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Exome Sequencing and Multigene Panel Testing in 1,411 Patients With Adult-Onset Neurologic Disorders

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Abstract

Background and Objectives

Owing to their extensive clinical and molecular heterogeneity, hereditary neurologic diseases in adults are difficult to diagnose. The current knowledge about the diagnostic yield and clinical utility of exome sequencing (ES) for neurologic diseases in adults is limited. This observational study assesses the diagnostic value of ES and multigene panel analysis in adult-onset neurologic disorders.

Methods

From January 2019 through April 2022, ES-based multigene panel testing was conducted in 1,411 patients with molecularly unexplained neurologic phenotypes at the Ghent University Hospital. Gene panels were developed for ataxia and spasticity, leukoencephalopathy, movement disorders, paroxysmal episodic disorders, neurodegeneration with brain iron accumulation, progressive myoclonic epilepsy, and amyotrophic lateral sclerosis. Single nucleotide variants, small indels, and copy number variants were analyzed. Across all panels, our analysis covered a total of 725 genes associated with Mendelian inheritance.

Results

A molecular diagnosis was established in 10% of the cases (144 of 1,411) representing 71 different monogenic disorders. The diagnostic yield depended significantly on the presenting phenotype with the highest yield seen in patients with ataxia or spastic paraparesis (19%). Most of the established diagnoses comprised disorders with an autosomal dominant inheritance (62%), and the most frequently mutated genes were *NOTCH3* (13 patients), *SPG7* (11 patients), and *RFC1* (8 patients). 34% of the disease-causing variants were novel, including a unique likely pathogenic variant in *APP* (Ghent mutation, p.[Asn698Asp]) in a family presenting with stroke and severe cerebral white matter disease. 7% of the pathogenic variants comprised copy number variants detected in the ES data and confirmed by an independent technique.

Discussion

ES and multigene panel testing is a powerful and efficient tool to diagnose patients with unexplained, adult-onset neurologic disorders.

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Glossary

ALS = amyotrophic lateral sclerosis; **CNVs** = copy number variants; **CSVD** = cerebral small vessel disease; **ES** = exome sequencing; **GS** = genome sequencing; **NBIA** = neurodegeneration with brain iron accumulation; **OGM** = optical genome mapping; **PME** = progressive myoclonic epilepsy; **RNDs** = rare neurologic diseases; **SCAs** = spinocerebellar ataxias; **SNVs** = single nucleotide variants; **SVs** = structural variants.

Diseases of the nervous system are associated with high morbidity and mortality and therefore place an important burden on health care. Neurologic disorders are the leading cause of disability and the second leading cause of death.[1] The prevalence of neurodegenerative disorders is increasing worldwide, in part because of an extended life expectancy.[2] An important part of these disorders have a genetic origin, with clinical and genetic (locus and allelic) heterogeneity complicating the diagnostic process. Receiving a diagnosis for previously unexplained neurologic symptoms not only has psychological consequences, in some cases it also alters therapeutic or surveillance strategies. Furthermore, it enables accurate counseling regarding prognosis, recurrence risk, and the identification of at-risk family members. Last but not least, patients can choose for prenatal or preimplantation genetic testing to prevent transmission of the disease-causing variant to their offspring.

To deal with the locus heterogeneity and unspecific or atypical clinical presentations of most of these rare neurologic diseases (RNDs), exome sequencing (ES) has become a widely used tool in the field of neurogenetics. The diagnostic yield of ES across different neurologic indications varies between 22% and 68%, with the highest yields reported in pediatric cohorts and patients presenting with neuromuscular phenotypes.[3,4] Evidence of the clinical utility of exome sequencing in adult patients with a variety of neurologic diseases in large patient cohorts is lacking. In this study, we report the diagnostic yield of exome-based gene panel testing in 1,411 predominantly adult (93%) patients presenting with ataxia, spastic paraplegia, leukoencephalopathy, movement disorders, paroxysmal or episodic disorders, neurodegeneration with brain iron accumulation (NBIA), progressive myoclonic epilepsy (PME), or amyotrophic lateral sclerosis (ALS).

Methods

This retrospective observational study aims to evaluate the diagnostic yield of large gene panel testing across a variety of adult-onset neurologic disorders. In 2019, 6 diagnostic gene panels have been introduced at the Center for Medical Genetics of the Ghent University Hospital to diagnose patients with neurologic disorders. The gene panels are ES-based and comprise genes that have been associated with 6 distinct neurologic phenotypic categories: 'Ataxia and Spasticity' (390 genes), 'Leukoencephalopathy' (266 genes), 'Movement Disorders' (269 genes), 'Neurodegeneration with Brain Iron Accumulation' (NBIA) (16 genes), 'Paroxysmal Episodic Disorders' (53 genes), and 'Progressive myoclonic epilepsy' (PME) (34 genes). A seventh and final gene panel for ALS-causing genes (35 genes) has been introduced in 2021. Current versions (v2) of the different gene panels are available in the supplementary data (eTable 1, links.lww.com/NXG/A601). There is an extensive overlap between the different panels. Taken together, they consist of 725 genes associated with Mendelian neurologic diseases. In this study, we retrospectively evaluated all cases in which gene panel testing for an unexplained neurologic disorder was requested between January 2019 and April 2022. Patients presenting with neuromuscular disorders or epilepsy with intellectual disability were not included in this study because the described gene panels were created to diagnose hereditary disorders of the CNS in adults specifically. Statistical analysis was performed using SPSS 28. Comparison of means was performed using an independent sample t test with a significance level (α) set at 0.05.

