Hereditary peripheral neuropathies diagnosed by next-generation sequencing

BACKGROUND Next-generation sequencing (NGS) is a genetic technique used to determine the order of nucleotides in DNA. The technique has proved to be more efficient than the traditional method, Sanger sequencing, for sequencing multiple genes. NGS is now being used to diagnose disorders in which multiple genes are involved. This study has examined whether next-generation sequencing produces a greater number of positive diagnoses than its traditional counterpart in patients with suspected hereditary peripheral neuropathy.

MATERIAL AND METHOD This study is a retrospective review of samples from 103 patients investigated for hereditary peripheral neuropathy, received by Telemark Hospital in the period 2012–14. After exclusion of duplication/deletion of *PMP22*, 96 samples were analysed by NGS with physical enrichment of 52 hereditary peripheral neuropathy genes.

RESULTS A genetic cause was identified in 35 patients (34 %) with peripheral neuropathy, of which 28 (27 %) were point mutations identified by NGS.

INTERPRETATION Of the pathogenic point mutations identified in this study, 12 were in genes that would previously have been analysed by Sanger sequencing in our department, whereas 16 were in genes that would not previously have been tested.

Helle Høyer
helle.hoyer@sthf.no
Øyvind L. Busk
Øystein L. Holla
Linda Strand
Section of Medical Genetics
Department of Laboratory Medicine
Telemark Hospital

Michael B. Russell
Centre for Research
Akershus University Hospital

Camilla F. Skjelbred
Geir J. Braathen
Section of Medical Genetics
Department of Laboratory Medicine
Telemark Hospital

MAIN POINTS

Samples from 96 patients were analysed with next-generation sequencing due to suspected hereditary peripheral neuropathy

Point mutations were identified in 28 patients

Next-generation sequencing produced more than double the number of genetic diagnoses in this patient population relative to the number that would have been obtained with a previously used method

DNA sequencing is used to read the nucleotide sequence in all or part of an organism's genetic material. The method «next-generation sequencing» (NGS) has revolutionised the speed and capacity of DNA sequencing, with the result that the human genome can now be sequenced in a few days, rather than the ten years required with traditional methods (Sanger sequencing) (1, 2).

NGS is the subject of some controversy. It is already used extensively in research, and is now being introduced into clinical genetic diagnostics. Two main variants are used: exome sequencing, which involves sequencing all coding genes (all exons) and gene-panel sequencing, which involves sequencing selected genes, such as those responsible for a given disease.

Sanger sequencing was previously used to examine selected genes sequentially (2–4). However, this method imposes a limit on the number of genes that it is feasible to sequence. The much greater capacity of NGS makes it more efficient for diagnosing genetically heterogeneous diseases such as hereditary peripheral neuropathies, epilepsy and cardiomyopathies (4–6).

Charcot-Marie-Tooth disease (CMT) is the most common hereditary peripheral neuropathy, with a prevalence in Norway of 40–80 per 100,000 population (7, 8). The disease is also referred to as hereditary motor and sensory neuropathy (HMSN).

Clinical symptoms often begin distally in the legs, with motor signs such as paresis and atrophy, and sensory signs such as loss of sensitivity to vibration and touch. The disease develops gradually and may affect the arms later in the disease course. With further progression patients often develop walking difficulties, claw foot (pes cavus) and hammer toes. Severity and age of onset vary, but the disease is usually slowly progressive (9–11). Inheritance may be autosomal dominant, autosomal recessive or sex-linked.

Charcot-Marie-Tooth disease is further classified on the basis of nerve conduction velocity (NCV) in the median nerve: demyelinating disease (CMT1): NCV < 38 m/s; axonal disease (CMT2): NCV > 38 m/s and intermediate disease: NCV = 25–45 m/s (10, 11). Charcot-Marie-Tooth disease is closely related to several less common peripheral neuropathies: distal hereditary motor neuropathy (dHMN), hereditary sensory neuropathy (HSN) and hereditary sensory and autonomic neuropathy (HSAN). These diagnostic groups may be viewed as a continuum, both clinically and genetically (Fig. 1).

After completion of the human genome project and the introduction of NGS, the number of genes associated with Charcot-Marie-Tooth disease and other inherited peripheral neuropathies increased rapidly. Today there are around 90 genes associated with this disease group, as shown in Figure 2 (6, 12–14).

