Next Generation Molecular Diagnosis of Hereditary Spastic Paraplegias: An Italian Cross-Sectional Study

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Hereditary spastic paraplegia (HSP) refers to a group of genetically heterogeneous neurodegenerative motor neuron disorders characterized by progressive age-dependent loss of corticospinal motor tract function, lower limb spasticity, and weakness. Recent clinical use of next generation sequencing (NGS) methodologies suggests that they facilitate the diagnostic approach to HSP, but the power of NGS as a first-tier diagnostic
INTRODUCTION

Hereditary spastic paraplegia (HSP) is a term used to refer to a group of rare (about 1.8 individuals per 100,000) (1) genetically heterogeneous neurodegenerative motor neuron disorders characterized by progressive age-dependent loss of corticospinal motor tract function, leading to lower limb spasticity, urinary bladder dysfunction, and weakness. Next generation sequencing (NGS) methods have recently emerged as the best approach for the genetic study of HSP (2), having allowed, over the past 4 years, the identification of more than 10 novel causative Spastic Gait (SPG) genes. To date, 85 different spastic gait disease loci, and 79 known causative SPG genes, have been identified (3, 4).

Although it is relatively easy, in appropriate clinical practice settings, to reach probable or possible clinical diagnoses of HSP, the high levels of genetic heterogeneity and different patterns of inheritance associated with this group of disorders make molecular diagnosis challenging. NGS, with its innovative technology, is a rapid, high-throughput and cost-effective approach for identifying the genetic background of Mendelian disorders. Target resequencing multigene panels (TRPs) represents the most cost-effective approach involving analysis of the coding exons of a restricted number of genes. Additional high-throughput NGS methods are whole exome sequencing (WES), covering the full set of DNA sequences encoding and whole genome sequencing (WGS) representing the most expensive, all-inclusive technique (5). However, the true informativeness and diagnostic power of these techniques in clinical settings is often limited due to difficulties in processing the considerable amount of information generated through deep sequencing, as well as imperfect genotype-phenotype correlations.

To ascertain the effective diagnostic power of NGS in a real-life neurogenetic setting, we carried out a cross-sectional study adopting a targeted resequencing gene panel (TRP) method already validated in research studies on HSP (6). Although sequencing a whole-exome (or the full genome) provides unparalleled genetic information, we reasoned that panel-based sequencing offers some advantages owing to cost savings and the ease and speed of data interpretation allowing more rapid translation at bedside. The study involved 239 consecutive patients presenting clinical signs of HSP, recruited over the past 4 years (September 2014–August 2018) in tertiary neurological or neuropaediatric centers in Italy; blood samples were analyzed at a single center. In reporting the results of this study, we describe different rates of molecular diagnosis in HSP, and discuss the advantages and disadvantages of our strategy as a first- or second-tier approach in the diagnostic workflow of motor neuron disorders, illustrating some unexpected results as well as major limitations.

MATERIALS AND METHODS

Patients and Study Design

With the help of the Italian Spastic Paraplegias and Ataxias Network (ITASPAX), we consecutively collected DNA samples from patients with a clinical diagnosis of spastic paraplegia. Each index case underwent a detailed neurological examination, carried out by a neurologist or neuropaediatric specialist at the clinical center to which the patient in question had been referred. This examination included application of the Spastic Paraplegia Rating Scale (SPRS) (7). Phenotypes were classified as pure HSP or complicated HSP according to the Harding criteria (8). Genes universally recognized to be responsible for HSP (Table S1) were investigated using a customized NGS TRP
strategy described elsewhere (6). The recruitment of patients \( n = 239 \) and collection of blood samples were performed between September 2014 and August 2018. Whenever possible, major clinical and demographic characteristics, presumed age at disease onset, disease duration and disease severity (SPRS) scores were recorded using a common clinical investigation form (case report form, CRF); these data were subsequently used for correlations with genes/variants identified in the study. For cross-sectional analyses of disease severity, the first documented SPRS score in each proband was selected, whereas all available SPRS scores were included for longitudinal analyses.

**Standard Protocol Approvals, Registrations, and Consent**

All the participants, including relatives involved in segregation studies, provided written informed consent in accordance both with Italian National Health System guidelines and with the Declaration of Helsinki. This consent was collected by the various clinical centers belonging to the ITASPAX network. The study was approved by the Tuscany Regional Pediatric Ethics Committee and also by the Ethics in Research Committee of IRCCS Fondazione Stella Maris (Pisa, Italy). Storage/handling of genetic and personal data complied with Italian National Health Institute (ISS) regulations on ethical and biomedical research and with relevant current legislation.

**Targeted Sequencing Workflow and Sequencing Analysis**

Genomic DNA was extracted from peripheral venous blood using the MagPurix Blood DNA Extraction Kit 200 designed for the MagPurix DNA Extractor (Zinexts, Taiwan).