Gene Panel Content

The 7 gene panels were composed by collating information from different databases and resources. We used the Genomics England Panelapp in combination with OMIM searches and PubMed literature searches to find more recent gene-phenotype associations.[5,6] An overview of the genes in the different gene lists is provided in eTable 1 (links.lww.com/NXG/A601).

Exome Sequencing

Exome sequencing (ES) was performed on the Illumina Hiseq 3000 and the Novaseq 6000 Platform after enrichment of gDNA with SureSelectXT Low Input Human All Exon v6 and v7 (Agilent Technologies). The BWA-MEM 0.7.17 algorithm was used for read mapping against the human genome reference sequence (NCBI, GRCh38/hg38) duplicate read removal, and variant calling. Variant calling and filtering were performed using Seqplorer, an in-house developed tool for the analysis of ES data. The position of the called variants was based on NCBI build GRCh38. A minimum of 90% of the interrogated genes has a coverage of >20x. Nucleotide numbering was according to the Human Genome Variation Society guidelines (HGVS). Variant filtering criteria in Seqplorer included a population frequency (gnomAD) <0.02, impact on the protein predicted to be moderate or high, minimal variant quality of 20, and minimal depth of 2 reads. Low-impact variants such as synonymous variants or intronic splice region variants >8 nucleotides away from the splice junction were not prioritized using standard settings. All variants with a quality score below 300 (SNVs) or 500 (small

Table 1 Description of the Patient Cohort

	Total patient cohort (%)	Diagnosed patients (%)
N	1,411	144
Age (y) (mean ± SD)	51 ± 20	50 ± 19
Younger than 18	97 (7)	10 (7)
Aged 18 or older	1,314 (93)	134 (93)
Sex		
Male	669 (47)	73 (51)
Female	742 (53)	71 (49)
Gene panel		
Leukoencephalopathy	535 (38)	44 (30)
Ataxia spasticity	365 (26)	70 (49)
Movement disorders	378 (27)	22 (15)
Paroxysmal episodic disorders	99 (7)	8 (6)
Progressive myoclonic epilepsy (PME)	7 (0)	0 (0)
Neurodegeneration with brain iron accumulation (NBIA)	11 (1)	0 (0)
Amyotrophic lateral sclerosis (ALS)	16 (1)	0 (0)

indels) were confirmed with Sanger sequencing. Seqplorer was not capable of calling variants in noncoding RNAs because of the absence of a predicted protein impact. Variant classification was performed using an in-house developed tool based on the ACMG and ACGS guidelines in the following classes: (1) benign, (2) likely benign (>95% certainty that the variant is benign), (3) variant of unknown significance, (4) likely pathogenic (>95% certainty that the variant is pathogenic), and (5) pathogenic.[7-11] Class 4 and class 5 variants were considered to be disease-causing. Potential CNVs were called using ExomeDepth, an algorithm which uses ES data to detect read depth differences in coding regions.[12]

Targeted Analysis *RFC1*

In patients with homozygosity for SNP rs2066782, in linkage disequilibrium with the intronic pathogenic pentanucleotide repeat expansion in *RFC1*, flanking PCR, and repeat-primed PCRs for the pathogenic AAGGG and nonpathogenic AAAGG or AAAAG repeat expansion in *RFC1* were performed as described in a study in 2019.[13] Positive cases were defined as samples showing no amplifiable product on flanking PCR and the presence of a decremental saw-tooth pattern on repeat-primed PCR for the pathogenic AAGGG repeat expansion.

Targeted Analysis of *PRNP* OPRI

The region containing the octapeptide repeat insertion was amplified using the forward primer 5′-GCAGTCATTATGGCGAACCTTGGCTG-3′ and the reverse primer 5′-TGCATGTTTTCACGATAGTAACGG-3′. PCR products were separated using gel electrophoresis. The amplicon of the wild-type allele consists of 460 bp. If an alternative larger band was detected, the amplified DNA was purified and sequenced using Sanger sequencing.