The most common cause of Charcot-Marie-Tooth disease is duplication (one additional copy) of the *PMP22* gene, as diagnosed in 14 % of patients in families with clinical disease in eastern Akershus county, Norway (7). After *PMP22* duplication, point mutations (single base changes in DNA) are the next most common cause of Charcot-

Marie-Tooth disease and other inherited peripheral neuropathies. In traditional clinical diagnostics, candidate genes are tested sequentially with Sanger sequencing. These candidate genes are selected by assessing clinical and neurophysiological features in the patient and the pattern of inheritance in the family (15, 16). Sanger sequencing is resource-intensive, and most clinical laboratories have only had the capacity to test a few of these genes (9, 12, 17). Matters are complicated further by genetic heterogeneity (one phenotype can be caused by different genotypes) and variable expressivity (one gene can produce different phenotypes).

In two Norwegian studies of patients with clinical Charcot-Marie-Tooth disease, one based on families in eastern Akershus and the other on a clinical population in Northern Norway, Sanger sequencing was used to test for point mutations in the six and seven genes, respectively, with presumed highest frequency (*GJB1*, *MPZ*, *MFN2*, *PMP22*, *LITAF*, *EGR2*, *NEFL*). Point mutations were identified in 11 % and 8 % of patients respectively (7, 18). International studies have reported a somewhat higher proportion of point mutations in these genes, 16–20 % (19–21). We believe there is a need for a more efficient assay, with the capacity to test more of the genes implicated in neuropathy. A specific genetic diagnosis can provide patients and their relatives with information about prognosis and recurrence risk and may be relevant to future gene-specific therapies (17, 22).

In a recent study, the families from eastern Akershus were tested for point mutations in 51 genes associated with hereditary neuropathy via gene-panel NGS. The discovery rate for point mutations increased from 11 % with Sanger sequencing in a previous study (7) to 30 % using NGS (6). Internationally, University College London Hospitals and Aarhus University Hospital are among those now offering NGS as part of clinical diagnostics for hereditary peripheral neuropathies. However, most research to date has described single families; there have been few large studies of the usefulness of this approach for patients.

We have now reviewed the results of gene-panel NGS for the first 103 patients tested in our clinic for suspected hereditary peripheral neuropathy. The aim was to examine whether NGS provides a greater number of positive diagnoses than its traditional counterpart in a clinical population of patients with suspected hereditary disease.

Material and method

This quality assurance study is based on a retrospective review of material obtained through standard practice at the Section of Medical Genetics, Telemark Hospital. The material includes specimens from all index patients with suspected hereditary peripheral neuropathy received between 1 March 2012 and 1 May 2014, a total of 103 patients.

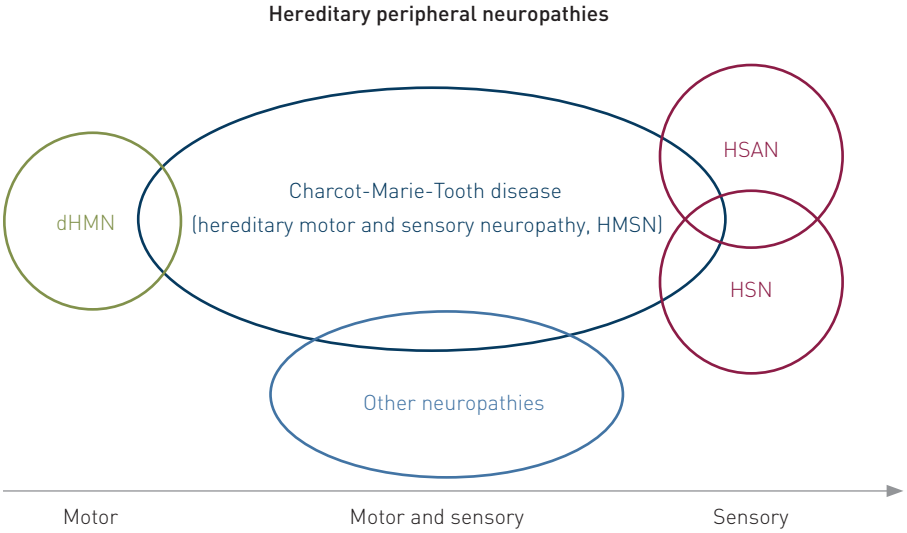

Figure 1 *Different groups of hereditary peripheral neuropathies have overlapping phenotypes. dHMN = distal hereditary motor neuropathy, HSAN = hereditary sensory and autonomic neuropathy, HSN = hereditary sensory neuropathy*

Samples were received after consultations with the departmental geneticist and neurologist (n = 47) or were sent in by 44 different external parties (n = 56). The latter were distributed as follows: neurological departments (n = 27), neurologists in private practice (n = 8), general practitioners (n = 8), genetics departments (n = 5), paediatric departments (n = 5), other (n = 3). CMT1 was suspected in ten patients, CMT2 in 16, CMTX (sex-linked) in three, unknown Charcot-Marie-Tooth disease in 44 and neuropathy in 30.