A library probe was then prepared according to the manufacturer’s instructions (Agilent Technologies, Santa Clara, CA, United States), and the 2200 TapeStation Assay kit (Agilent Technologies) was used to validate and quantify this library preparation. Following the manufacturer’s recommendations, two different customized TRPs were designed over the course of this study. One of them, termed Spastoplex, contained 72 genes and was designed using Haloplex technology (Agilent Technologies) for the Illumina Sequencing system (Illumina Inc., San Diego, CA); it was employed as a second-/third-tier test in 91 index cases in whom absence of punctate mutations and gene deletions/duplications in test in 91 index cases in whom absence of punctate mutations (case report form, CRF); these data were subsequently used for correlations with genes/variants identified in the study. For cross-sectional analyses of disease severity, the first documented SPRS score in each proband was selected, whereas all available SPRS scores were included for longitudinal analyses.

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investigated using STRING (https://string-db.org) in order to highlight possible functional protein-protein interactions.

**Confirmation of Variants**
Putatively deleterious variants were validated by PCR-based standard capillary Sanger sequencing, both in probands and in relatives available for segregation studies, also to determine whether the mutations were inherited or occurred de novo. Segregation analyses contributed to the definition of pathogenic variants. Indeed, whenever more than one change had been prioritized in the index case, study of affected ($n = 113$ individuals) and unaffected relatives ($n = 124$) helped us to determine which variants were more likely to be disease causative.

**MLPA Testing**
Multiplex ligation-dependent probe amplification (MLPA) was always performed to detect gene deletions/duplications in SPAST/SPG4 (13, 14), and in cases that also presented a single mutation in a relatively frequent recessive gene (SPG7, SACS or KIAA1840/SPG11), in order to detect possible second mutations. We used the commercially available Salsa Kits P165-C2 (for SPAST/SPG4 and ATL1/SPG3A), P213-B2 (for REEP1/SPG31 and SPG7), P306-B1 (for KIAA1840/SPG11), and P441-A1 (for SACS), according to the manufacturer’s instructions (MRC-Holland, Amsterdam). MLPA results were analyzed using Coffalyser v.140721.1958 software (MRC-Holland, Amsterdam).

**RESULTS**

**Clinical Findings**
Over a 4-year period, we collected 476 samples: 239 from index patients (median age at latest examination 35 years, range 1–82 years), and 237 from patients’ affected or unaffected relatives. The average age at disease onset in the entire group of investigated patients was 18 years ± 18.7 (SD), and the vast majority of the cases were Italians with Italian parents. Around half of the cases were men (131/239, 55%) and 72% were adults (> 16 years).

Predictably pathogenic mutations were identified in 70 index patients (34 men and 36 women), whose clinical information is summarized in Table 1. Of these patients, assigned a positive molecular diagnosis, 23% showed a dominant pattern of inheritance and 27% clear recessive inheritance; the largest proportion (44%) comprised apparently sporadic patients, while X-linked forms accounted for the smallest proportion (6%, 4 patients). Complex forms were more frequent than pure HSP (65 vs. 35%) and they were more common among sporadic or autosomal recessive (AR) HSP cases, as also reported by others (3).
The patients with a conclusive diagnosis had an average age at disease onset of 17.5 years ± 18.7 (SD) years with a disease duration of 20.3 (1.2) years at first examination. The time to diagnosis was calculated as the months between the appearance of the first medical sign requiring neurological evaluation and molecular diagnostic confirmation. This time could be ascertained with certainty in only 28 patients, all of whom underwent SpastiSure3.0 analysis, and it was, on average, 25.4 months (range: 5.2–37.8).

The clinical presentation of HSP was found to be heterogeneous. Whilst lower limb spasticity was detected in almost all the patients with a clear molecular diagnosis of HSP, brisk reflexes were found in 75%, and bilateral clonus in about 70%. Sensory and cerebellar ataxias were rarely observed (33%), whereas deep sensation was impaired in 68%. Neurophysiological investigations were significantly impaired in 75% of the cases and urinary sphincter disturbances were recorded in about 62%. Abnormal brain MRI findings were seen in 49% of the 70 molecularly defined cases, mostly consisting of cerebellar atrophy or thinning of the corpus callosum, or both. Spine MRI abnormalities were documented in 15% of the index cases. Behavioral abnormalities, including panic disorder, major depression, substance abuse and attention deficit hyperactivity disorder, were observed in five cases. Other co-morbidities included cluster headache (n = 2), optic atrophy (n = 1), lower motor neuron disease (n = 3), early ovarian failure (n = 3), myoclonic seizures (n = 2), and grand mal seizures (n = 1).

