetotic movements. Both brothers are non-verbal, and neither is currently able to walk independently or feed themselves.

Diagnostic tests and investigations for the brothers included normal Initial investigations included; cardiac echocardiography, EMG/NCS, brain MRI, and hearing exams. Vitamin E, urine MPS and oligosaccharides, arylsulfatase, beta-hexosaminidase, beta-galactosidase,

biotinidase, phytanic acid, serum acylcarnitine, and transferrin isoelectric focusing were unremarkable.

Skin and muscle biopsies for case did not show any significant abnormalities or evidence of storage disease. Muscle analysis for case 6b was suggestive of reduced mitochondrial abundance with a mild reduction in complex I + III enzyme activity. NGS analysis of mitochondrial DNA revealed no deletions; however, there were two homoplasmic variants of uncertain significance; *MT-ND1*:m.4084G>A; p.Val260Ile) and *MT-ND2*:m.5095T>C; p.Ile290Thr) identified as homoplasmic in his twin brother and mother. Negative single gene testing included; aCGH, *POLG1*, *SCN1A*, Fragile X, Smith-Magenis, and Angelman syndrome. WES (quad) in 2015 identified two likely clinically unrelated variants of uncertain significance in both probands; a maternally inherited heterozygous VUS was identified in *IDH2* (c.439G>C; p.Val147Leu) and a paternally inherited heterozygous VUS was identified in *MYH2* (c.2963T>C; p.Met998Thr). WGS (quad) was completed and revealed the same variants that were identified by WES. RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Case 7

This was a 29-y-old woman born full term after an uncomplicated pregnancy and delivery. At six wk of age she presented with nystagmus and suspected blindness. By three y of age she had an ataxic gait and progressive incoordination. At eight y of age she had a mildly abnormal EEG showing interhemispheric asymmetry with intermittent right sided slowing posteriorly and slow background for age. She was diagnosed with retinitis pigmentosa at 15 y of age and had severe weakness requiring a wheelchair almost full time. Brain MRI at 22 y of age were suspicious for neurodegeneration with brain iron accumulation (NBIA)/fatty acid hydroxylase-associated neurodegeneration (FAHN).

Single gene testing was negative for *NPC1*, *SCA1*, *SCA2*, *SCA3*, *SCA6*, *SCA7*, *SCA8*, *SCA17*, *CYP7B1*, *SPG11*, *SACS*, *SPG7*, *SPG15*, *PNPLA6*, *SPG20*, *SPG21*, *CCT5*, and *SPG44*. Muscle biopsy of her right triceps revealed a non-specific type 2 atrophy. mtDNA sequencing and deletion/depletion analysis from muscle was negative. EMG/NCV, ECHO, and ECG were unremarkable.

In 2014, WES (trio) identified four maternally inherited variants. A single pathogenic heterozygous variant in *USH2A* (c.2276G>T; p.Cys759Phe), a likely pathogenic heterozygous variant in *PANK2* (c.1052C>T; p.Ser351Leu), a heterozygous variant of uncertain clinical significance in *WFS1* (c.2453G>A; p.Arg818His), and a heterozygous VUS in candidate gene *ACBD5* (c.925G>T; p.Gly309Ter). Deletion/duplication analysis was negative for *PANK2* and *USH2A* and no second variant was identified.

WGS (trio) was diagnostic for a newly described peroxisomal disorder associated with the *ACBD5* gene. The results identified the previously found maternally inherited *ACBD5* variant as well as a paternally inherited heterozygous deletion of exon 4 of *ACBD5*. Recent case reports suggest that loss of function variants in *ACBD5* impair peroxisomal very long chain fatty acid

metabolism (β – oxidation). (Ferdinandusse et al., 2017, Yagita et al., 2017). Individuals have been described with retinal dystrophy/rod-cone dystrophy, abnormal eye movements, developmental delays, spastic paraparesis, hypomyelination, and signal abnormalities in the brainstem and deep white matter. (Abu-Safieh et al., 2013 Ferdinandusse et al., 2017, Yagita et al., 2017).