Most patients lived in Eastern Norway, in the counties Vestfold (n = 21), Oslo (n = 18), Akershus (n = 12), Buskerud (n = 12), Telemark (n = 12), Østfold (n = 6), Oppland (n = 5) and Hedmark (n = 3). A total of 14 patients lived in other parts of Norway. Patients from our previous study (6) could potentially be part of this sample, but we believe that this applies to very few, if any.

The data were anonymised, and the study was approved by the Norwegian Social Science Data Services (NSD), which granted exemption from the need to obtain informed consent. The method used was standard diagnostic practice at the hospital over the period concerned, and the approval of the regional ethics committee was therefore not sought for this study. Patients have access to information about the method via the results sent to the requisitioning doctor, and on the hospital's website.

All patients were first tested for duplication/deletion of *PMP22*, if not already done, using Multiplex Ligation Probe Amplification (MLPA). Patients with negative results underwent further testing with NGS. Prior to sample work-up, a gene panel comprising 52 genes associated with peripheral neuropathy was designed for physical enrichment and NGS. The gene list is shown in Fig. 2. DNA was extracted from blood samples and the sample work-up was performed according to a standard protocol from Illumina (Illumina Inc, San Diego, CA). The laboratory methods and bioinformatic analysis have been described in more detail previously (6).

Variants were classified as follows: class 5 – clearly pathogenic, class 4 – likely to be pathogenic, class 3 – unknown significance, class 2 – unlikely to be pathogenic, class 1 – clearly not pathogenic. The classification of variants is partly a manual process, based on the study of normal frequencies, conservation in other organisms, data from internal controls and previously published literature. Variants assigned to classes 4 and 5 are reported to the patient as likely and clearly pathogenic respectively. Class 3 variants are not reported with full nomenclature, but as variants of unknown significance. In the case of class 3 variants, samples will often be requested from family members in an attempt to clarify the variant's significance. Patients with class 3–5 variants are advised to undergo genetic counselling, in which their clinical signs and symptoms are considered alongside the genetic data and an effort is made to clarify variants of unknown significance. Class 1 and 2 variants are not reported to the patient.