Disease severity was ascertained on the basis of disability scores (where 1 = normal, 2 = walks but cannot run, 3 = walks with aids, 4 = wheelchair bound), and corresponded to an average score of 2.6, with less than one third of the patients found to be wheelchair bound at the time of the study. Disease severity, also expressed as the mean SPRS score, was 18.5/52 points (data available for 48 cases), whereas the cross-sectional progression rate, obtained by dividing the SPRS score by the disease duration in each individual, was on average 0.92 SPRS points per year (data available for 42 patients). Segregation analyses in families documented intrafamilial variability in terms of age at disease onset, clinical features and disease progression, as already illustrated by us and others (15–17).

Molecular Findings

In this study, the known HSP-related SPG genes were analyzed, using a validated NGS TRP strategy, in a large cohort of patients. Over 95% of the bases in targeted regions showed an excellent quality value (QC >30) and >99% coverage of the targeted region with a read depth of at least 100X. Spastoplex was applied as a second- or third-line investigation in 91 index patients for molecular confirmation of HSP, whereas 148 patients underwent TRP analysis, with SpastiSure3.0, immediately, as a first-tier approach. Bioinformatic filtering and allele frequencies in public and internal databases were used to prioritize variant types. It was felt that critical, multicenter re-evaluation of clinical presentation, age at disease onset, and segregation in the families might help to establish whether variants could be annotated as disease associated. On the basis of such re-evaluation, and annotation of variants and their confirmation by Sanger sequencing, 85 known or probable pathogenic variants (12 homozygous and 73 heterozygous) were identified in a total of 70 cases, corresponding to a global positive diagnostic yield of 29% (Table 2). VUS were found in 86/239 patients (36% of the full cohort) and the majority of these were detected with the first TRP approach. Once we had adopted a more stringent bioinformatic filters than those described before by us and others (6), 83 patients (35% of the full cohort) were still without a molecular diagnosis (Figure 2) at the end of our study.

Overall, loss-of-function mutations accounted for 43% and missense mutations for 57% of the variants detected (Figure 3). Only four mutations causing a premature stop codon were identified, whereas no large deletions/duplications were detected. As regards the patterns of inheritance among the diagnosed patients, 54% (38/70) had a documented autosomal dominant (AD) pattern of transmission, while 40% (28/70) presented either AR HSP or were sporadic cases in which AR inheritance had not previously been demonstrated. The remaining four cases had X-linked HSP (Figure 4).

Eight patients harbored mutations in SPAST (8/38), which appeared to be the most common mutated AD gene identified in our study. Other mutated AD genes were considerably rarer. In our cohort, about half of the mutations in AR HSP genes

### Table 1: Clinical features in patients with molecular diagnostic confirmation.

| Feature                        | Percentage |
|-------------------------------|------------|
| M/F                           | 34/36      |
| Familial/sporadic             | 39/31      |
| Dominant/recessive/X-linked   | 16/19/4    |
| Age at onset, y, mean ± SD (n) | 17.5 ± 18.7 (56) |
| Duration, mean ± SD (n)       | 20.3 ± 1.2 (69) |
| Disability, mean ± SD (n)     | 2.4 ± 1.6 (63) |
| SPRS, average score (n)       | 18.5 ± 2.4 (48) |

**DISABILITY**

- Stick use                          62.9%
- Wheelchair use                     28.5%

**UPPER LIMB**

- Hypertonia                         25.5%
- Hyperreflexia                      24.5%

**LOWER LIMB**

- Abnormal vibration sense           68.2%
- Amyotrophy                        9.5%
- Pes cavus                         26.9%
- Bilateral clonus                  71.4%
- Urinary dysfunction               61.7%

**ABNORMAL MEP**

- Lower limbs                       73.2%
- Upper limbs                        55.5%