Sanger Sequencing of Noncoding RNAs *SNORD118* and *RNU7-1*

Variants in noncoding genes can only be detected through ES if the region of interest is enriched prior to sequencing. Using the SureSelectXT Low Input Human All Exon v6 and v7 (Agilent Technologies) kits, we saw enrichment for *RNU7-1* but not for *SNORD118*. Variants in *RNU7-1* were confirmed using Sanger sequencing as described in a study in 2022.[14] The variants in *SNORD118* were detected using Sanger sequencing. Primer sequences are available on request.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

Standard Protocol Approvals

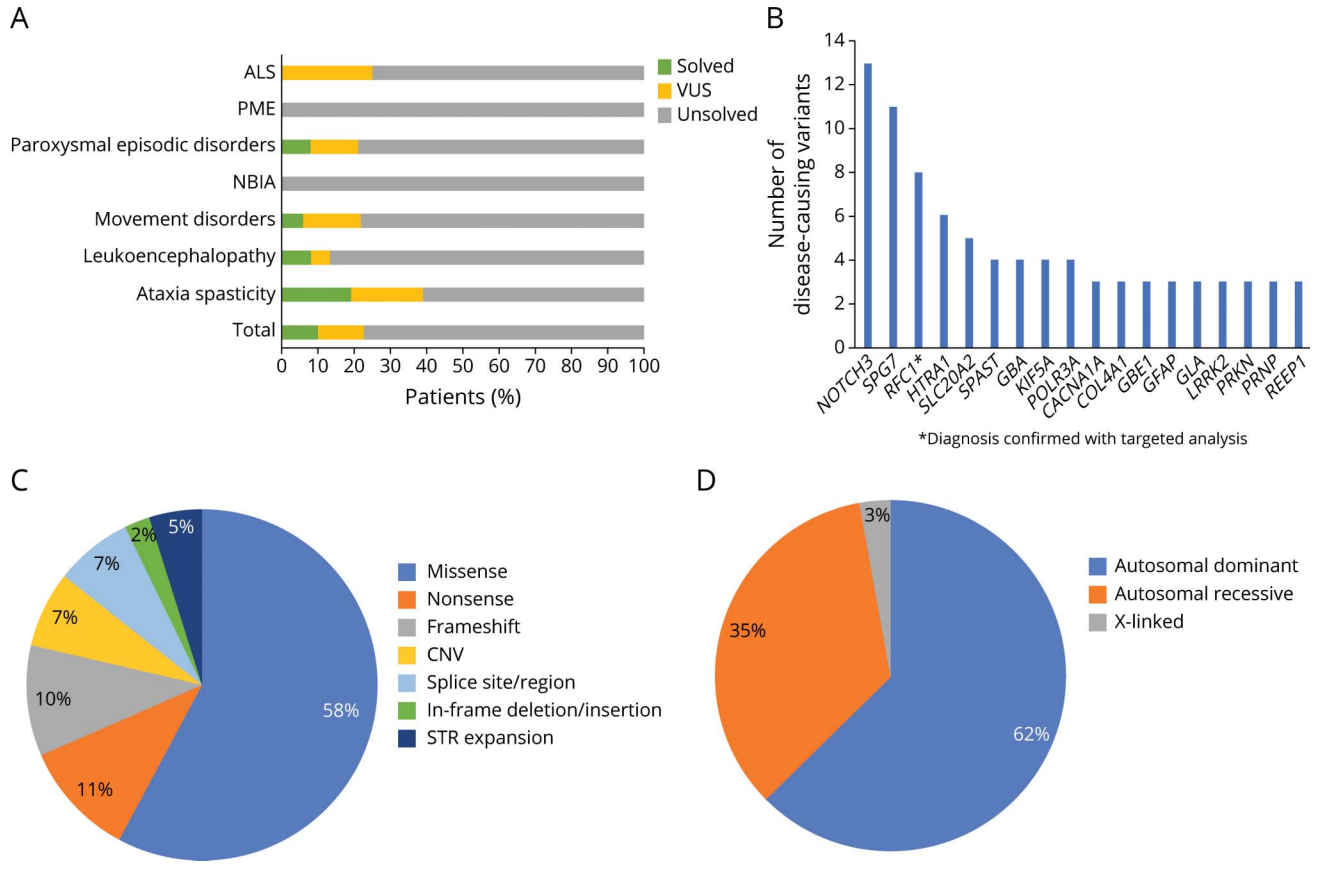
All clinical and genetic data were gathered during routine diagnostic and clinical activity. Clinical data were provided to the principal investigator by the referring physicians. The study complies with retrospective studies regulations and was approved by the Ethics Committee of the University Hospital of Ghent (project BC-07055).

Results

Description of the Patient Cohort

Since the implementation of the 7 multigene panels in 2019, exomes of 1,411 patients have been analyzed. Six hundred sixty-nine patients (47%) were male; 1,314 patients (93%) were aged 18 years or older. The mean age of the analyzed patient cohort was 51 years (Table 1). In most of the patients (38%), the 'leukoencephalopathy' gene panel was requested, followed by the 'movement disorders' (27%) and the 'ataxia spasticity' gene panel (26%). In 7%, the 'paroxysmal and episodic disorders' gene panel was requested. The ALS, NBI, and PME panels were analyzed in 1%, 1%, and 0%, respectively, of the patient cohort (Table 1). In 51 patients (4%), more than 1 gene panel was requested. For the sake of simplicity, we only took the gene panel into account which was the closest match to the patient's phenotype. In 144 of 1,411 patients (10%), a definite molecular diagnosis was established through the identification of (a) class 4 (likely pathogenic) or class 5 (pathogenic) variant(s) (eTable 2, links.lww.com/NXG/A602). The diagnostic rate of exome sequencing in the small pediatric subcohort was 10% (10 of 97), similar to the yield obtained in adults (10%, 134 of 1,314) (Table 1). There was no significant age difference between the group of diagnosed patients (50 ± 19 years) and the undiagnosed group (51 ± 20 years) ($p = 0.46$, 95% CI: −2.14 to 4.74). In 68 of the 144 diagnosed patients (47%), there was

