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Genetic spectrum of hereditary neuropathies with onset in the first year of life

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Early onset hereditary motor and sensory neuropathies are rare disorders encompassing congenital hypomyelinating neuropathy with disease onset in the direct post-natal period and Dejerine–Sottas neuropathy starting in infancy. The clinical spectrum, however, reaches beyond the boundaries of these two historically defined disease entities. *De novo* dominant mutations in *PMP22*, *MPZ* and *EGR2* are known to be a typical cause of very early onset hereditary neuropathies. In addition, mutations in several other dominant and recessive genes for Charcot–Marie–Tooth disease may lead to similar phenotypes. To estimate

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mutation frequencies and to gain detailed insights into the genetic and phenotypic heterogeneity of early onset hereditary neuropathies, we selected a heterogeneous cohort of 77 unrelated patients who presented with symptoms of peripheral neuropathy within the first year of life. The majority of these patients were isolated in their family. We performed systematic mutation screening by means of direct sequencing of the coding regions of 11 genes: *MFN2*, *PMP22*, *MPZ*, *EGR2*, *GDAP1*, *NEFL*, *FGD4*, *MTMR2*, *PRX*, *SBF2* and *SH3TC2*. In addition, screening for the Charcot–Marie–Tooth type 1A duplication on chromosome 17p11.2-12 was performed. In 35 patients (45%), mutations were identified. Mutations in *MPZ*, *PMP22* and *EGR2* were found most frequently in patients presenting with early hypotonia and breathing difficulties. The recessive genes *FGD4*, *PRX*, *MTMR2*, *SBF2*, *SH3TC2* and *GDAP1* were mutated in patients presenting with early foot deformities and variable delay in motor milestones after an uneventful neonatal period. Several patients displaying congenital foot deformities but an otherwise normal early development carried the Charcot–Marie–Tooth type 1A duplication. This study clearly illustrates the genetic heterogeneity underlying hereditary neuropathies with infantile onset.

Keywords: early onset hereditary neuropathies; congenital hypomyelinating neuropathy; Dejerine–Sottas neuropathy; genotype–phenotype correlations; Charcot–Marie–Tooth disease

Abbreviations: EGR2 = early growth response 2; FGD4 = FYVE, RhoGEF, and PH domain-containing protein 4; GDAP1 = ganglioside-induced differentiation-associated protein 1; MFN2 = mitofusin 2; MPZ = myelin protein zero; MTMR2 = myotubularin-related protein 2; NEFL = neurofilament light chain; PMP22 = peripheral myelin protein 22; PRX = periaxin; SBF2 = set-binding factor 2; SH3TC2 = SH3 domain and tetratricopeptide repeat domain 2.

Introduction

Hereditary motor and sensory neuropathies with onset in infancy are rare disorders that were first described by Dejerine and Sottas, 1893 in the late 19th century as a separate disease, distinct from the more commonly occurring Charcot–Marie–Tooth neuropathy (Gabreels-Festen, 2002; Plante-Bordeneuve and Said, 2002). Today, we know that these early onset hereditary neuropathies are not a single entity but rather represent a broad clinical and genetic spectrum of disorders that is still incompletely understood (Ryan and Ouvrier, 2005).

Presenting symptoms in the neonate can be severe and often include hypotonia and respiratory insufficiency (Phillips *et al*., 1999; Smit *et al*., 2008). In other patients, clinical presentation consists of early foot deformities, delay in early motor milestones, distal sensory loss and weakness with progressive gait difficulties (Plante-Bordeneuve and Said, 2002; Wilmshurst *et al*., 2003; Burns *et al*., 2009). These early and severely affected patients are often isolated in their family, obscuring the inheritance pattern; either *de novo* mutations in dominant genes or recessive alleles inherited from unaffected parents are at play (Gabreels-Festen, 2002; Plante-Bordeneuve and Said, 2002; Parman *et al*., 2004).

Two historically described forms have remained in current literature, although they are now known to represent elements of the broader disease spectrum of demyelinating neuropathies rather than distinct entities (Gabreels-Festen, 2002, 2005; Scherer, 2006). These are congenital hypomyelinating neuropathy with disease onset in the direct post-natal period and Dejerine–Sottas neuropathy, starting in infancy. Typically, *de novo* mutations in *myelin protein zero (MPZ)*, *peripheral myelin protein 22 (PMP22)* and *early growth response 2 (EGR2)* are described as the cause of congenital hypomyelinating neuropathy and Dejerine–Sottas neuropathy. Clinical and genetic variability of hereditary motor and sensory neuropathy reaches beyond these boundaries however.

The currently understood genetic spectrum of early onset hereditary motor and sensory neuropathy is very heterogeneous, including both dominant and recessive mutations in multiple genes (Ryan and Ouvrier, 2005). As already mentioned, *de novo* and occasionally also inherited dominant mutations in *EGR2*, *MPZ* and *PMP22* may occur (Plante-Bordeneuve and Said, 2002; Smit *et al*., 2008). Recessive mutations in *myotubularin-related protein 2 (MTMR2)*, *set-binding factor 2 (SBF2)* and *FYVE, RhoGEF, and PH domain-containing protein 4 (FGD4)* are typically found in patients with redundant myelin loops or myelin outfoldings on sural nerve biopsies (Bolino *et al*., 2000; Nelis *et al*., 2002*a*; Azzedine *et al*., 2003; Fabrizi *et al*., 2009). Recessive mutations in *periaxin (PRX)*, *SH3 domain and tetratricopeptide repeat domain 2 (SH3TC2)* and *ganglioside-induced differentiation-associated protein 1 (GDAP1)* are also associated with very early disease onset (Nelis *et al*., 2002*b*; Takashima *et al*., 2002; Senderek *et al*., 2003). Mutations in *mitofusin 2 (MFN2)*, *GDAP1*, *EGR2* and *neurofilament light chain (NEFL)* can behave both as dominant and recessive traits (Timmerman *et al*., 1999; Nicholson *et al*., 2008; Yum *et al*., 2009; Zimon *et al*., 2011). Since many patients still remain without a molecular diagnosis, the above-mentioned list of genes is by no means exhaustive.