Case 8

This case is a 31-y-old female with mild intellectual disability, optic atrophy, spasticity, and dystonia. Visual deficiencies were first noted at three and a half y of age and omnidirectional nystagmus was noted at 10 y of age, shortly after she was in a motor vehicle accident. At 15 y of age she continued to have progressive visual deficiencies along with declines in motor function. She experienced decreased mobility with sore knees, lower back pain, an inability to run, and a tendency to fall. Neurological examination at 22 y of age confirmed optic atrophy, ataxia, peripheral neuropathy, generalized weakness, and lower limb spasticity. Multiple repeated urine organic acid analysis showed a reproducible increase in lactate and pyruvate and significant increase in 3-methylglutaconic acid.

Investigations included a swallowing study which revealed moderate oropharyngeal dysphagia. Brain MRI revealed bilateral symmetric abnormal signals. Muscle pathology and mtDNA sequencing was unremarkable, but metabolic enzyme testing was revealed a mild reduction in complex I + III. Genetic testing was normal for *OPA1*, *OPA3*, *ARSA* (MLD) and *GALC* (Krabbe disease).

WES (trio) was performed in 2015 and was negative. WGS (trio) was negative. RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Case 9

This is a 30-y-old female who first presented at 18 mo of age with global developmental delays. Over the ensuing year she developed dysarthria and progressive ataxia. She began using a wheelchair full-time around 12 y of age due to the progressive ataxia and weakness in her lower limbs. Coenzyme Q10 (CoQ10) concentration in muscle tissue was found to be low (11.09 ng/g fresh tissue, N: >15 ng/g fresh tissue) and she was started on supplementation of CoQ10. Brain MRI at 12 y of age was unremarkable at the time. EMG was suggestive of a pure motor neuropathy or neuronopathy, suspicious for a Spinal- Muscular Atrophy (SMA)-like condition. Genetic testing for *SMN1*, *COQ2*, *PDSS1*, *PDSS2*, *CABC1*, *CACNA1A*, *SYNE1*, and *COQ9* was negative.

In 2016, WES (trio) was not diagnostic but found one paternally inherited variant of uncertain clinical significance in *PLEKHG5* (c.2695G>A; p.Gly899Ser). Pathogenic variants in *PLEKHG5* are associated with autosomal recessive Charcot-Marie-Tooth (CMTRIC) (OMIM: 615376) and distal spinal muscular atrophy type 4 (DSMA4) (OMIM: 611067). Deletion/duplication analysis of *PLEKHG5* was negative; consequently, this was considered to not likely be pathogenic.

WGS (trio) identified a homozygous VUS in *EXOSC8* at c.98G>A; p.Arg33Lys. Pathogenic variants in *EXOSC8* have been associated with autosomal recessive pontocerebellar hypoplasia, type 1C (OMIM: 616081). This variant was not identified through WES because of poor read coverage in this area. Based on the patient phenotype a diagnosis of autosomal recessive pontocerebellar hypoplasia, type 1C was established (OMIM: 616081). RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Cases 10a and 10b.

The proband (Case 10a) is a 23-y-old woman with intellectual disability, progressive ataxia, vision loss, spasticity, dysarthria, and mild proximal weakness. She was born at term to distantly consanguineous parents of Syrian descent. Parents reported normal milestones until around five y of age. By eight y of age she had experienced a regression of her fine and gross motor skills, dysmetria, nystagmus with saccadic eye movements, scanning speech, dysarthria, and progressive spinocerebellar ataxia and decreased visual acuity. By her late teenage y she was experiencing progressive dysphagia, severe truncal titubation, worsening bowel and bladder incontinence, increased tremor, lower limb spasticity, and further declines in balance and cognition.

Case 10b is the 18-year-old younger brother of Case 10a. He presented with an identical history of intellectual disability, progressive ataxia, vision loss, spasticity, dysarthria, and mild proximal weakness. His first symptoms appeared around eight y of age with generalized weakness. By 10 y of age he was unable to run and a positive Gower's sign on examination. Similar to his sister, he developed dysarthria, dysphagia, scanning speech, ataxia with truncal titubation, saccadic eye movements, brisk reflexes, upgoing toes, and dysmetria. He also had moderate cognitive impairment and nighttime enuresis. At 18 y of age he was diagnosed with osteoporosis.