**BRAIN IMAGING FEATURES**

- MRI abnormalities                 68%
- Cerebellar atrophy                22%
- Hyperintense WM                   12%
- Thin corpus callosum              9%
| Index case | Gene   | NM_RefSeq    | cDNA       | NP_Ref   | Protein     | Allelic frequency | References |
|------------|--------|--------------|------------|----------|-------------|-------------------|------------|
| Pt1        | CYP2U1 | NM_183075    | c.1168C>T  (hom) | NP_88898 | p.Arg390Ter  | 1.219e-5         | (19)       |
| Pt2        | CPT1C  | NM_00199752  | c.2133+1G>A (het) | NP_001186681 | /             | 9.018e-6         | This work  |
| Pt3        | CYP7B1 | NM_004820    | c.338insT  (hom) | NP_004811 | p.Phe1145Ter3 | /                 | This work  |
| Pt4        | DDHD1  | NM_00160148  | c.1429C>T  (hom) | NP_001153620 | /             | p.Arg477Ter      | (19)       |
| Pt5        | OPA1   | NM_130836    | c.1180G>A  (hom) | NP_570849 | p.Ala394Thr  | 3.253e-5         | (20)       |
| Pt6        | FA2H   | NM_024306    | c.1051A>G  (het) | NP_077282 | p.Ser351Gly  | /                 | (21)       |
| Pt7        | FA2H   | NM_024306    | c.805C>T  (het) | NP_077282 | p.Arg269Cys   | /                 | (21)       |
| Pt8        | KIF1A  | NM_001244006 | c.440_443delCAinsC  (het) | NP_004811 | p.Gly147Ala+148Lys | / | This work |
| Pt9        | KIF1C  | NM_006612    | c.1046G>A  (het) | NP_006603 | p.Arg349His   | 1.082e-5         | This work  |
| Pt10       | PLP1   | NM_001128834 | c.210T>G  (het) | NP_001122306 | /             | /                 | (22)       |
| Pt11       | FA2H   | NM_024306    | c.103G>T  (het) | NP_077282 | p.Asp35Tyr    | 1.22e-8          | (23)       |
| Pt11       | FA2H   | NM_024306    | c.193C>T  (het) | NP_077282 | p.Pro65Ser    | /                 | (21)       |
| Pt12       | SPG11  | NM_025137    | c.2833A>G  (het) | NP_079413 | p.Arg945Gly   | 3.12e-8          | (24)       |
| Pt12       | SPG11  | NM_025137    | c.128delC  (het) | NP_079413 | p.Ser435fsTer15 | / | This work |
| Pt13       | CYP7B1 | NM_004820    | c.1108C>G  (het) | NP_004811 | p.Arg370Gly   | /                 | This work  |
| Pt13       | CYP7B1 | NM_004820    | c.887A>G  (het) | NP_004811 | p.Asn296Thr   | /                 | This work  |
| Pt14       | DDHD2  | NM_015214    | c.1978G>C  (hom) | NP_066029 | p.As660His    | 6.493e-5         | (25)       |
| Pt15       | CYP2U1 | NM_183075    | c.343G>A  (het) | NP_88898 | p.Gly115Ser   | 6.625e-5         | This work  |
| Pt15       | CYP2U1 | NM_183075    | c.1151G>T  (het) | NP_88898 | p.Arg384ile  | 0.002300         | This work  |
| Pt16       | DDHD2  | NM_015214    | c.759delT  (het) | NP_056029 | /             | /                 | This work  |
| Pt17       | SPG7   | NM_003119    | c.1A>T  (het) | NP_003110 | p.Metarg391Leu | 1.148e-5         | (26)       |
| Pt18       | FA2H   | NM_024306    | c.340_363del (het) | NP_077282 | /             | /                 | (21)       |
| Pt19       | FA2H   | NM_024306    | c.1055C>T  (het) | NP_077282 | p.Thr352Ile   | /                 | (21)       |
| Pt19       | KIF1A  | NM_001244006 | c.760C>T  (het) | NP_001230937 | p.Arg254Trp  | /                 | (27)       |
| Pt20       | KIF1A  | NM_001244006 | c.1048C>T  (het) | NP_001230937 | p.Arg350Trp  | /                 | This work  |
| Pt21       | WASHC5 | NM_014846    | c.2504+1G>A  (het) | NP_056661 | /             | /                 | This work  |
| Pt22       | SLP1   | NM_014846    | c.1625A>G  (het) | NP_056761 | p.Asp542Gly   | 0.0004141        | (28)       |
| Pt23       | CPT1C  | NM_001199752 | c.1802C>T  (het) | NP_001186681 | p.Thr601Met  | 1.219e-5         | This work  |
| Pt24       | TRPV4  | NM_021625    | c.1981C>T  (het) | NP_067838 | p.Arg661Cys   | 2.031e-5         | This work  |
| Pt25       | ERLN2  | NM_007175    | c.187C>A  (het) | NP_090916 | p.Q63K       | /                 | This work  |
| Pt26       | WASHC5 | NM_014846    | c.1924A>G  (het) | NP_056661 | p.Ile642Val   | /                 | This work  |
| Pt27       | KIF1A  | NM_001244006 | c.4927G>A  (het) | NP_001230937 | p.Asp1643Asn | 0.0003241       | This work  |
| Pt27       | KIF1A  | NM_001244006 | c.155T>C  (het) | NP_001230937 | p.Phe522Ser  | /                 | This work  |
| Pt28       | SPAST  | NM_014946    | c.1245+4_1245 +12delAGCTGCTTCTG (het) | NP_056761 | /             | /                 | This work  |
| Pt29       | SPG7   | NM_003119    | c.850_851delTTinsC (het) | NP_003110 | p.Phe284ProfsTer46 | / | (25)       |
| Pt30       | CAPN1  | NM_001198868 | c.618_619delAG (hom) | NP_001185797 | p.G208fsTer7  | 0.0000005        | This work  |
| Pt31       | IFIH1  | NM_0022168   | c.1524T>G  (het) | NP_071451 | /             | /                 | This work  |
| Pt32       | SPG7   | NM_003119    | c.1013G>T  (het) | NP_003110 | p.Gly338Val  | /                 | This work  |
| Pt33       | SETX   | NM_001351527 | c.6122T>C  (het) | NP_001338456 | p.Ile2041Thr  | 0.0001383        | This work  |
| Pt34       | SPG11  | NM_025137    | c.1203delA  (het) | NP_079413 | p.Lys401fsTer15 | / | This work |
| Pt34       | SPG11  | NM_025137    | c.6754+50C>A  (het) | NP_079413 | /             | /                 | This work  |
| Pt35       | L1CAM  | NM_000425    | c.3628G>C  (hom) | NP_000416 | p.Asp1210His  | 5.653e-6         | This work  |
| Pt36       | SPAST  | NM_014946    | c.323_328delTGCGG (het) | NP_055761 | p.V108_109del | /                 | This work  |