The majority of the early onset hereditary neuropathies represent demyelinating phenotypes with sometimes profoundly slowed motor nerve conduction velocities, indicative of severe demyelination or even amyelination. Noteworthy examples are *PRX* and *FGD4* mutation carriers (Takashima *et al*., 2002; Fabrizi *et al*., 2009) and also congenital onset forms with mutations in *MPZ*, *PMP22* and *EGR2* (Gabreels-Festen, 2002). Nerve conduction studies in *MTMR2*, *SBF2*, *SH3TC2* and *NEFL* mutation carriers typically also show slowed nerve conduction velocity in the demyelinating range (Nelis *et al*., 2002*a*; Azzedine *et al*., 2003; Senderek *et al*., 2003; Parman *et al*., 2004; Yum *et al*., 2009). In addition to the demyelinating phenotypes, severe early onset axonal neuropathies can be due to *MFN2* or *GDAP1*

mutations (Nelis *et al*., 2002*b*; Nicholson *et al*., 2008; Feely *et al*., 2011).

In very young children presenting with neuropathy in the absence of a remarkable familial history, acquired causes of peripheral neuropathy such as inflammatory neuropathies, toxic causes and nutritional deficiencies have to be considered. The disease history and electrophysiology may provide hints in that direction. Acquired causes, however, are proportionally less common than hereditary causes (Connolly, 2001; Wilmshurst *et al*., 2003). More often, peripheral neuropathies in very young children may be seen as part of a syndromic (metabolic) hereditary disorder. Although other clinical features than those of the peripheral neuropathy usually dominate the phenotype, severe demyelinating neuropathy leading to motor development delay may be observed as the initial and only clinical sign in these disorders (Wilmshurst *et al*., 2003; Ryan and Ouvrier, 2005; Scherer, 2006; Landrieu *et al*., 2011).

Detailed electrophysiological testing is essential to confirm the clinical diagnosis and further subdivide patients into demyelinating and axonal phenotypes. Performing such examinations is challenging in very young children, and normal values vary significantly in the first 5 years of life due to ongoing maturation of the PNS and limb growth. For this reason, results of nerve conduction studies must be interpreted with utmost caution (Garcia *et al*., 2000; Wilmshurst *et al*., 2003).

In general, the need for diagnostic sural nerve biopsies has drastically decreased due to the extensive knowledge of the various genetic causes of hereditary neuropathies and the increasing availability of molecular testing. However, in the specific case of early onset neuropathies, nerve biopsies may still be diagnostically meaningful in selected patients (Wilmshurst *et al*., 2003).

Hereditary neuropathies are most often of mixed motor and sensory type, both in adults and children. Some forms of hereditary motor neuropathy and hereditary sensory and autonomic neuropathy can start in infancy as well (Dierick *et al*., 2008; Rotthier *et al*., 2009). These subforms can be distinguished through careful analysis of clinical presentation, electrophysiology and if possible neuropathology. As an important differential diagnosis, Infantile spinal muscular atrophy with respiratory distress type 1 (SMARD1) (spinal muscular atrophy with repiratory distress type 1) due to mutations in *immunoglobulin mu binding protein 2 (IGHMBP2)* should be ruled out because these patients may initially be diagnosed as early onset hereditary motor sensory neuropathy due to very low motor nerve conduction velocities in the upper and lower limbs (Pitt *et al*., 2003). Additional assessment of at least one sensory nerve that is usually normal in these patients may help to distinguish this disorder from typical early onset hereditary motor sensory neuropathy 1.

Since the first description by Dejerine and Sottas, many detailed studies have been published on clinical, electrophysiological and neuropathological aspects of early onset hereditary neuropathies (Dejerine and Sottas, 1893). Molecular genetic studies so far have not focused extensively on systematic genetic screenings but rather on single patient reports, smaller patient series or gene identification studies. These studies offer only a partial view on the genetic variability and the correlation with the clinical phenotypes of childhood neuropathies.

Early onset neuropathies pose a particular diagnostic challenge both to the treating child neurologist confronted with often severely affected patients and to geneticists seeking to provide molecular diagnosis and appropriate genetic counselling.

In the current study, we report on the findings from the systematic screening of 11 relevant genes in a cohort of 77 unrelated patients with presumed hereditary motor and sensory neuropathy with onset in the first year of life. Several of these patients have been reported before, either in smaller case reports or as part of gene identification studies. Patients presenting with pure hereditary motor neuropathy or hereditary sensory and autonomic neuropathy have been studied previously and were therefore not included (Dierick *et al*., 2008; Rotthier *et al*., 2009). These genotype–phenotype correlations provide important insights that are of particular relevance in the context of molecular diagnostics of rare hereditary neuropathies with an early disease onset.

Patients and methods

Patient cohort

In this study, 77 unrelated index patients were included who presented with symptoms of motor and sensory neuropathy within the first year of life. In order to grasp the full spectrum of phenotypes, we did not apply exclusion criteria regarding electrophysiology, inheritance pattern, associated clinical features or neuropathology. Presenting symptoms varied widely ranging from early neonatal hypotonia with feeding and breathing difficulties over progressive delay of motor milestones to early foot deformities in the first 12 months of life. A diagnosis of demyelinating neuropathy was made for 45 patients and axonal neuropathy for 15 patients. For the remaining 17 patients, no clear distinction could be made. Detailed electrophysiology was available for 50 patients. For a total of 29 patients, neuropathological examination of a sural nerve biopsy was performed. In total, 61 patients were isolated in their family, in 10 patients a dominantly inherited phenotype was seen in the family and in six patients familial history was suggestive of a recessive trait. For 21 out of the 77 index patients consanguinity was noted; 18 of these were isolated in their family.

Patients were referred for genetic diagnosis from various European countries, the Middle East and the USA and were clinically evaluated by neuro-paediatricians with expertise in the field of rare neuromuscular disorders. Parents or legal representatives of all patients signed an informed consent form prior to enrolment. The local institutional review boards approved the study.

Mutation screening

Genomic DNA was extracted by means of standard protocols from blood samples obtained from patients, healthy unrelated controls and, if available, family members. The coding regions and exon–intron boundaries of 11 genes (*MFN2*, *PMP22*, *MPZ*, *EGR2*, *GDAP1*, *NEFL*, *FGD4*, *MTMR2*, *PRX*, *SBF2* and *SH3TC2*) were polymerase chain reaction amplified using primer oligonucleotides designed with Primer3 (Rozen and Skaletsky, 2000) Primer sequences and polymerase chain reaction conditions are available on request.