Initial investigations included brain MRIs for both siblings. The MRI of case 10a revealed white matter changes. An MRI in Syria for case 10b showed signs of leukomalacia in the periventricular areas. Repeat MRI for case 10b at 18 y of age revealed white matter changes, mild atrophy and an elevation of choline levels and iron deposition in globus pallidi and bilateral thalami suggestive of a neurodegenerative disease such as neurodegeneration with brain iron

accumulation (NBIA). Other investigations for the siblings, such as EEGs, ECHOs, EGCs, EMG/NCV, muscle biopsy, measurement of CoQ10 levels in skeletal muscle, mtDNA sequencing, and metabolic analysis were unremarkable. Genetic testing included; aCGH, karyotype, *NPCI*, *NPC2*, *CABCI*, Friedrich ataxia, SCA 1, 2 3, 6, 7, 8, and 17, and a hereditary spastic paraplegia panel.

WES for both siblings (duo) was completed in 2015 and was negative. WGS (quad) was completed and identified a homozygous deletion of exon 3 of *SLC44A1* in both siblings (Chr8:108,066,751-108,095, 501x0 (GRCh37)). *SLC44A1* encodes for choline transporter-like protein 1. This deletion was predicted to result in a frameshift at amino acid position 90 and subsequent termination of 18 codons thereafter. Through GeneMatcher (Sobreira et al., 2015) a number of other individuals with biallelic variants in *SLC44A1* have been identified with a similar childhood-onset neurodegenerative condition with progressive ataxia, dysphagia, tremors, cognitive decline, dysarthria, and optic atrophy (Fagerberg et al., 2020) . This was supported by *in vitro* work investigating choline transporter defects (Taylor, 2019). RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Case 11

This case is a 16-year-old boy who was referred to the neuromuscular clinic with a complex neurological disorder characterized by severe distal axonal peripheral neuropathy, progressive dysphagia, global developmental delay, congenital cataracts, glaucoma, corneal dystrophy, nystagmus, and retinal detachment.

Testing included mtDNA sequencing, electron transport chain enzyme analysis in skeletal muscle, mass spectrometry and lab quantification of guanidinoacetate and creatine, and sequencing of *CTDP1*. Sequencing of *POLG* in 2008 identified a variant of uncertain significance (c.2852A>C; p.Tyr951His). The variant was not maternally inherited, and no paternal sample was available for family studies.

WES (duo) in 2014 identified several variants of uncertain significance, including the same *POLG* variant identified previously. In 2012 the same *POLG* variant had been reported in another patient with distal myopathy with cachexia (Pitceathly et al., 2012). The *POLG* variant was felt to explain part of the proband's phenotype (e.g. long-standing progressive muscle wasting and weakness, cataracts, and borderline CK elevation), but was not felt to explain the entirety of his phenotype. For example, there was no evidence of a large fiber peripheral neuropathy or ocular findings in the other reported case. Family studies to investigate the other variants identified by WES were not informative. This included biallelic variants of uncertain significance in *ZNF469* (c.8260C>T; p.His2754Tyr and c.1457G>A; p.Arg486His) (Brittle cornea syndrome 1, OMIM: 229200), and a maternally inherited variant in *DNM2* (c.2228C>T; p.Pro743Leu) (Centronuclear myopathy 1, OMIM: 160150).

Because the entirety of this patient's phenotype was not felt to be explained by the *POLG* variant, additional genetic testing was pursued. WGS (duo) in 2018 again identified the same

likely pathogenic heterozygous variant in *POLG* (c.2852A>C; p.Tyr951Ser). A recent publication by Castiglioni et al. in 2018 describes an expansion of the *POLG* phenotype which matches most of Case 11's clinical symptoms.

In addition to the *POLG* variant, WGS also identified the biallelic variants of uncertain significance in *ZNF469* discussed above as well as a heterozygous variant in *EPHA2* of unknown parental origin (c.1457G>A; p.Arg486His). Pathogenic variants in *EPHA2* have been associated with autosomal dominant cataracts type 6, multiple types (OMIM: 116600). It is presently unclear if the variants in *ZNF469* or *EPHA2* are playing any role in the complex eye phenotype of this patient. RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Case 12

The case is a four-year-old girl with global developmental delay, microcephaly, hypotonia, hypermobility, and focal epilepsy. She was born by C-section at 38 weeks due to breech positioning and oligohydramnios. Reduced fetal movements were reported during the pregnancy. She presented at 4 mo of age with failure to thrive and hypotonia. Her head circumference was 39 cm, just below the second percentile and she had a tent-shaped mouth. She was unable to sit independently at 18 mo of age but could lie prone and push up via arm extension. She was admitted at 2 y 6 mo for generalized, tonic-clonic status epilepticus with multiple febrile seizures within a 24-hour period, this was her first recorded occurrence. She had the ability to walk with the assistance of a walker but could not crawl or walk independently, and her development plateaued at 3.5 y of age. She is non-verbal and her communication is limited to shaking her head for “no”.