Figure 1 ES and Multigene Panel Testing in 1,411 Patients Yields a Diagnosis in 10% of the Cases



(A) Detection rate of causal variants and variants of unknown clinical significance (VUS) per gene panel (expressed in percentages). (B) Overview of the most frequently implicated genes across all gene panels ranked according to the burden of (likely) pathogenic variants. (C) Distribution of causal variant type. (D) Inheritance pattern associated with the molecular diagnoses.

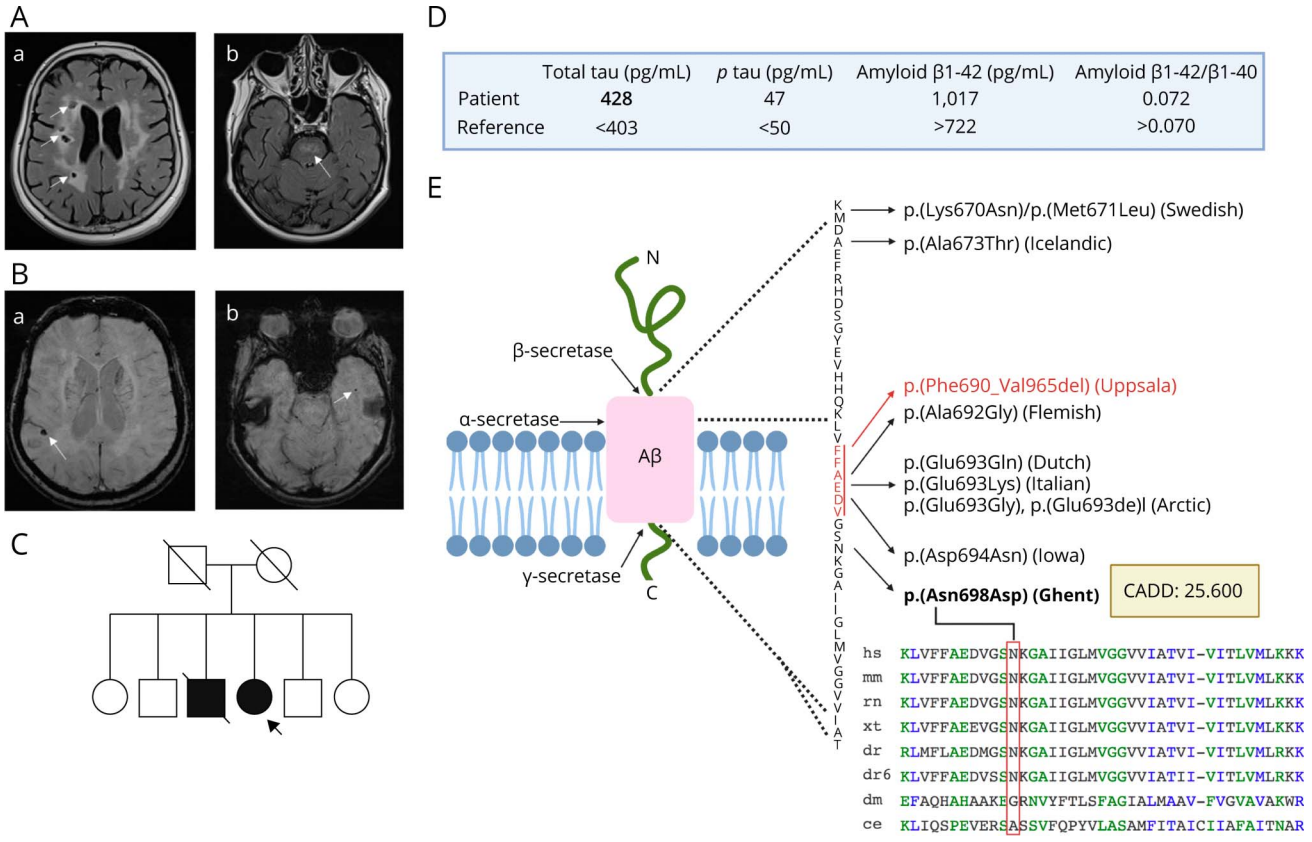
a positive family history (eTable 2). The mean age at onset of the first symptoms in the group of diagnosed patients was 37 years, pointing toward a diagnostic delay of more than 14 years on average. When taking only adult patients (at time of request of genetic test) into account, the average age at symptom onset was 39 years and the median age was 45 years. Of the diagnosed patients presenting ataxia and/or spasticity, 33% (22 of 66) underwent previous genetic testing for the dominant spinocerebellar ataxias (SCAs), Friedreich ataxia, or fragile X tremor/ataxia syndrome (FXTAS) (eTable 2). The large majority (96%) of the cases were analyzed as singleton (1,349 of 1,411), 2% (30 of 1,411) was analyzed in duo with an affected family member, and 2% (32 of 1,411) was analyzed as a trio (patient + parents). The genetic analyses were requested by 250 different clinicians, most of which neurologists but also pediatricians and geneticists requested gene panel testing.