(Continued)
occurred in four genes, namely KIAA1840/SPG11, FA2H/SPG35, CYP7B1/SPG5, and DDHD2/SPG54 (Figure S1). In the X-linked forms, two patients carried different missense variants in ABCD1, one had a missense variant in L1CAM/SPG1, and a single case harbored a nonsense change in PLP1/SPG2 (Figure 5).

Interestingly, 26/70 patients (36%) with a clear molecular diagnosis of HSP also showed VUS in other genes, especially KIAA1840/SPG11 and SAC5. However, STRING analysis of these cases did not reveal obvious protein-to-protein interactions that might have partly explained a more complex genotype (data not shown).

## Table 2 (Continued)

| Index case | Gene   | NM_RefSeq | cDNA        | Protein | Allelic frequency | References |
|-----------|--------|-----------|-------------|---------|------------------|------------|
| Pt38      | SPAST  | NM_014946 | c.1130G>A (het) | NP_056761 p. Gly377Glu | /  | (33)           |
| Pt39      | HSPD1  | NM_002156 | c.188T>C (het) | NP_002147 p.Ile63Thr | /  | This work      |
| Pt40      | ERLN2  | NM_007175 | c.860_873dupAGG CCATTGCTTCC (hom) | NP_009106 | / | This work      |
| Pt41      | IFIH1  | NM_002168 | c.1583T>G (het) | NP_071451 p.Leu528Arg | 0.0004600 | This work      |
| Pt42      | SPAST  | NM_014946 | c.164delA (het) | NP_056761 p.Tyr55fsTer5 | /  | This work      |
| Pt43      | PNPLA6 | NM_00116611 | c.3585C>G (het) | NP_00110983 p.Asp195Glu | /  | This work      |
| Pt44      | PNPLA6 | NM_00116611 | c.2398G>A (het) | NP_00110983 p.Val797Met | 0.001776 | This work      |
| Pt45      | ADAR   | NM_001111 | c.164T>C (het) | NP_0011102 p.Pro55Leu | 0.0001119 | This work      |
| Pt46      | DDHD1  | NM_001160148 | c.2189dupT (het) | NP_001153820 p.Leu730fsTer23 | /  | This work      |
| Pt47      | GC1H   | NM_000161 | c.510-1G>C (het) | NP_000152 | /  | This work      |
| Pt48      | AP5Z1  | NM_014855 | c.1302-1G>T (het) | NP_056760 | /  | This work      |
| Pt49      | AP5Z1  | NM_014855 | c.2287G>A (het) | NP_056760 p.Val763Met | 0.0001060 | This work      |
| Pt50      | ADAR   | NM_001111 | c.2159T>C (het) | NP_001102 p.Val720Ala | 2.165e-5 | This work      |
| Pt51      | ERLN2  | NM_007175 | c.502G>A (het) | NP_009106 p.Val169Met | /  | This work      |
| Pt52      | BCD2   | NM_001003800 | c.783A>G (het) | NP_01003800 p.Met265Val | 2.525e-5 | This work      |
| Pt53      | ABCD1  | NM_000033 | c.836T>C (het) | NP_000024 p.Leu279Pro | (31) | This work      |
| Pt54      | DDHD2  | NM_015214 | c.38delA (het) | NP_056029 p.Gln135fsTer16 | /  | This work      |
| Pt55      | DDHD2  | NM_015214 | c.864A>C (het) | NP_056029 p.Ile288Leu | 0.0003393 | This work      |
| Pt56      | GC1H   | NM_000161 | c.454-2A>T (het) | NP_000152 | /  | This work      |
| Pt57      | ZFYVE7 | NM_001002261 | c.149A>G (het) | NP_01002261 p.Tyr50Cys | 3.968e-5 | This work      |
| Pt58      | SPAST  | NM_014946 | c.1728+1G>A (het) | NP_055761 | /  | This work      |
| Pt59      | SPG11  | NM_025137 | c.675+1G>A (hom) | NP_079413 | /  | This work      |
| Pt60      | ABCD1  | NM_000033 | c.1165C>T (rm1) | NP_000024 p.Arg389Cys | 4.634e-5 | (33)           |
| Pt61      | SETX   | NM_001351527 | c.3992C>T (het) | NP_00138456 p.Pro1331Leu | 0.0004152 | This work      |
| Pt62      | REEP1  | NM_01164730 | c.542-4A>G (het) | NP_001158202 | /  | This work      |
| Pt63      | TUBB4A | NM_001289129 | c.1072C>T (het) | NP_001276058 | /  | This work      |
| Pt64      | WASHC5 | NM_014846 | c.1550A>G (het) | NP_055661 p.Asn517Ser | 2.922e-5 | This work      |
| Pt65      | POLR3A | NM_007055 | c.1031G>T (het) | NP_008986 p.Asp344Leu | /  | This work      |