Initial investigations included an EEG which was mildly abnormal with some bursts of 4 Hz sharp waves suggestive of propensity for focal seizures in this area. Brain MRI revealed a small pituitary cystic structure (4.6 mm), periventricular leukomalacia, prominence of supratentorial CSF space with moderate dilation of supratentorial ventricles and moderate cerebral volume loss in the parieto-occipital region. Microarray, EMG/NCV, CK, and a congenital muscular dystrophy panel testing were negative.

WES (trio) was done in 2017 and identified a paternally inherited heterozygous pathogenic variant in *TSEN54* (c.919G>T; p.Ala307Ser). Deletion/duplication analysis of *TSEN54* did not identify a second variant and her MRI was not consistent with pontocerebellar hypoplasia. WGS (trio) identified the same *TSEN54* variant, but no other findings felt to explain her phenotype. RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript

levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Case 13

This case is a 6-y-old female born to non-consanguineous parents. At birth, she weighed 5 lbs with microcephaly (measurement not available). She presented with her first seizure at four mo of age and an EEG at eight mo of age showed modified hypsarrhythmia consistent with infantile spasms. At five y of age her epilepsy worsened, and she was diagnosed with probable Lennox-Gastaut syndrome. MRI studies at fifteen mo of age showed microcephaly (HC = 43 cm, <3rd percentile), marked cerebral atrophy, and high T2 signal in the basal ganglia and cerebellum. At four y of age she was diagnosed with cortical blindness. Developmental regression and reduced hearing was noted at five and a half y of age, resulting in the loss of previously gained abilities including gross and fine motor skills and overall social interaction. Examination found spasticity and brisk reflexes in all four limbs. Chromosome aCGH was negative.

In 2014, WES (trio) identified a single heterozygous paternally inherited variant in *RMND1* (c.713A>G; p.Arg238Ser). Pathogenic biallelic variants in *RMND1* are associated with (Combined Oxidative Phosphorylation Deficiency 11 (COXPD11), OMIM: 614922). No second variant in *RMND1* was identified. Also found were two compound heterozygous variants of uncertain clinical significance in candidate gene *TKT* (c.133G>A; p.Ala45Thr and c.976C>T; p.Arg326Cys). Her TKT enzyme activity was reported as 7.3 nmol/mg protein/minute (controls: 12.1—17.8). This activity of TKT in fibroblasts is decreased to approximately 50% of normal, which was not felt to be sufficient to be disease causing. WGS (trio) identified the previously reported variants in *RMND1* and *TKT*. No additional variants were identified. RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Case 14

This case 14 is a 17-y-old woman born to consanguineous parents with intellectual disability, myoclonus, dystonia, chorea, supranuclear vertical gaze palsy, and dystonia. The initial onset of her movement disorder was observed at four mo of age and involved her arms, legs, hands, and tongue. These movements did not involve her face and did not occur in her sleep but worsened when participating in tasks. Her developmental milestones were globally delayed, she began to walk at the age of three and demonstrated speech and language delays. Assessment at age 12 y revealed that she was at a 6-y intellectual level. Her head circumference at this time was 56.5 cm (>97th percentile). She exhibited a supranuclear gaze palsy, oculomotor apraxia, and facial myoclonus with dystonia. Her speech articulation was affected by her movement disorder and vocal cord nodules. Her chorea was slowly progressive, and she reported occasional episodes of dysphagia when she had increased myoclonus. Nystagmus was noted on physical examination with slow-moving saccades in the horizontal plane, and she showed an inability to look downward past the midline. Examination at 17 y of age revealed dystonia in the hands with rapidly alternating movement and dystonia in her legs and feet. She had significant balance problems with a slightly broad ataxic gait.

Initial investigations included a brain MRI which showed subtle diffuse periventricular white matter high signals on T2 weighted images. Urine metabolic screen, urine organic acids, serum copper, ceruloplasmin, lactate, and plasma amino acids were unremarkable. Molecular genetic analysis of *NPC1*, *NPC2*, and microarray were negative.