Polymerase chain reaction products were purified with the Exonuclease I-Shrimp Alkaline Phosphatase enzymes (USB). Mutation screening was performed by bidirectional sequencing using the BigDye®

Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Fragments were electrophoretically separated on an ABI3730xl DNA Analyser (Applied Biosystems). Sequence analysis was performed with the SeqMan™II (DNASTAR Inc.) programme. The nucleotide numbering was based on the published online protein and messenger RNA sequence of the respective genes (www.ncbi.nlm.nih.gov). The conventions of the Human Genome Variation Society nomenclature (http://www.hgvs.org/mutnomen) were used for the description of the identified mutations. Sequence variants were confirmed by an independent polymerase chain reaction and sequencing of the original or new stock DNA samples. Segregation of the mutations was performed when DNA samples of family members were available. For newly identified mutations, a minimum of 360 control chromosomes were screened.

Copy number variation studies

The Multiplex Amplicon Quantification technique was applied (Sleegers *et al*., 2006) in order to investigate the presence of pathogenic copy number variations on the second allele in patients with heterozygous variations in *MTMR2* and *PRX*. The same technique was used to screen for the CMT1A duplication/HNPP deletion in the 17p11.2-12 region.

Multiplex amplicon quantification consists of a multiplex polymerase chain reaction amplification of fluorescently labelled target and control amplicons, followed by fragment analysis on an ABI3730 DNA Analyser (Applied Biosystems). In this assay, target amplicons are located in and around the exons of the corresponding genes and eight control amplicons are located at randomly selected genomic positions outside the corresponding target regions and other known copy number variations. These amplicons were polymerase chain reaction amplified in a single reaction containing 20 ng of genomic DNA. Peak areas of the target amplicons were normalized to these of the control amplicons. Comparison of normalized peak areas between patients and reference individuals resulted in a dosage quotient for each target amplicon, calculated by the Multiplex Amplicon Quantification software package (www.multiplicon.com). Dosage quotient values <0.75 were considered indicative for a deletion.

To further confirm an identified partial duplication of *PMP22*, the commercially available Multiplex Ligation-dependant Probe Amplification kit (P033-CMT1) was used. The same technique was used to check for additional partial deletions of *MFN2* (P143-MFN2-MPZ). Manufacturer's instructions were applied (MRC).

Results

Genetic findings

Extensive mutation screening of 11 genes in a large cohort of 77 unrelated patients with neuropathy with onset in the first year of life revealed pathogenic sequence variants in 35 patients, representing 45% of the total cohort. Sequence variants were found in all screened genes. The genetic findings are presented per gene of interest in Table 1 and mutation frequencies per gene are shown in Fig. 1.

Some patients have been reported previously as part of gene identification studies or smaller case series; the relevant references are provided in Table 1 (Raeymaekers *et al*., 1991; Nelis *et al*., 1994, 2002*b*; Sorour *et al*., 1997; Timmerman *et al*., 1999; Ceuterick-de Groote *et al*., 2001; Takashima *et al*., 2002; Senderek *et al*., 2003; Stendel *et al*., 2007; Fabrizi *et al*., 2009).

A total of five patients carried a *de novo* heterozygous mutation in *MPZ*, *EGR2*, *PMP22* or *MFN2*. Four isolated patients were heterozygous for mutations in *NEFL* or *MPZ*, the CMT1A duplication or a partial *PMP22* duplication; for these, however, the *de novo* character could not be confirmed due to lack of DNA samples from additional family members. Mutations in *MPZ* and the CMT1A duplication were transmitted as a dominant trait in five patients; 20 patients inherited recessive mutations in *GDAP1*, *MTMR2*, *SBF2*, *FGD4*, *PRX* or *SH3TC2* from their unaffected parents. In one isolated patient (CMT-756.01), a novel heterozygous Arg400Pro mutation in *MFN2* was identified. Low levels of the same mutant allele in the electropherogram of the unaffected mother suggest a parental mosaicism for this mutation. This mutation targets a highly conserved amino acid and is probably damaging for the protein as predicted by a high Polyphen2 score (Adzhubei *et al*., 2010).

Additional Multiplex Amplicon Quantification and Multiplex Ligation-dependant Probe Amplification assays in the patients with heterozygous sequence variants in *PRX, MTMR2* and *MFN2* did not provide evidence for partial intragenic deletions.

Clinical findings

Detailed clinical and electrophysiological findings in patients carrying pathogenic mutations are presented in Tables 2 and 3. The data from the nerve conduction studies have to be interpreted with caution since the age at which electrophysiological testing was performed varied widely among patients making the application of one standardized set of normal values impossible (Garcia *et al*., 2000).

All patients with pathogenic mutations presented with symptoms suggestive of peripheral neuropathy within the first year of life. Presenting symptoms were nonetheless variable. A small number of patients present very soon after birth with hypotonia, occasionally associated with breathing difficulties. A second group of patients present with congenital or very early foot deformities often in combination with progressive delay in motor milestones in the first year of life, after an otherwise normal neonatal period.

Discussion

We performed a broad and systematic genetic screening in a large cohort of patients presenting with infantile onset hereditary neuropathy. Overall, a molecular diagnosis could be reached in 45% of patients by screening for mutations in a total of 11 genes and for the CMT1A duplication in the 17p11.2-12 region (Fig. 1). Although the overall mutation frequency was high, the genetic heterogeneity is extensive resulting in a diagnostic yield per gene that rarely exceeds more than a few per cent of the total cohort. Both dominant and recessive mutations are implemented in this age group with the recessive ones being slightly more prevalent in our series (20 index patients versus 15). Most common were the CMT1A duplication and mutations in *MPZ*,

Table 1 Overview of genetic findings in 35 patients with hereditary neuropathy with onset in the first year of life