Multigene Panel Analysis After ES in Patients With RND Results in a Diagnostic Rate of 10%

The overall diagnostic yield, across the 7 gene panels, was 10% (144/1,411) (Figure 1A). The diagnostic rate differed according to the analyzed gene panel and was the highest for the 'ataxia spasticity' gene panel with the identification of (a) causal pathogenic variant(s) in 19% of the patients (70 of 365). Both the 'paroxysmal and episodic disorders' panel (8 of 99) and the 'leukoencephalopathy' gene panel (44 of 535) yielded a diagnosis in 8% of participants, followed by the 'movement disorders' gene panel with a diagnostic rate of 6% (22 of 378). Analysis of the smaller gene panels NBIA, PME, and ALS did not result in definite diagnoses up to now (Figure 1A). In 177 patients (13%), a variant of unknown clinical significance (VUS, class 3 variant) was identified which could potentially explain the patient's phenotype (Figure 1A, eTable 3, links.lww.com/NXG/A603).

The most frequently implicated genes in patients presenting with leukoencephalopathy were *NOTCH3* (cerebral arteriopathy with subcortical infarcts and leukoencephalopathy, type 1, MIM: 125310) (13 patients) and *HTRA1* (cerebral arteriopathy with subcortical infarcts and leukoencephalopathy, type 2, MIM: 616779) (6 patients). In patients for which the 'ataxia spasticity' gene panel was requested, biallelic *SPG7* mutations (spastic paraplegia 7, MIM: 607259) were the most common underlying cause (11 patients). Heterozygous *GBA* mutations (Parkinson disease, late-onset, susceptibility to,

Figure 2 Novel APP Likely Pathogenic Missense Variant (p.[Asn698Asp], Ghent Variant), Associated With Cerebral Amyloid Angiopathy



(A and B) Brain MRI of our patient showing signs of extensive microangiopathy with diffuse confluent leukoencephalopathy (A1) lacunar infarctions (A1, indicated by arrows) and involvement of basal ganglia, pons (A2), and right cerebral peduncle (fluid-attenuated inversion recovery (FLAIR) imaging) and several cerebral microbleeds (susceptibility weighted imaging (SWI) (B1-2, indicated by arrows). (C) Pedigree of the patient in which the novel variant was identified. The proband is indicated with an arrow. (D) Results of patient CSF analysis and reference values for total and phosphorylated tau, $A\beta_{42}$, and the ratio of $A\beta_{42}/A\beta_{40}$. (E) Schematic of the transmembrane amyloid precursor protein. The arrows indicate the sites of secretase activity. The amino acid sequence of the Aβ domain is depicted, together with the pathogenic variants that have been identified in this region. The novel Ghent variant (p.[Asn698Asp]), with a CADD score of 25.6, is depicted in bold. The asparagine residue is moderately conserved but is located in a highly conserved region.

MIM: 168600) and biallelic *PRKN* mutations (Parkinson disease, juvenile, type 2, MIM: 600116) were the most frequently identified molecular causes (respectively, 4 and 3 patients) in patients presenting with a movement disorder (Figure 1B).

The disease-causing variants comprised missense variants (58%), nonsense variants (11%), frameshift variants (11%), splice site or region variants (7%), copy number variants (CNVs) (7%), in-frame small deletions or insertions (2%), and short tandem repeat (STR) expansions (5%) (Figure 1C). One hundred thirty-seven unique (likely) pathogenic variants were identified, 46 of which (34%) had no ClinVar entry and were never reported in the literature before (eTable 2, links.lww.com/NXG/A602).[15] 62% of the molecular diagnoses were associated with autosomal dominant inheritance. In 35%, autosomal recessive inheritance was seen, and in 3%, the disease was transmitted through X-linked inheritance (Figure 1D, eTable 2). In 7 patients, a heterozygous pathogenic variant in the *ATM* gene was reported as an incidental finding, associated with an increased risk to develop breast cancer.