WES (trio) was negative. WGS (duo) in 2018 detected a homozygous variant of uncertain significance in *SCN3A* (c.2204A>G; p.Asn735Ser). Pathogenic variants in *SCN3A* have been associated with an autosomal dominant familial focal epilepsy with variable foci (FFEVF4),

(OMIM: 604364). Since neither the proband nor either of her parents had a history of epilepsy this variant was felt to not explain the patient's phenotype. RNA-Seq of the entire transcriptome from fibroblasts did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patients.

Case 15

This case is a 9-y-old boy born at 41.5-wk gestation after a pregnancy complicated by polyhydramnios and decreased fetal movements. He had bilateral talipes equinovarus requiring serial casting and tenotomy of the left foot. He was delayed in meeting motor milestones. At age two he was able to sit with no supports and crawl independently. He was able to pull to stand but unable to support his full weight when standing. By four y he was able to walk with a walker. He had reduced fine motor skills due to significant distal weakness in his hands. Feeding was an issue due to choking spells when consuming fluids without a straw and swallowing studies revealed thin fluid aspiration and limited penetration without trace aspiration. His speech was impacted by vocal weakness/hypophonia.

Nerve conduction studies and EMG revealed median and ulnar velocities of approximately 10 m/s and a diagnosis of congenital hypomyelinating neuropathy was suspected. Sequencing of *EGR2*, *PRX*, *ENFL*, *LITAF*, *GDAP1*, *FGD4*, *FIG4*, *MTMR2*, *SH3TC2*, *NDRG*, *PMP22*, and *MPZ* were negative.

In 2014, WES (trio) identified two variants in genes possibly associated with his reported phenotype. A heterozygous *de novo* variant at c.23C>T; p.Thr8Met) in *SETX* and a heterozygous paternally inherited VUS at c.2176G>A; p.Val726Met in *IGHMBP2*. Heterozygous pathogenic variants in *SETX* have been associated with (juvenile ALS type 4 (OMIM: [602433](#)) while biallelic pathogenic variants in *SETX* have been associated with ataxia-oculomotor apraxia type 2 (OMIM: 606002). Clinically it was felt that his phenotype was earlier onset and more severe than the phenotype described in juvenile ALS Type 4. Biallelic pathogenic variants in *IGHMBP2* are associated with spinal muscular atrophy with respiratory distress type 1 (SMARD1) (OMIM: 604320); however, deletion/duplication analysis of *IGHMBP2* was negative and no second

variant was identified. *SETX* has a 42% shared homology with *IGHMBP2* which also raised the possibility of a synergistic interaction between the variants identified in these two genes. WGS (trio) was completed and revealed the same variants identified by WES. No additional variants of interest were identified. RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Since these results have been returned, we have been made aware of a few other cases of children with the same *de novo* variant in *SETX* (c.23C>T; p.Thr8Met) and identical phenotype. Further work is being done to better characterize and publish this possible expansion in phenotype.

Case 16

This case 16 is a 10-y-old girl born at 37 wk gestation after a pregnancy complicated by oligohydramnios and reduced fetal movements. She presented at six mo of age due to delays in meeting her gross and fine motor milestones. She gained the ability to walk independently at two y of age but continues to use a walker due to difficulty with balance. Evaluation at seven y of age found truncal hypotonia, intention tremor, balance problems that appeared cerebellar in nature, and spasticity and weakness in the lower extremities. She had moderate intellectual disability with roughly 30 words in her vocabulary and would use just two words together at a time. She was not toilet trained. Bilateral hearing loss was also documented at this time. A brain MRI at 9 y of age showed a complex septate pineal gland cyst and persistent diffuse cerebellar volume loss prominent in the vermis.

Initial investigations included normal; lactate, ammonia, electrolytes, ALT, AST, CK, cholesterol, 7-dehydrocholesterol, homocysteine, alpha-fetoprotein, acylcarnitine profile, plasma amino acids, total and free carnitine, vitamin B12, transferrin isoelectric focusing, and uric acid levels. Muscle biopsy of the right quadriceps skeletal muscle showed a slight enlargement of mitochondria with no paracrystalline inclusions. Respiratory chain enzyme activities and fibroblast studies for pyruvate dehydrogenase complex deficiency and pyruvate carboxylase activity were unremarkable.