Gene	Mutation	Individual	Inheritance	Segregation	Ethnicity	Consanguinity	Reference/additional remark
CMT1A	Duplication	CMT-AII2_5.1	Autosomal dominant	+	Belgian	–	Raeymaekers et al. (1991)
		PN-1745.1	Isolated case	–	Belgian	–	
		PN-491.1	Autosomal dominant	+	Austrian	–	
		PN-908.3	Autosomal dominant	+	Spanish	–	
PMP22	Partial duplication exon 4	CMT-127.04	Isolated case	–	European	–	
EGR2	Arg359Trp	PN-27.1	Isolated case, *de novo*	+	Ashkenazy Jewish	–	Timmerman et al. (1999)
MFN2	**Arg104Trp**	CMT-797.01	Isolated case, *de novo*	+	Greek	–	
	Arg400Pro	CMT-756.01	Isolated case,	+	Italian/Irish	–	Parental mosaicism in asymptomatic mother
MPZ	His81Arg	CMT-65.07	Autosomal dominant	+	British	–	Sorour *et al.* (1997). Two additional family members born with clubfeet
	Ala209GlufsX24	PN-1540.1	Isolated case, *de novo*	+	Belgian	–	
	Asp134Glu	PN-506.1	Autosomal dominant	+	Belgian	–	Nelis *et al.* (1994). Two additional family members with delayed motor milestones
	Arg98Cys	PN-752.1	Isolated case	–	Belgian	–	
	Arg98Cys	PN-966.4	*De novo*	+	Austrian	–	Similarly affected identical twins
NEFL	Asn98Ser	PN-1385.1	Isolated case	–	Finnish	–	
PMP22	Ser72Leu	PN-750.3	Isolated case, *de novo*	+	Belgian	–	Ceuterick-de Groote *et al.* (2001)
FGD4	Tyr587fsX14 hmz	CMT-190.01	Isolated case	+	Italian	+	Fabrizi *et al*,(2009)
	Arg224stop hmz	CMT-230.01	Isolated case	+	Turkish	+	Stendel *et al.* (2007)
GDAP1	Ser194stop hmz	PN-860.3	Autosomal recessive	+	Moroccan	+	Nelis *et al.* (2002)
MTMR2	**His416ArgfsX6 hmz**	CMT-201.01	Isolated case	–	Turkish	–	
PRX	**Gly1258ThrfsX124 hmz**	PN-1699.1	Isolated case	–	Druze (Israel)	+	
	Ala244ArgfsX69 hmz	PN-2175.1	Isolated case	+	Moroccan	+	
	Cys715stop hmz	PN-44.1	Autosomal recessive	+	Belgian	+	Takashima *et al.* (2002)
	Leu83CysfsX14 hmz	PN-761.3	Isolated case	+	Maghreb	+	Takashima *et al.* (2002)
SBF2	**Gln513Stop hmz**	CMT-194.01	Isolated case	–	Turkish	+	
	Tyr1594Stop hmz	CMT-220.01	Isolated case	+	Turkish	+	
	Leu1316PhefsX9 + Arg1433Ser	PN-1101.2	Isolated case	+	Polish	–	
SH3TC2	Arg954Stop + **Ala878Asp**	CMT-191.01	Isolated case	+	Italian	–	
	Arg954Stop hmz	CMT-192.01	Isolated case	+	Italian	–	
	Arg529Gln hmz	CMT-133.01	Isolated case	+	Turkish	+	Senderek *et al.* (2003)
	IVS5-2A>G hmz	CMT-189.V.5	Autosomal recessive	+	Italian	+	Senderek *et al.* (2003)
	Glu657Lys hmz	CMT-234.01	Isolated case	+	Turkish	+	Senderek *et al.* (2003)
	Arg583AlafsX586 hmz	CMT-235.01	Isolated case	+	Turkish	+	Senderek *et al.* (2003)
	Leu832HisfsX839 hmz	PN-1289.1	Isolated case	–	Iranian	+	Senderek *et al.* (2003)
	Arg954stop hmz	PN-1321.1	Isolated case	+	Belgian	–	
	Arg954stop hmz	PN-754.3	Isolated case	+	Dutch	–	

Mutations are heterozygous unless stated otherwise, novel mutations are shown in bold, hmz, homozygous.

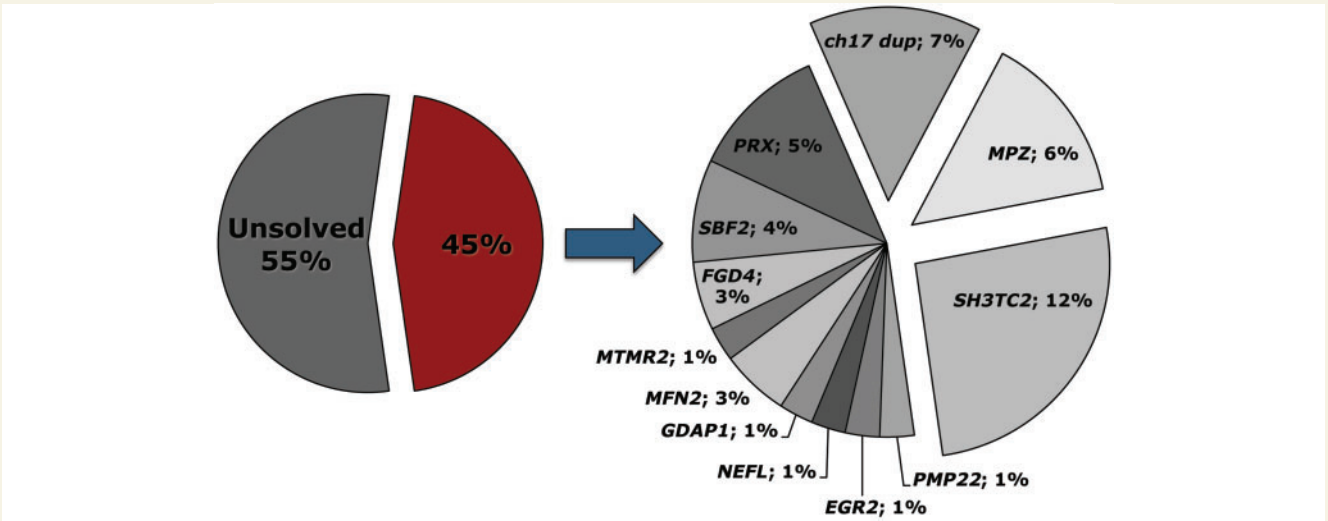

Figure 1 Gene distribution among 35 patients with pathogenic mutations out of a cohort of 77 unrelated index patients.

PMP22, *PRX* and *SH3TC2*, accounting together for 69% of the identified pathogenic variations.