Disease-Causing Copy Number Variants (CNVs) in RNDs

For many RNDs, it is well-established that CNVs can contribute to their etiology. In the past, CNV calling from exome data was not routinely performed in our center. For this patient cohort, ExomeDepth[5] was used to detect disease-causing CNVs and confirmation was done using qPCR, MLPA (multiplex ligation–dependent probe amplification), or shallow whole genome sequencing (CNV sequencing). In 11 patients (0.8%), a definite diagnosis was made after analysis of the read depth using ExomeDepth (Figure 1C). Pathogenic CNVs included previously reported CNVs such as a ~240 kb deletion affecting *ITPR1* (spinocerebellar ataxia 15, MIM: 206700)[16] and single or multiple exon deletions in *SPAST* (spastic paraplegia 4, MIM: 182601)[17] and *PRKN* (Parkinson disease, juvenile, type 2, MIM: 600116).[18] In addition, novel CNVs were detected including an 11 Mb duplication

containing *SNCA* (Parkinson disease 1, MIM: 168601), a 2.6 Mb deletion including the *FGF14* gene (spinocerebellar ataxia 27, MIM: 609307), a ~1.2 Mb deletion comprising *KIF1A* (spastic paraplegia 30, autosomal dominant, MIM: 610357), and lastly single (exon 1) or multiple (exon 10–11) exon deletions in *SPG7* (spastic paraplegia 7, autosomal recessive, MIM: 607259) (eTable 2, links.lww.com/NXG/A602).

Shortcomings of ES in RND Diagnostics

In 9 patients, an independent technique was required to confirm the diagnosis because ES is not well suited to detect certain types of sequence variation such as STR expansions or indels in repeat regions because of the fact that short reads are generated, and introns and noncoding regions are not enriched.

In 8 unrelated patients (eTable 2, links.lww.com/NXG/A602; p.135–p.142) presenting with a highly similar phenotype characterized by cerebellar atrophy, sensory neuropathy and vestibular areflexia ES showed homozygosity for the SNP rs2066782 in exon 19 of the *RFC1* gene, known to be in nearly complete linkage disequilibrium with the pathogenic pentanucleotide (AAGGG) repeat expansion.[19] The repeat region was analyzed for aberrant findings in the BAM files, but the second intron of *RFC1* was not covered. Sanger sequencing of the intronic pentanucleotide repeat expansion in the *RFC1* gene was performed after repeat-primed and long-range PCR amplification confirming the diagnosis of cerebellar ataxia, neuropathy, and vestibular areflexia syndrome (CANVAS, MIM: 614575) (eFigure 1, A–C, links.lww.com/NXG/A600).

A 48-year-old man (eTable 2, links.lww.com/NXG/A602; p.144) was referred to our hospital because of acute cognitive decline with hallucinations, ataxic gait, and myoclonic jerks, reminiscent of Creutzfeldt-Jakob disease (CJD) (MIM: 123400). Analysis of the movement disorders gene panel did not reveal high-impact or medium-impact single nucleotide variants (SNVs) or small indels in the *PRNP* gene, encoding the prion protein. In depth analysis of the octapeptide repeat region encoding codons 51 to 91 revealed the presence of a synonymous nucleotide change in the R3 octapeptide sequence, indicative of the presence of the R3g octapeptide, present in pathogenic octapeptide repeat insertions (OPRI's). PCR fragment analysis followed by Sanger sequencing showed the presence of a heterozygous 4-OPRI, which has shown to be associated with a rapidly progressive CJD phenotype (eFigure 1, D and E, links.lww.com/NXG/A600).[20]

In a 20-year-old male patient (eTable 2, links.lww.com/NXG/A602; p.143) with a clinical diagnosis of Aicardi-Goutieres syndrome presenting with severe intellectual disability, periventricular leukomalacia, and cerebral calcifications, analysis of the leukoencephalopathy gene panel initially did not result in a diagnosis. After *RNU7-1*, a gene encoding a component of the replication-dependent histone pre-mRNA–processing complex, was described as a novel cause of Aicardi-Goutieres syndrome 9,[14,21] we reanalyzed the data and found compound heterozygosity for 2 pathogenic variants in *RNU7-1* (n.27dup; n.40_47del) by analyzing the BAM files. The variants were not prioritized by our in-house developed tool because it prioritizes variants based on predicted impact on protein. It should be noted that noncoding genes are not routinely enriched using the commercially available enrichment kits. *RNU7-1* is located within the first intron of the protein coding gene *C12orf57* (NM_001301839.2) or just upstream of it (NM_138425.4) and was sufficiently covered by ES to detect biallelic pathogenic variants (eFigure 1F, links.lww.com/NXG/A600).

In a patient with a typical clinical and radiologic presentation of leukoencephalopathy with brain calcifications and cysts (LCC, MIM: 614561), ES failed to identify variants in the small nucleolar RNA *SNORD118* (box C/D snoRNA U8) because of insufficient coverage. *SNORD118* is located in the 5′UTR of *TMEM107* (NM_183095.4) in a region which was not enriched with the used kit (Agilent SureSelectXT v6) (eFigure 1E, links.lww.com/NXG/A600). Targeted sequencing of *SNORD118* identified compound heterozygous pathogenic variants (NR_033294.1: n.74G>A; n.*10G>A), thereby confirming the clinical diagnosis.