A myopathy panel identified a heterozygous *de novo* variant of uncertain significance in the *RMND1* gene (c.759dup; p.His254Thrfs*2). Pathogenic variants in this gene have been associated with autosomal recessive combined oxidative phosphorylation deficiency (COXPD11) (OMIM: 614922). No second variant was identified. mtDNA sequencing, a

hereditary spastic paraplegia (HSP) panel, karyotype, microarray, and SCA 1, 2, 3, 6, 7, 8, 17 were all negative.

WES (trio) in 2016 was negative. WGS (trio) identified the same *de novo RMND1* variant seen previously as well as a paternally inherited heterozygous likely pathogenic variant in *SLC12A6* (c.2230C>T; p.Arg744Ter). Neither variant was felt to definitively explain the patient's phenotype. RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Case 17

This case is a 12-y-old girl born at 39 wk gestation by caesarean section after a pregnancy was complicated by mild placenta abruption at 27 weeks. Nystagmus was observed at birth and she was diagnosed with laryngomalacia and gastroesophageal reflux disease (GERD) during the neonatal period. Her first unprovoked seizure occurred at three mo of age with upward rolling eyes, full body convulsions, and apnea. EEG showed bifrontal epileptiform charges. Brain MRI showed delayed myelination and enlarged ventricles with prominent bifrontal extra-axial CSF spaces with cerebellar atrophy and diffuse volume loss.

She was able to sit independently at three y of age and gained the ability to walk with a walker by age six y. She has limited fine motor skills and is not able to grasp objects. She is primarily non-verbal with the exception of “yes” and “no”. aCGH, Rett syndrome, metabolic screen, and routine bloodwork were unremarkable. She has multiple dysmorphic features such as a small and smooth philtrum pectus carinatum, high arched palate, bilateral pes planus, 2-3 digit syndactyly, and bilateral arachnodactyly.

WES (trio) was negative. WGS (trio) revealed two variants of uncertain significance; one heterozygous maternally inherited variant in *RANBP2* (c.5579A>C; p.Lys1860Thr), and a heterozygous paternally inherited variant in *CHRNA2* (c.859_862delinsC; p.Phe287_Tyr288delinsHis). RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Case 18

This case is a 17-y-old woman with features of both spinocerebellar ataxia (SCA) and hereditary spastic paraplegia (HSP). She was born at full-term to non-consanguineous parents after an uncomplicated pregnancy and delivery. She met normal developmental milestones until 8 mo of age when her development stagnated. Developmental regression was noted at age 10 mo as she was losing her balance and no longer cruising around furniture. By 15 mo she could no longer crawl or stand and was non-verbal. Her head circumference was 48 cm (>95th percentile). Brain MRI at 15 y showed mild cerebellar atrophy and VEP was indicative of abnormal visual evoked responses with slowing. Examination found clonus in both feet, upgoing toes, and excessive drooling. She eventually re-gained the ability to cruise along furniture, developed a pincer grasp, could point to objects she was interested in, and use monosyllables. At 15 y of age her balance was notably better, and she no longer required any gait assisting devices and was independent for all activities of daily living (ADLs).

Initial investigations included an unremarkable EEG. Lactate, carboxyltransferase, electrolytes, CBC, TSH, glucose, very long chain fatty acids, cholesterol, triglycerides, ceruloplasmin, urine organic acids, plasma amino acids, urine purine/pyrimidine profile, guanidinoacetate, and creatine/creatinine ration were negative.

Muscle pathology on a biopsy of skeletal muscle from the right vastus lateralis was unremarkable. Mitochondrial respiratory chain enzyme analysis on skeletal muscle revealed a mild complex I+III deficiency. mtDNA deletion analysis of skeletal muscle tissue and mtDNA sequencing on cultured fibroblasts were both negative. Genetic testing for Rett syndrome, GAMT deficiency, Friedreich ataxia, SCA 1, 2, 3, 6, 7, 8, 17, a panel of nuclear genes associated with mitochondrial disease (N = 406), and aCGH were unremarkable negative.