Classification of sequence variants is

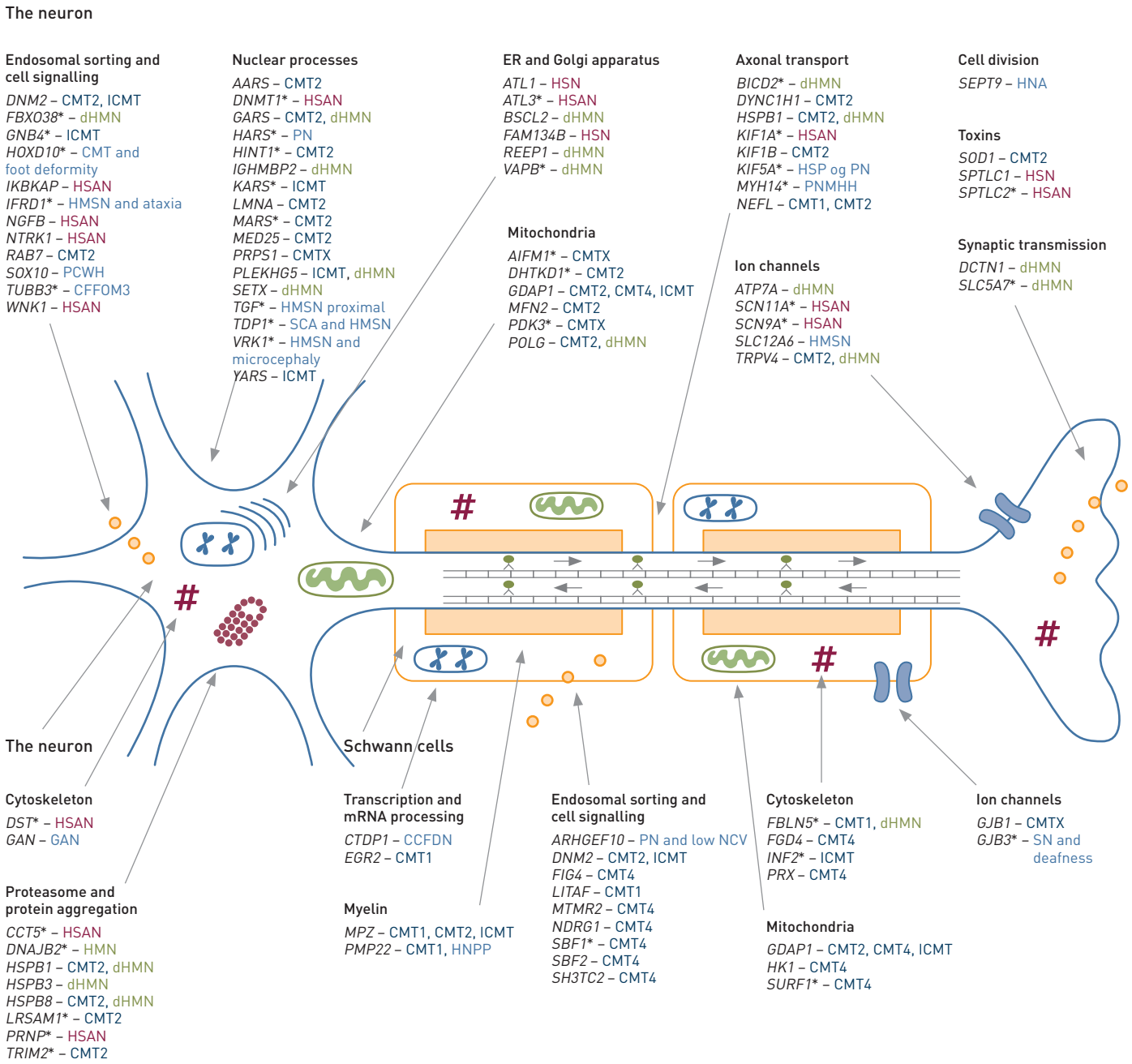

Figure 2 *Genes associated with hereditary peripheral neuropathy, their associated phenotypes and presumed pathogenic mechanisms (6, 12–14). Genes marked with an asterisk are not part of the gene panel used in the current study, but were included in the diagnostic gene panel in our department from autumn 2014. Colour codes for phenotype as in Figure 1.* DNM2 *and* GDAP1 *are listed twice since they have roles in both neurons and Schwann cells. Abbreviations: CCFDN = congenital cataracts, facial dysmorphism, and neuropathy; CFFOM = congenital fibrosis of the extraocular muscles; CMT = Charcot-Marie-Tooth disease; CMTX = Charcot-Marie-Tooth disease type X (sex-linked); dHMN = distal hereditary motor neuropathy; ER = endoplasmic reticulum; GAN = giant axonal neuropathy; HMSN = hereditary motor and sensory neuropathy; HNA = hereditary neuralgic amyotrophy; HNPP = hereditary neuropathy with liability to pressure palsies; HSAN = hereditary sensory and autonomic neuropathy; HSN = hereditary sensory neuropathy; HSP = hereditary spastic paraplegia; ICMTA = intermediate Charcot-Marie-Tooth disease; NCV = nerve conduction velocity; PCWH = peripheral demyelinating neuropathy, central dysmyelination, Waardenburg syndrome and Hirschsprung disease; PN = peripheral neuropathy (for genes not specified in another class); PNMHH = peripheral neuropathy, myopathy, hoarseness and hearing loss; SCA = spinocerebellar ataxia; SN = sensory neuropathy (for genes not specified in another class)*

based partly on judgement, but for a variant to be assigned to class 5, it must have been reported as pathogenic in at least two independent cases. In addition, functional studies must have shown an effect on protein structure/function, and the genotype and phenotype of the patient must be consistent with existing reports in the literature. Variants assigned to class 4 have often been reported as pathogenic in one previous case or are located in the vicinity of other pathogenic variants. Further details are given in Table 1. This classification is based on recommended criteria from the Association for Clinical Genetic Science (23). Further information on the classification of patients in this study can be obtained by contacting the authors.

All variants in classes 4 and 5 were verified by Sanger sequencing. Family members were often tested with Sanger sequencing for variants in classes 3–5.

The method is accredited in accordance with ISO 15189.

Results

The study material included 55 women (53 %) and 48 men (47 %). The average age was 48 years (SD 20) at sample receipt.

Prior to NGS, seven patients (7 %) were diagnosed with duplication or deletion of *PMP22* via MLPA. The remaining 96 patients underwent NGS of 52 peripheral neuropathy genes. All 96 samples met accredited quality-control standards. On average, 99.1 % (SD 0.9) of all relevant nucleotides had a coverage of more than 30x.