As far as dominant mutations are concerned, these are often *de novo* events, as was previously described in patients with congenital hypomyelinating neuropathy or Dejerine–Sottas neuropathy (Gabreels-Festen, 2002; Plante-Bordeneuve and Said, 2002). However, we also show a few cases of inherited dominant traits (mainly for *MPZ* and the CMT1A duplication) that can also produce severe early onset phenotypes in some patients, in spite of the generally milder phenotype in the other family members (Raeymaekers *et al*., 1991; Nelis *et al*., 1994; Sorour *et al*., 1997). These young patients represent the far end of the disease spectrum associated with the various demyelinating types of classical Charcot–Marie–Tooth disease. In these instances, the familial history is useful in guiding the molecular testing, but one has to be equally alert for such mutations in isolated patients. It is important to note that more mildly affected or even asymptomatic individuals are still at risk of having offspring with a more severe onset phenotype. This was recently shown for other dominantly inherited Charcot–Marie–Tooth variants caused by mutations in *TRPV4* and *GDAP1* (Zimon *et al*., 2010, 2011). There are also reports in the literature of transmitted cases of severe Charcot–Marie–Tooth phenotypes (Smit *et al*., 2008). For the dominant genes, mutations in *MPZ* were equally frequent as patients carrying the CMT1A duplication. In the particular case of patient CMT-756.01 with the novel heterozygous Arg400Pro mutation in *MFN2*, a possible parental mosaicism was identified. This finding has important implications towards genetic counselling and emphasizes the importance of critical analysis of DNA samples of clinically unaffected parents.

Mutations in recessive genes are often found in isolated patients, many of whom are the product of a consanguineous union. The cohort used for this study contained a total of 21 index patients from such consanguineous families; this may have influenced the total frequency of recessive forms to some extent. It is, however, important to note that of the 20 index patients with recessive mutations, at least five stem from non-consanguineous European families (PN-1101.2, CMT-191.01, CMT-192.01, PN-1321.1 and PN-754.3). Therefore, a certain degree of suspicion for recessive forms of neuropathy is warranted in non-consanguineous populations, especially since the inheritance pattern would not be evident in small kinships.

Overall, we found *SH3TC2* to be the most commonly mutated recessive gene in this cohort. *SH3TC2* is associated with variable phenotypes and is known to be relatively common among the recessive Charcot–Marie–Tooth variants (Houlden *et al*., 2009).

From a phenotypic point of view, it is important to note that even within this group of patients with neuropathy with a very early disease onset, there is considerable clinical variability. A few different subgroups of patients can be delineated that in turn correspond to selected subsets of the genes screened in this study (Fig. 2).

The first group comprises genuinely congenital onset phenotypes presenting soon after birth with symptoms such as hypotonia and breathing difficulties. In these patients, *de novo* mutations in *MPZ*, *EGR2* and *PMP22* are typically found (Phillips *et al*., 1999; Timmerman *et al*., 1999; Ceuterick-de Groote *et al*., 2001). In addition, we identified a heterozygous *NEFL* mutation that was previously reported to cause severe early onset phenotypes (Yoshihara *et al*., 2002). More surprisingly, we detected a previously unreported partial duplication of exon 4 of *PMP22* in an isolated patient presenting with a congenital onset phenotype. Although the vast majority of copy number variations in the 17p11.2-12 region is the recurrent 1.4 Mb CMT1A duplication/deletion, some atypical non-recurrent copy number variations have been reported including the entire *PMP22* gene, single *PMP22* exons or regulatory sequences (Zhang *et al*., 2010). The *de novo* character of this variation in patient CMT-127.04 could not be proven due to lack of the parent's DNA samples; it may therefore still be a benign polymorphism. However, this *PMP22* exonic duplication may well be pathogenic through such mechanisms as exon shuffling or insertional translocation

Table 2 Overview of clinical findings in 35 patients with hereditary neuropathy with onset in the first year of life

Gene	Individual	Onset age	Symptoms at onset	Age at last examination (yrs)	Motor delay	Respiratory insufficiency	Foot deformities	Walking	Nerve pathology	Additional features
CMT1A dup	CMT-AII2_5.1	Congenital	Foot deformities	65	Absent	Absent	Present	Never walked normally		Early scoliosis, bilateral deafness
	PN-1745.1	Congenital	Club feet	2	Present	Absent	Present	Gait difficulties, walked at 22 m		Foot surgery at 6 m
	PN-491.1	Congenital	Foot deformities	4	Absent	Absent	Present	Progressive gait difficulties (4 y)		
	PN-908.3	Congenital	Foot deformities	19	Absent	Absent	Present	Unsteady gait with steppage, unaided	Focally folded myelin, rare OBF (8 y)	Multiple foot surgery, younger brother similar phenotype, father mildly affected
PMP22 exon 4 duplication	CMT-127.04	Congenital	Hypotonia	13	Present			Never walked independently, wheelchair bound by age 7 y	Hypomyelination, classic and basal lamina OBF and very short myelinated internodes	Severe scoliosis requiring surgery, Parents and two sibs asymptomatic and normal nerve conduction velocities
EGR2	PN-27.1	Congenital	Hypotonia with breathing difficulties	13	Present	Present		Disturbed, walked unaided at 3 y	Severe fibre loss, demyelination and focally folded myelin	Died at 16 y due to pneumonia
MFN2	CMT-797.01	<1 y	Developmental delay	5	Present	Absent	Absent	Walked without assistance >39 m, AFOs		
	CMT-756.01	<1 y	Delayed motor milestones	10	Present	Absent	Present	Delayed walking, pronounced steppage gait		Brisk tendon reflexes, no clinical sensory loss
MPZ	CMT-65.07	Congenital	Clubfeet	28	Absent	Absent	Present	Progressive walking difficulties in primary school		Sensory ataxia, no papillary accommodation reflex
	PN-1540.1	Congenital	Hypotonia, breathing difficulties	27	Present	Present	Present	Never walked unsupported, wheelchair bound since age 6 y	Hypomyelination	Severe scoliosis requiring surgery
	PN-506.1	<1 y	Delayed motor milestones	16	Present	Absent	Present	Walked with support at 21 m, wheelchair bound since age 15 y	Severe demyelination	
	PN-752.1	<1 y	Delayed motor milestones	38	Present	Absent	Present	Started walking at 33 m	Demyelination	Scoliosis, nystagmus, sensory ataxia
	PN-966.4	<1 y	Delayed motor milestones	7	Present	Absent	Absent	Started walking at 30 m, never walked normally		Proximal weakness, identical twin with similar phenotype
NEFL	PN-1385.1	3-4 m	Hypotonia, growth retardation	4	Present	Absent	Absent	Started walking at 25 m		

(continued)