WES in 2014 was negative. WGS (trio) identified a few variants of uncertain significance in genes with autosomal recessive inheritance. One was a paternally inherited heterozygous likely pathogenic variant in *KIAA056* (c.922C>T; p.Gln308Thr). Pathogenic variants in this gene are associated with autosomal recessive Joubert syndrome 26 (OMIM: 616784). A maternally inherited heterozygous likely pathogenic splice site variant in *PNPT1* (c.1441+2T>A). Pathogenic variants within this gene are associated with autosomal recessive combined oxidative phosphorylation deficiency-13 (COXPD13) (OMIM: 614932). A third heterozygous likely pathogenic variant was seen in *SLC39A8* (c.610G>; p.Gly204Cys) and was also of maternal origin. Pathogenic variants in this gene have been associated with autosomal recessive congenital disorder of glycosylation type II_n (OMIM: 616721). No second variant was identified in any of these three genes. RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Case 19

This case is a 49-y-old woman with a complex neurodegenerative disorder and neuropsychiatric features. Parents report normal development until she experienced a significant regression with her cognition, speech, and gait after undergoing corrective surgery for scoliosis at 13 y of age. She required full-time use of a wheelchair by 15 y of age and was dependent on her parents for all ADL's. At age 43 y she began to experience visual hallucinations, anxiety, and behavioural problems such as increased violence.

MRI was suggestive of mitochondrial cytopathy with diffuse cerebral and cerebellar atrophy, basal ganglia loss, and T2 hyperintensity in the transverse pontine fibers. Increased choline and decreased *N*-acetylaspartate (NAA) were observed in basal ganglia. Genetic testing of *NPCI*, *NPC2*, SCA 1, 2, 3, 6, 7, 8, 17, and Friederichs ataxia were all negative. Acylcarnitine profile, plasma amino acids, serum lactate, urine metabolic screen, and urine organic acids were unremarkable.

WES in 2014 revealed compound heterozygous variants *in trans* in *VPS13A* (c.5884C>T; p.Arg1962Cys and c.8676C>T, p.Ile289Ile). *VPS13A* has been associated with autosomal recessive choreoacanthocytosis (OMIM: 200150), however this diagnosis was felt to not fit her clinical picture due to an absence of acanthocytes in her blood. WGS (duo) was negative. RNA-Seq of the entire blood transcriptome did not identify any significant changes in transcript levels or splicing patterns in genes believed to be related to the clinical presentation of this patient.

Case 20

This case 20 is a 38-y-old woman with spinocerebellar ataxia, optic atrophy, and intellectual disability. She exhibited ataxia early in childhood and did not walk independently until after 18 mo of age. Her ataxia was slowly progressive, and she continues to have difficulties with ambulation requiring a wheelchair for longer distances. At 24 y of age she was diagnosed with bilateral optic atrophy and visual evoked potentials were abnormal with no observable peripheral or central responses. Physical exam found limited downgaze, horizontal nystagmus, ptosis, oculomotor apraxia, significant intention tremor, mild scanning speech, and truncal titubation.

Initial investigations included a brain MRI at 14 y which was normal but by 28 y of age her MRI showed a prominence of the cerebellar fovea, vermis and CSF spaces in the posterior fossa. Nerve conduction studies showed evidence of a predominantly axonal sensorimotor polyneuropathy. Biopsy of the right sural nerve revealed axonal neuropathy and demyelination. Biochemical testing of skeletal muscle revealed normal levels of CoQ10 with a reduction in the enzyme activity of complex I + III.

Genetic testing included molecular analysis for Friedreich ataxia, SCA 1, 2, 3, 6, 7, 8, 17, *POLG*, *TWINK*, microarray, and mtDNA sequencing in skeletal muscle tissue. Testing of cerebrospinal fluid found normal glucose, protein, and lactate levels.

WES (singleton) was completed in 2017 and identified a number of variants of uncertain significance, including compound heterozygous variants (*in trans*) in candidate gene *SLC25A10* (c.G449A; p.R150H and c.C574T; p.P192S). *SLC25A10* encodes for a mitochondrial protein carrier, however no association with human disease had been reported to date.

In 2018, WGS (trio) highlighted a homozygous likely pathogenic splice site variant in *ACAD9* (*c.244+3A>G*). Review of WES data showed that this variant was identified but was not felt to be diagnostic because it was not felt that there was a strong phenotype match. However, RNA-Seq in blood confirmed that exon splicing was affected by this variant. Pathogenic variants in *ACAD9* are associated with mitochondrial complex I deficiency type 20 (MC1DN20). The classic phenotype is muscle weakness, hypotonia, cardiomyopathy, liver failure, encephalopathy, and Reye-like episodes in infancy/childhood. Ataxia is still a rarely reported phenotype of *ACAD9*-related mitochondrial complex I deficiency and it's likely this patient represents a milder phenotype of this condition.