Identification of Novel Disease-Causing Variants and New Genotype-Phenotype Correlations

Owing to its unbiased approach, ES and large gene panel analysis allows for the identification of novel disease-associated variants and novel genotype-phenotype correlations. 34% of the causative variants have not been classified in ClinVar, nor have they been reported in the literature. As such, 46 novel (likely) pathogenic variants are reported (eTable 2, links.lww.com/NXG/A602).

We highlight the identification of a novel missense variant in the *APP* gene (c.2092A>G, p.[Asn698Asp]), encoding the amyloid beta-A4 precursor protein, in a 75-year-old female patient (p.98) with recurrent stroke and diffuse leukoencephalopathy consistent with cerebral small vessel disease (CSVD) (Figure 2, A and B). Family history was positive with an older brother who suffered from recurrent stroke with periventricular leukodystrophy and multiple lacunar infarcts associated with rapid cognitive decline (Figure 2C). CSF analysis in the patient showed Aβ42 within the normal range of non-Alzheimer disease controls but a slightly elevated level of total tau (Figure 2D). The variant substitutes the well conserved asparagine residue at codon 698 to an aspartic acid residue at position 27 within the β-amyloid (Aβ) peptide, is absent from population database GnomAD, and in silico predictions point toward pathogenicity with a CADD score of 25.6 (Figure 2E). Pathogenic *APP* variants are associated with Alzheimer disease 1 (MIM: 104300) and cerebral amyloid angiopathy (CAA) (MIM: 605714). The *APP* p.(Asn698Asp) variant is located close to a stretch of 5 residues (position 690–695) constituting a hotspot of pathogenic variants known to attenuate α-secretase cleavage and thereby promoting the accumulation of Aβ.[22] We hypothesize that *APP* p.(Asn698Asp) is associated with a CAA-related stroke

phenotype although the pathologic and biochemical details remain to be elucidated.

Discussion

In this study, we assessed the clinical utility of ES and multigene panel testing in 1,411 patients with predominantly adult-onset neurologic diseases. ES has proven its utility as a first-tier genetic test in pediatric patient cohorts, presenting with intellectual disability (ID),[23-26] but evidence in adults is scarce and derived from data on small patient cohorts.[3,27,28] The currently available literature focuses on the utility of ES for specific disease entities such as ID and epileptic encephalopathy, neuromuscular disorders, or specific patient populations such as children or consanguineous families.[3] The cohorts on which the analyses were performed ranged from 24 to 314 patients, and the obtained diagnostic yields ranged from 22 to 68%.[3] With this study, we are the first to report the clinical utility of ES in a large (n > 1,000) predominantly adult patient cohort (93%) with a variety of late-onset neurologic disorders. We showed that ES in adults with RNDs yields a diagnosis in 10% of patients. Only 7% of the cases comprised pediatric patients in which the diagnostic yield was similar to the adult cohort (10%). The 7 gene panels included in this study predominantly contain genes associated with late-onset neurologic diseases and are not suited to diagnose the most common pediatric neurologic disorders such as the epilepsy/intellectual disability disorders. Patients presenting with ataxia or spastic paraplegia were most likely to obtain a definite diagnosis (19%). In patients presenting with unexplained cerebral white matter disease (38% of the entire cohort), a molecular diagnosis was identified in 8%. The obtained diagnostic yield of 10% in patients with neurodegeneration or adult-onset RNDs is significantly lower than the yields reported for other or more specific disease entities.[3] We believe that 2 levels of diagnostic complexity are to be taken into account in the context of RNDs. First, RNDs are known to be associated with clinical heterogeneity caused by nonpenetrance, reduced penetrance, differences in expressivity, and pleiotropy.[29,30] In our cohort, this is demonstrated by the fact that a positive family history was seen in 47% of the solved cases, yet over 60% of the diagnoses are known to be associated with autosomal dominant inheritance. Although we have little information on the rate of de novo variants because of the lack of trios, we hypothesize that nonpenetrance, age-related penetrance, and differences in expressivity explain this discrepancy. In addition, genetic RNDs often have clinical presentations that are (very) similar to the more common, sporadic disorders such as Parkinson disease, Alzheimer disease, and ALS. In addition, accurate phenotyping, for example, in patients presenting with movement disorders can be challenging, especially in early disease stages. The second level of complexity is situated on a molecular level. We showed that the disease-causing variants in our patient cohort consisted of SNVs and small indels (81%), splice site and deeper intronic splice region variants (7%), CNVs (7%), and STR expansions (5%). Only 33% of the diagnosed patients presenting with ataxia and/or spasticity were first tested for the SCAs, Friedreich ataxia, or FXTAS, which probably points toward an underdiagnosis of these more common causes of (spastic) ataxia caused by repeat expansions. It is well-established that ES has its limitations and is not suited to diagnose certain types of genomic alterations such as CNVs, STR expansions, noncoding variants, mtDNA variants, and methylation alterations. It has been shown in the literature that 33% of rare disease diagnoses were not solved through ES but required different methods[31] and that a diagnostic ceiling is reached for ES in ataxias and neurologic disorders.[32] However, an increasing number of tools are being made available to get more out of ES data than just the SNVs and small indels. Using ExomeDepth, we found pathogenic CNVs in 7% of the solved cohort (1% of the entire cohort). ExomeDepth and other tools such as Conifer[33] and Vargenius[34] are being used to detect read depth differences in NGS data of patients with neurologic diseases with reported yields between 1% (dystonia cohort) and 9% (epilepsy/ID).[35-37]