Table 2 Continued

Gene	Individual	Onset age	Symptoms at onset	Age at last examination (yrs)	Motor delay	Respiratory insufficiency	Foot deformities	Walking	Nerve pathology	Additional features
PMP22	PN-750.3	Congenital	Hypotonia	4	Present	Absent	Present	Walks with aid at 2 y, never walked independently	Hypomyelinating neuropathy on skin biopsy	
FGD4	CMT-190.01	<1 y	Delayed motor milestones	21	Present	Absent	Present	Walked with aid at 17 m, unsteady gait with steppage ever since	Hypertrophic demyelinating neuropathy with focally folded myelin	Scoliosis
	CMT-230.01	<1 y	Delayed motor milestones	30	Present	Absent	Present	Delayed walking		
GDAP1	PN-860.3	2 m	Foot deformity	7	Present	Absent	Present	Started walking at 16 m with limp, progressive deterioration	Large myelinated axons Absent, regenerating clusters, rare OBF	
MTMR2	CMT-201.01	8 m	Delayed motor milestones	8	Present	Absent	Present	Delayed and disturbed from the onset	Fibre density↓, focally folded myelin, OBF	
PRX	PN-1699.1	Congenital	Hypotonia	11	Present	Absent	Present	Delayed, only walked with aid at the age of 4 y, walks unsteady at 11 y		Moderate proximal weakness, 47XXX karyotype
	PN-2175.1	<1 y	Delayed motor milestones	4	Present	Absent	Absent	Delayed, walked with assistance at 16 m, without assistance at 30 m		
	PN-44.1	<1 y	Delayed motor milestones	50	Present	Absent	?	Delayed	Loss of myelinated axons, OBF, myelin outfoldings, no septate-like junctions in paranodal myelin	Hearing loss, scoliosis
	PN-761.3	<1 y	Delayed motor milestones	6	Present	Absent	?	Delayed, stood with support at 4 y, walked at 5 y	Severe loss large myelinated fibres, basal lamina OBF	
SBF2	CMT-194.01	<1 y	Delayed motor milestones	15	Present	Absent	Present	Delayed, walked unsupported at 2, 5–3 y, frequent falls	Loss of myelinated axons, small OBF, focally folded myelin	Hypophonia
	CMT-220.01	<1 y	Delayed motor milestones	8	Present	Absent	Present	Delayed, walked unsupported at 2, 5–3 y		
	PN-1101.1	<1 y	Delayed motor milestones	10	Present	Absent	Present	Delayed, walked with support at 2 y	Focally folded and uncompacted myelin, OBF	Anisocoria, facial weakness

(continued)

Table 2 Continued

Gene	Individual	Onset age	Symptoms at onset	Age at last examination (yrs)	Motor delay	Respiratory insufficiency	Foot deformities	Walking	Nerve pathology	Additional features
SH3TC2	CMT-191.01	<1 y	Delayed motor milestones	10	Present	Absent	Present	Delayed walking, unsteady gait, frequent falls, steppage, walks with AFO	Hypertrophic de-remyelinating neuropathy with focally folded myelin and basal-lamina OBF	Severe scoliosis since age 10 y, requiring surgery at age 16 y, short stature
	CMT-192.01	<1 y	Hypotonia	12	Absent	Present	Present	Started walking at 13 m but with frequent falls, wheelchair bound at 12 y	Hypertrophic de-remyelinating neuropathy with focally folded myelin and basal-lamina OBF	Severe scoliosis since age 2 y requiring surgery at age 12 y, bilateral facial weakness
	CMT-133.01	<1 y	Delayed motor milestones	8	Present	Absent	Absent	Walked at 30 m	Branching of SC on nerve biopsy	
	CMT-189.V.5	<1 y	Delayed motor milestones	15	Present	Present	Absent	Walked at 24 m, progressive worsening, wheelchair bound at 15 y		Pronounced scoliosis at 7 y, progressive over time with respiratory difficulties at 30 y
	CMT-234.01	<1 y	Delayed motor milestones	25	Present	Absent	Present	Delayed walking, walks with aid		Nystagmus
	CMT-235.01	<1 y	Delayed motor milestones	19	Present	Absent	Present	Walked with aid at 18 m	OBF, SC branching	
	PN-1289.1	<1 y	Delayed motor milestones	17	Present		Present	Started walking at 24 m		Congenital nystagmus, scoliosis requiring surgery
	PN-1321.1	<1 y	Delayed motor milestones	42	Present	Absent	Present	Delayed walking at 26 m, progressive over time, wheelchair dependency in adulthood	Hypertrophic demyelinating neuropathy with basal lamina OBF	Pronounced scoliosis with short stature, sensorineural hearing loss
	PN-754.3	<1 y	Delayed motor milestones	10	Present	Absent	Present	Started walking at 20 m	Demyelinating neuropathy	Pronounced scoliosis

Foot deformities include pes cavus, pes planus, hammer toes and club feet.
AFO = ankle foot orthosis; m = months; OBF = onion bulb formation; SC = Schwann cell; y = years.

Table 3 Overview of nerve conduction studies in 35 patients with hereditary neuropathy with onset in the first year of life