| Pt66      | POLR3A | NM_007055 | c.1909+22G>A (het) | NP_008986 | /  | This work      |
| Pt67      | POLR3A | NM_007055 | c.758T>C (het) | NP_056274 p.Ile253Thr | /  | This work      |
| Pt68      | POLR3A | NM_007055 | c.3201_3202delGC (het) | NP_008986 | /  | This work      |
| Pt69      | POLR3A | NM_007055 | c.1909+22G>A (het) | NP_008986 | /  | This work      |
| Pt70      | POLR3A | NM_007055 | c.460G>T (het) | NP_01230937 p.Val154Phe | /  | This work      |
| Pt71      | POLR3A | NM_007055 | c.1362A>T (het) | NP_056869 p.Gln728Arg | /  | This work      |
| Pt72      | POLR3A | NM_007055 | c.344C>T (het) | NP_004811 p.Ala455CysfsTer17 | 4.063e-6 | This work      |
| Pt73      | POLR3A | NM_007055 | c.5014G>T (het) | NP_004811 p.Ser115Phe | 4.106e-6 | This work      |
| Pt74      | POLR3A | NM_007055 | c.3122_3124delGAC (het) | NP_079413 | /  | This work      |
| Pt75      | POLR3A | NM_007055 | c.3122_3124delGAC (het) | NP_079413 | /  | This work      |
FIGURE 2 | The graphs show the results obtained separately from the two panels used for the study: Spastoplex (A) and Spastisure3.0 (B). The dotted area indicates patients who received a genetic diagnosis; the gray area those found to harbor variants of unknown significance (VUS); and the striped area the cases that remained unsolved. The third graph (C) displays the final result of the cross-sectional study, obtained by averaging the data from the various panels. The VUS section contains a small striped segment, representing the patients (21%) found to carry a single variant in genes that, if mutated, can give rise to their phenotype.

FIGURE 3 | This pie chart shows the different mutation types and their relative frequency among the pathogenic ones identified in this study. As expected, the missense type (thin stripes) is the most frequent, followed by the Ins/Del/Dup (dark gray), splicing (dots), and finally nonsense (thick stripes) types.

FIGURE 4 | This pie chart shows the frequency of HSP inheritance patterns: autosomal dominant HSP is the most frequent (dots), followed by the autosomal recessive (squares) and the X-linked (dark gray) forms.

Overall, most VUS occurred in a set of four genes, namely KIAA1840/SPG11, SACS, AP5Z1/SPG48 and LYST (Figure S2). Among the 86 patients presenting VUS, we enlisted 18 (21%) who are still under investigation because they harbor a single variant in at least one gene that, if mutated, is known to give rise to the clinical conditions they display (Figure 2C). However, since we lack complete CRF data for these cases and/or segregation analysis could not be performed in them, patients were considered *bona fide* members of the VUS group in spite of lacking a complete molecular study. The other 68 HSP cases with VUS showed no molecular findings correlating directly with their clinical features; however, it cannot be excluded that some of the genetic changes they show, as well as new (as yet unknown) genetic changes, play a role in causing the phenotype. Eighty-three index cases resulted molecularly undefined in our NGS study (35% negative yield). Full dataset of gene variants generated for this study can be found in the ClinVar genetic
The wide clinical and genetic heterogeneity of HSP and the increasingly thin line between the various ataxic-spastic and spastic-dystonic forms mean that it is still difficult to arrive at a rapid and precise diagnosis of this condition. NGS approaches are increasingly being used for genetic diagnostics in routine clinical settings, and different published papers report successful use of this technology in HSP (36–39), with positive yields ranging from 20% in adult cases to 52.5% in child cohorts (3, 39–43).