To detect STR expansions, repeat-primed PCR and Southern Blot techniques are still gold standard because of the difficulty to detect repeat expansions in short read NGS data and the occurrence of expansions in noncoding regions, which are not usually enriched in diagnostic ES. Bioinformatic tools such as ExpansionHunter,[38] GANGSTR,[39] and STRetch[40] are increasingly used to detect repeat expansions from short and long read NGS data and have proven their utility.[41] We did not apply these algorithms on our patient cohort. We specifically searched for homozygosity of the SNP (rs2066782), known to be in linkage disequilibrium with the pathogenic intronic *RFC1* repeat, and diagnosed 8 patients with CANVAS. To detect STR expansions and indels in repeat regions (such as the OPRIs in *PRNP*), long read sequencing techniques are promising and will probably replace currently used time-consuming and labor-intensive strategies.[42,43] Another layer of molecular complexity in RNDs is the occurrence of pathogenic variants in the mtDNA. Exome capture kits in diagnostic settings generally do not include mtDNA. However, it was shown that mtDNA sequence can be extracted and reassembled from ES data using tools such as Mitoseek,[44] mito-matic,[45] or MtoolBox,[46] referred to as indirect or untargeted mtDNA sequencing yielding a diagnosis in 0.2% of a large undiagnosed disease cohort.[47] We did not look for mtDNA variants in our patient cohort. Finally, we identified 4 patients with spastic ataxia caused by biallelic *POLR3A* variants, one of which is the intronic c.1909+22G>A, known to be a hypomorphic allele, and frequently implicated in adolescence-onset spastic ataxia.[13,48] Our variant prioritization and annotation pipeline Seqplorer prioritizes high-impact and medium-impact variants including intronic splice region variants within less than 8 nucleotides away from the splice junction. The variant at position +22 was initially not prioritized but is a well-known pathogenic variant. This latter points toward the fact that deeper intronic splice region variants might account for a significant part of the missing heritability in RNDs because these variants are often not prioritized by variant calling pipelines or not sufficiently covered by ES.

Ideally, all pathogenic sequence variants (SNVs, CNVs, structural variants (SVs), noncoding variants, STR expansions, and mtDNA variants) should be able to be detected using 1 single genetic test. With the advent of (long read) genome sequencing (GS), which does not include an enrichment step in the library preparation, we might be heading in the right direction.[49] In addition, techniques to identify structural variation with high resolution such as optical genome mapping (OGM) might help to reduce the missing heritability in RNDs.[50]

A few limitations of this study deserve attention. First, the use of virtual gene panels limits the burden of variants to be interpreted and classified but might be associated with a lower diagnostic yield compared with analysis of the Mendeliome or whole exome. The likelihood of obtaining a diagnosis depends on accurate phenotyping of patients and subsequently the gene panel chosen by the referring physician. This study enabled us to evaluate the performance of the gene panels and showed, for example, that more than 50% of the diagnosed adult cases (23 of 41) with cerebral white matter disease could be attributed to pathogenic variants in *NOTCH3*, *HTRA1*, or *COL4A1/2*. A two-tier strategy in which these genes are analyzed before sequencing or looking at the entire exome might increase diagnostic efficiency. To date, the smaller gene panels associated with more specific phenotypes such as NBIA and PME did not result in diagnoses and were not regularly requested, presumably because of the clinical rarity of these disorders and because of the fact that there is an important overlap with the larger gene panels which are preferred by physicians requesting genetic testing. The ALS gene panel was introduced later and was requested only in familial ALS cases (10%) in which *C9orf72* has been excluded as the underlying cause (causative in 50%).[51] Second, the interpretation and classification of variants depended on the phenotypic information provided on request of the genetic test which was often insufficient. In addition, for the interpretation of class 3 variants, we requested samples of healthy and/or affected family members to perform segregation analysis for reclassification which was not always performed. As such, a diagnostic yield of 10% undoubtedly is an underestimation given the fact that a significant part of the class 3 variants might be disease-causing. Third, we showed that the repeat expansions causing the dominant SCAs and FXTAS were tested only in the minority of the patients presenting with spastic ataxia. We did not evaluate whether there were cases, undiagnosed after ES, with such a triplet repeat expansion underlying the phenotype. Finally, we cannot exclude that the composition of our patient cohort and distribution of the analyzed gene panels were subject to referral bias.

We conclude that ES-based genetic testing is well suited to diagnose hereditary neurologic diseases in adults demonstrated by a diagnostic yield of 10% but also has its shortcomings inherent to its technological limitations. Future studies are necessary to evaluate the utility of novel technologies such as (long read) GS in diagnostics of patients with RND.

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Appendix 1 *(continued)*

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Appendix 1 *(continued)*

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