Gene	Patient	Age	Median motor		Ulnar motor		Peroneal motor		Tibial motor		Median sensory		Ulnar sensory		Sural sensory	
			Amp	CV	Amp	CV	Amp	CV	Amp	CV	Amp	CV	Amp	CV	Amp	CV
CMT1A dup	CMT-AII2_5.1	–	–	17.0	–	20.0	–	–	–	–	–	23.0	–	–	–	–
	PN-1745.1	20 m	–	–	–	–	2.0	18.0	4.6	20.0	–	–	–	–	–	–
	PN-491.1	4 y	–	–	–	–	1.5	17.5	–	–	–	–	–	–	–	–
	PN-908.3	19 y	5.5	19.3	–	–	0.05	12.3	–	–	–	–	–	–	A	A
PMP22 exon 4 duplication	CMT-127.04	13 y	A	A	A	A	A	A	A	A	A	A	A	A	–	–
EGR2	PN-27.1	6 y	1.5	8.0	–	–	0.4	8.0	–	–	A	A		–	A	A
MFN2	CMT-797.01	–	↓	–	↓	–	↓	–	↓	–	A	A	A	A	A	A
	CMT756.01	9 y	–	–	–	–	A	A	↓	–	–	–	–	–	N	–
MPZ	CMT-65.07	–	–	11.1	–	–	–	–	–	–	A	A	–	–	–	–
	PN-1540.1	20 y	1.4	2.8	A	A	A	A	A	A	A	A	–	–	A	A
	PN-506.1	4 y	0.6	7.0	1.2	17.0										
	PN-752.1	38 y	0.2	5.0	0.7	7.0	–	5.0	0.02	6.0	–	–	–	–	–	–
	PN-966.4	–	–	11.0	–	6.0	–	–	–	–	–	–	–	–	–	–
NEFL	PN-1385.1	5 y	–	29.0	–	–	–	24.0	–	–	A	A	A	A	A	A
PMP22	PN-750.3	2 y	A	A	–	–	A	A	A	A	A	A	–	–	A	A
FGD4	CMT-190.01	11 y	1.6	6.2	2.9	8.4	A	A	A	A	A	A	A	A	A	A
	CMT-230.01	–	–	5.0	–	–	–	–	–	–	A	A	–	–	–	–
GDAP1	PN-860.3	3 y	1.9	42.0	1.5	50.0	A	A	–	–	A	A	–	–	A	A
MTMR2	CMT-201.01	7 y	0.6	13.0	–	–	A	A	0.6	13.0	A	A	–	–	A	A
PRX	PN-1699.1	11 y	–	–	–	–	A	A	A	A	–	–	–	–	A	A
	PN-2175.1	3 y	1.9	6.9	–	–	–	–	–	–	–	–	–	–	–	–
	PN-44.1	41 y	1.1	3.0	0.5	3.0	–	–	–	–	A	A	–	–	–	–
	PN-761.3	5 y	A	A	–	–	–	–	–	–	–	46.3	–	–	A	A
SBF2	CMT-194.01	8 y	–	–	–	26.0	–	–	–	–	–	–	–	–	–	–
	CMT-220.01	9 y	–	16.0	–	22.0	–	–	–	–	–	–	–	–	–	–
	PN-1101.1	10 y	–	–	–	21.0	–	14.0	–	–	–	–	–	–	–	–
SH3TC2	CMT-191.01	10 y	9.1	34.5	6.2	25.0	1.4	18.4	–	–	–	–	–	–	0.2	29.4
	CMT-192.01	10 y	0.2	39.6	4.5	30.0	0.1	19.5	–	–	–	–	–	–	7.0	40.6
	CMT-133.01	–	–	27.0	–	37.0	–	24.0	–	–	A	A	–	–	–	–
	CMT-189.V.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	CMT-234.01	–	–	12.0	–	–	–	–	–	–	–	–	–	–	–	–
	CMT-235.01	–	–	26.0	–	–	–	21.0	–	–	–	34.0	–	–	A	A
	PN-1289.1	20 y	3.9	31.6	4.3	37.9	0.1	26.5	–	–	A	A	–	–	–	–
	PN-1321.1	33 y	0.7	11.9	1.2	11.7	0.8	22.6	0.2	12.7	–	33.0	–	–	–	–
	PN-754.3	2 y	1.5	27.0	–	–	1.0	23.0	–	–	15.0	42.0	–	–	–	–

A = absent response; Age = age at examination; Amp = amplitude (motor: in millivolt; sensory: in microvolt); CV = conduction velocity (in metre per second); N = normal; – = not available; ↓ = reduced.

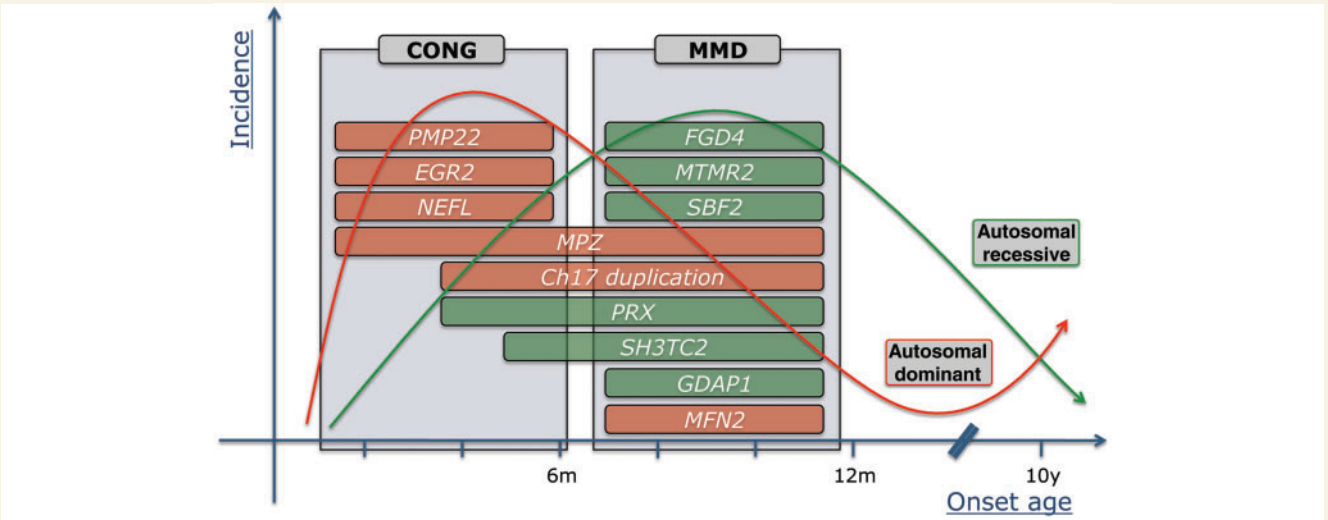

Figure 2 Gene distribution for patients with a known pathogenic mutation in function of disease onset, inheritance pattern and phenotypic subgroup. The *x*-axis represents the age at onset in months and years, the *y*-axis displays the estimated incidence of patients with dominant (red line) and recessive (green line) subtypes of hereditary neuropathy. CONG = congenital onset phenotype characterized by neonatal hypotonia ± breathing and feeding difficulties; MMD = motor milestone delay phenotype with or without early foot deformities and progressive delay in motor milestones within the first year of life; m = month; y = year.

(Zhang *et al*., 2010). No other sequence variants were found in patient CMT-127.04. To date, this partial duplication of exon 4 had not been encountered in >400 samples that were screened for diagnostic purposes with the same Multiplex Amplicon Quantification assay.

Recessive genes are far less prominent in the subgroup of the congenital cases with only two patients with mutations in *PRX* and *SH3TC2*, respectively.

The second phenotypic subgroup consists of patients who display early and progressive delay in motor development in the first year, often in combination with early foot deformities after an otherwise normal neonatal period. Recessive mutations in *FGD4*, *PRX*, *MTMR2*, *SBF2* and *GDAP1* are strongly represented in this subgroup. A few patients with dominant mutations in *MPZ* and *MFN2* further broaden the spectrum (Fig. 2).