To our knowledge, the present cross-sectional study of 239 HSP cases is among the largest in Europe and Italy to have used TRP analysis. The present study, which addressed “many genes in many patients” and investigated practically all the known genes related to HSP and similar motor neuron disorders, was expected to give a higher positive diagnostic yield than it actually did; furthermore, at least 60% of the patients were investigated underwent NGS testing as a first-tier approach, in order to reflect modern procedures in diagnostic laboratories. However, initial expectations notwithstanding, the overall findings showed a 29% diagnostic yield, a rate similar to those reported in other studies, including more recent ones that present population-specific data (44, 45). There are two possible reasons for this unexpected result. First, the molecular criteria and filtering options used in the present study to define positive cases were perhaps too stringent. Had we used looser criteria and disregarded absence of clinical and segregation data, we would have identified 18 additional cases. Second, about 20 further patients who harbored single potential pathogenic mutations were classified as VUS, since we did not specifically look for second mutations. For example, in two cases we identified, respectively, the p.Ala510Val mutation in SPG7, which is the most common alteration of the paraplegin gene (46), and a nonsense variant in FA2H/SPG35, but did not look for deep intronic changes or gross genomic rearrangements by additional methodologies such as customized array-CGH. Moreover, we decided to exclude variants with >1 homozygous count in gnomAD even those predicted to be deleterious and reported in the Human Gene Mutation Database (HGMD, www.hgmd.cf.ac.uk/). Thus, we can reasonably assume that our diagnostic yield is at least 10–15% lower than the true rate, and therefore can hardly be considered a complete molecular picture of the cohort. The higher yield we obtained when using TRP for first-tier analysis could be attributed to the fact that our collaborators’ clinical data collection became more precise and complete once we had adopted common criteria for inclusion in the study (a common CRF), or it may indicate that new TRP designs offer improved “genotypability,” or even both. Our study is offering results in line with current literature of diagnostic power in HSP (see Table 3). Previous studies have adopted TRP methods (either alone or followed by WES analyses) to corroborate a clinical diagnosis of HSP and the range of diagnostic yield varied from 19 to 62% (36, 39, 41–44, 47–52).

The study we presented also identified a large set of novel variants (see Table 2); indeed, <40% of the 85 pathogenic mutations detected in this research had previously been reported in the literature, or are listed in HGMD. These findings therefore further corroborate the larger-than-expected allelic heterogeneity of the Italian HSP population previously reported in the literature. We confirmed that mutations in SPAST/SPG4 account for more than 20% of solved cases, followed by other relatively less common AD SPG genes (namely, KIF1A and WASHC5). It is of note that we found few variants in REEP1/SPG31 but did not detect mutations in ATL1/SPG3A, which has previously been reported to be the second most common genes responsible for AD HSP, and among those frequently involved in early-onset pure forms (53). However, in our cohort only 16 patients developed pure HSP before

**DISCUSSION**

**FIGURE 5 |** Pie charts showing gene mutation rates by pattern of inheritance in 70 patients.

repository (https://www.ncbi.nlm.nih.gov/clinvar/) (submission code SUB4568040).
involvement with bipolar disorder in a daughter harboring the same mutation in KIF1A. Given that we are expanding the ways in which we genotype HSP patients, we should no longer be surprised to encounter an increasing variety of features associated with the condition.

Finally, it is worth dwelling on one particular result emerging from this study. Around 36% of the patients studied presented VUS, and were therefore left with molecularly undefined or uncertain diagnoses. Clinical manifestations in this group (see Table S2). We're not significantly different from the index cases with a molecular definition. Various technical limitations of the present study, the failure to investigate large gene rearrangements or regulatory intron regions, and even the rare presence of mosaicisms are all factors that might account for these incomplete diagnoses. Alternative explanations, such as very rarely, different zygosity for a known gene, the presence of clinical phenocopies, or the involvement of new HSP genes yet to be discovered, can also apply to this group of patients, as well as to the 83 index cases who were undiagnosed. Nonetheless, we feel that this apparent “missing heritability” in HSP is unlikely to be attributable solely to mutations in as yet unidentified genes. New genes emerged in the past 4 years account for less than 1% of the unsolved cases. It is far more likely that mutations not amenable to current standard approaches including structural variants and variants outside of annotated coding exons account for a substantial share of unexplained cases. Although full use of exome sequencing in clinical diagnosis is an option (44), we therefore propose that the next TRP to be designed should consider complete gene regions and use better bioinformatics tools in order to detect structural variants and uncover the significance of changes in regulatory regions.