The third phenotypic subgroup is largely overlapping with the second and consists of patients with a CMT1A duplication presenting with early and often congenital foot deformities, a more or less normal early motor development and progressive gait difficulties later on in childhood. These patients represent the far end of the severity spectrum of classical CMT1A. This finding underscores the importance of ruling out the CMT1A duplication in any patient with a demyelinating neuropathy, even in very young children (Fig. 2).

The electrophysiological findings in our cohort show that early onset hereditary neuropathies are more frequently of a demyelinating type rather than axonal. Severe slowing of nerve conduction velocity is seen in patients with mutations in *PRX* and *FGD4* and also in the myelin-associated genes *MPZ*, *EGR* and *PMP22*. A more variable range of slowing in motor nerve conduction velocity is observed in demyelinating neuropathies caused by mutations in *MTMR2*, *SBF2* and *SH3TC2*. Although axonal forms are proportionally less common, early onset neuropathies cannot be restricted to demyelinating neuropathies alone. We identified three index patients with axonal neuropathies carrying mutations in *GDAP1* and *MFN2*. Of note is that a sural nerve biopsy performed in Patient PN-860.03 (homozygous *GDAP1* mutation) showed limited (secondary) demyelinating changes in addition to manifest signs of axonal neuropathy (Nelis *et al*., 2002*b*). The direct comparison of the results of the nerve conduction studies presented here is troublesome because of the broad range of ages at which the electrophysiological exams were performed and the problem of normative values in young children (Garcia *et al*., 2000). However, the general trends in these electrophysiological findings are clinically meaningful and hold true despite the above-mentioned drawbacks. Thorough electrophysiological testing remains the cornerstone of the diagnosis in the context of hereditary neuropathies and should, in this particular instance, be performed by electrophysiologists specialized in paediatric neuromuscular disorders (Wilmshurst *et al*., 2003; Pitt, 2011). To differentiate from metabolic diseases, routine screening should be performed such as biochemical markers for lysosomal storage disorders in urine, very long chain fatty acids, isoelectric focusing of serum transferine and organic acids (Landrieu *et al*., 2011).

The current study focuses on patients with early onset forms of hereditary motor and sensory neuropathy. Overlap with pure motor forms (hereditary motor neuropathy) has been described, however, especially in axonal forms of hereditary motor and sensory neuropathy. In that context, it is important to note that recently, *MFN2* mutations were found in various patients with pure motor phenotypes (Feely *et al*., 2011). Therefore, it may be useful to extend future mutation screenings of *MFN2* to patients with early onset forms of hereditary motor neuropathy.

In general, nerve biopsies are considered invasive procedures that are best avoided if a diagnosis can be established using other methods. In this series, in 19 of 35 patients with proven mutations, neuropathological examination of a sural nerve or skin biopsy was performed. This indicates that such a procedure

may still be important in the diagnosis of hereditary neuropathies in young children for a number of reasons (Vallat *et al*., 2011). First, a nerve (and muscle) biopsy can be helpful in differentiating peripheral neuropathy from other causes of severe hypotonia in newborns. Secondly, patients with early onset neuropathies are often isolated in their family, making the differentiation from other rare acquired causes more difficult, especially in the absence of consanguinity among the parents. In addition, electrophysiological testing may be inconclusive with regard to discerning demyelinating from axonal forms due to technical limitations and severe denervation (Wilmshurst *et al*., 2003). Finally, pathology may unveil certain specific findings that can help to orient molecular testing. Myelin outfoldings and redundant myelin loops, for example, are a conspicuous feature that suggests possible mutations in the myotubularin genes *MTMR2* and *SBF2* (Nelis *et al*., 2002*a*; Azzedine *et al*., 2003). However, these findings should be interpreted cautiously, because in the current study, and in previous reports, similar changes were also observed in patients with mutations in *FGD4*, *SH3TC2*, *PRX* and *MPZ* (Takashima *et al*., 2002; Kochanski *et al*., 2004; Fabrizi *et al*., 2009; Houlden *et al*., 2009). Basal lamina onion bulbs are in turn considered a feature suggestive of *SH3TC2* mutation carriers (Houlden *et al*., 2009).

The current study contributes to our understanding of the clinical and genetic basis of hereditary neuropathies with disease onset in the first year of life. A molecular diagnosis could be reached in 45% of patients from a heterogeneous screening cohort of 77 unrelated index patients leaving more than half of the patients without genetic diagnosis to date. This further underscores the fact that other still unknown mutations must exist in addition to yet unreported phenotypic variants associated with known or unknown disease-associated genes.

Organizing rational molecular diagnostic testing in the specific case of early onset neuropathies is cumbersome due to the low mutation frequency per gene and the broad genetic heterogeneity (Ryan and Ouvrier, 2005). Some directions can be drawn up based on the findings of this study. In patients with a congenital disease onset, mutations in dominant genes are more likely to be the cause, while mutations in recessive genes are more probable in patients with progressive delay in motor development in the first year of life, especially if parents are consanguineous. Axonal subtypes are probably restricted to a smaller subset of genes that may be tested preferentially. Other findings such as myelin outfoldings on nerve pathology may help to prioritize molecular testing to some extent. Likewise, severe and early scoliosis, although very suggestive of *SH3TC2* mutations (Houlden *et al*., 2009), may also be seen in patients carrying mutations in other genes and can in fact be considered a feature potentially to be found in many types of severe and progressive neuropathy of early childhood.

In conclusion, reaching a correct genetic diagnosis in children with severe early onset hereditary motor and sensory neuropathy remains a major challenge with conventional screening techniques. A future solution for this diagnostic conundrum may lie in the more systematic diagnostic application of recently developed technologies for massive parallel sequencing. While operational costs of these methods plummet, capacity and overall robustness grows exponentially. These technologies allow for the simultaneous screening of larger sets of genes and eventually of patient's entire exome or even genome (Hoischen *et al*., 2010; Lupski *et al*., 2010; Montenegro *et al*., 2011). By doing so, mutations in one of the myriad of known genes can be identified quickly and also new genetic risk factors can be scrutinized on the same datasets. Such approaches would ultimately allow geneticists to base the entire process of molecular diagnosis in a patient on a single test. The downside of these technologies is that they will yield numerous sequence variants of unknown significance. Although further validation of sequence variants in known genes may still be relatively straightforward, the identification of pathogenic mutations in novel genes will be more problematic and will require pooling of multiple unrelated patients and the use of robust bioinformatics tools (Depristo *et al*., 2011).

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