Considering the limitations of our study, the increasing clinical overlap between the neurogenetic conditions in different motor neuron diseases (4), and the still large number of HSP patients who remain unsolved, it should be asked whether modern NGS approaches in clinical diagnostic laboratories should switch to more comprehensive technologies. Whilst genome sequencing is increasingly “knocking at the door” of routine clinical diagnostic practice, most national health systems cannot afford to implement this technique fully. In this setting, exome analyses seem to be a practical option. Use of clinical exome sequencing has shown its value in guiding practical patient management, and its diagnostic yield, ranging from 16 to 40% (5, 36), is similar to that of TRP, though reimbursements of costs is not similarly obvious in most health services. Furthermore, coding exon sequencing appears to be a feasible as first-tier diagnostic approach in naive HSP patients. It also seems reasonable to speculate that only full genome studies, or a combination of whole-exome sequencing with RNA studies, are likely to increase diagnostic yield beyond the present rates, once their costs are more affordable on a large scale.

**CONCLUSION**

The advent of the NGS technique in the last decade has revolutionized the way we approach neurogenetic diagnoses in

| TABLE 3 | Relative frequency of diagnostic yield in NGS analyses of patients with hereditary spastic paraplegia. |
|---|---|---|---|---|
| NGS method | Genes analyzed | Number of patients | Diagnostic rate (%) | References |
| TRP 16 | 31 | 15/31 (48%) | (39) |
| TRP 60 | 42 | 13/51 (25%) | (38) |
| TRP 12 | 29 | 14/29 (48%) | (42) |
| TRP (I) 34 | 25 | 8/25 (32%) | (44) total 20% |
| TRP (II) 70 | 73 | 12/73 (16%) | (43) |
| TRP 159 | 105 | 20/105 (19%) | (47) |
| TRP 113 | 47 | 29/47 (62%) | (48) |
| TRP 62 | 55 | 34/55 (62%) | (49) |
| TRP 149 | 99 | 47/99 (47%) | (49) |
| TRP + WES 58 | 97 | 25/97 (26%) | (41) |
| TRP+WES 51 | 37 | 150/526 (28.5%) | (60) |
| Clinical exome 2,731 | 9 | 6/9 (67%) | (39) |
| Clinical exome 4,813 | 66 | 18/66 (27%) | (40) |
| Clinical exome unknown | 48 | 8/48 (17%) | (45) |
| WES / 9 | 13/51 (25%) | (38) |
| WES / 12 | 6/12 (50%) | (61) |
| WES / 21 | 13/21 (62%) | (62) |

TRP: targeted resequencing panel; WES, whole exome sequencing. *Considering the whole cohort of patients; **29%, if also variants of unknown significance were considered; ***33%, considering also probably causative variants.
general, and the diagnosis of HSP in particular. The results of the present study show that the TRP method, used as the first step in clinical diagnostic laboratories, was able to provide information of real clinical significance in about 30–40% patients tested, and it emerged as a relatively inexpensive option (<200 euros per sample). Deeper phenotyping of patients in the clinic, integrated with more rapid use of functional validation (in vitro or in silico), should be mandatory, whether one prefers to use exome sequencing or, instead, larger TRPs, investigating beyond the coding exons. The development of new and more precise sequencing tools combined with universal data sharing, such as multinational initiatives as in GENESIS2.0 (55), and stringent bioinformatics criteria, could also be helpful for annotating variants, especially those classified as VUS. In the era of medical genomics and precision medicine, which has brought the first randomized clinical trials in HSP (56) and a deeper approach in modern neurorehabilitation (57), high levels of genetic heterogeneity should no longer prolong the time to diagnosis and preclude access to new treatment and care opportunities.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Regione Toscana Ethic Committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Regione Toscana Ethic Committee.

AUTHOR CONTRIBUTIONS

AD drafted the manuscript and critically revised molecular results. AD, AT, and MB performed molecular studies and bioinformatics analyses. CaC, MD, AR, GS, RB, CB, CrC, CD, GD, VD, MF, CF, SG, GG, CG, RG, FiG, AK, ML, ChaM, AIM, PM, FM, LM, MM, RM, OM, EP, APe, API, FP, MP, MS, FrS, FT, AT, MV, AA, GA, ChrM, AM, AIP, IR, and SR Contributed clinical information, assessed severity score, and contributed significantly to genotype/phenotype correlations. AlF and AnF revised the clinical strategy and edited the manuscript. FeG and FiS conceived the study, oversaw data acquisition, directed genotype/phenotype correlations, supervised the initial draft and critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2018.00981/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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