Among the 96 samples that underwent NGS, nine variants (9 %) were classified as clearly pathogenic, 19 (18 %) as likely to be pathogenic and ten (10 %) as being of unknown significance. Patients were informed about any clearly pathogenic and likely pathogenic variants. A total of 35 patients (34 %) thus received a genetic diagnosis, distributed among 15 different genes. In all, 28 (27 %) patients had point mutations identified by NGS (Table 2). One patient had pathogenic variants in two genes.

Discussion

A genetic cause of suspected hereditary peripheral neuropathy was identified in 35 of the 103 patients in this study. Seven had duplication/deletion of *PMP22*, while clearly/likely pathogenic point mutations were identified with NGS in 28 (27 %). By comparison, our former approach of Sanger sequencing seven genes (due to capacity constraints), would have detected pathogenic point mutations in 12 % of the patients in this material (Table 2). In our earlier study of families with clinical Charcot-Marie-Tooth disease, performed using the same laboratory method, point mutations were identified in 30 % of families (6)

The material in this study consists of persons referred for genetic testing and is thus quite highly selected. The results must therefore be interpreted with caution. In all, 46 % of patients (n = 47) were referred for testing by the departmental geneticist or neurologist, while testing of the other patients (n = 56) was ordered by 44 different requisitioners. Most of the latter tests, 86 % (n = 48), were requested by the specialist health service, of which 73 % (n = 35) were ordered by neurologists. This ensures a population that has been thoroughly assessed beforehand to exclude any differential diagnoses. The patients whose tests were requested by general practitioners had in

Table 2 Variants classified as certain and likely pathogens, detected by next-generation sequencing of samples sent to the Section of Medical Genetics, Telemark Hospital, due to suspected hereditary peripheral neuropathy (N = 103). Results are presented alongside those from other Norwegian studies. Note that the studies include different populations and have different designs. For example, the earlier studies included patients with suspected Charcot-Marie-Tooth disease, whereas the present study includes patients with suspected hereditary peripheral neuropathies in general. The studies cannot therefore be compared directly. No point mutations were detected in the following genes, which have therefore been excluded from the table: *ATP7A*, *CTDP1*, *DCTN1*, *EGR2*, *FAM134B*, *FIG4*, *GAN*, *GARS*, *GDAP1*, *HK1*, *HSPB3*, *HSPB8*, *IKBKAP*, *LITAF*, *MED25*, *MTMR2*, *NDRG1*, *NGF*, *NTRK1*, *PLEKHG5*, *PMP22*, *POLG*, *PRPS1*, *PRX*, *RAB7*, *SBF2*, *SEPT9*, *SLC12A6*, *SOX10*, *SPTLC1*, *TRPV4*, *WNK1*, *YARS*

Gene	Braathen et al. 2011 (7) N = 81 Per cent (number)	Østern et al. 2013 (18)[1] N = 435 Per cent (number)	Høyer et al. 2014 (6)[1, 2] N = 81 Per cent (number)	This study[1] N = 103 Per cent (number)
PMP22 duplication[3]	14 (11)	6 (26)[4]	14 (11)	2 (2)[4]
PMP22 deletion[3]	–	–	–	5 (5)
GJB1	6 (5)[5]	5 (20)	7 (6)	7 (7)
SH3TC2	–	–	5 (4)	5 (5)
MFN2	4 (3)[5]	2 (7)	5 (4)	2 (2)
SOD1	–	–	1 (1)	3 (3)
HSPB1	–	–	1 (1)	2 (2)
MPZ	1 (1)	1 (6)	1 (1)	2 (2)
DNM2	–	–	1 (1)	1 (1)
LMNA	–	–	2 (2)	0 (0)
AARS/ATL1[6]	–	–	0 (0)	1 (1)
ATL1	–	–	0 (0)	1 (1)
ARHGEF10	–	–	1 (1)	0 (0)
BSCL2	–	–	0 (0)	1 (1)
DYNC1H1	–	–	1 (1)	0 (0)
FGD4	–	–	0 (0)	1 (1)
IGHMBP2	–	–	0 (0)	1 (1)
KIF1B	–	–	1 (1)	0 (0)
MPZ/MFN2[3]	–	0 (1)	1 (1)	0 (0)
NEFL	–	0 (0)	0 (0)	1 (1)
REEP1/SETX[6]	–	–	1 (1)	0 (0)
Total mutations	25 (20)	14 (60)	44 (36)	34 (35)
Total point mutations	11 (9)	8 (33)	30 (24)	27 (28)

[1] Only variants judged to be certain or likely pathogens are listed

[2] Study based on the same patients as Braathen et al. 2011

[3] Duplication or deletion studied with Multiplex Ligation Probe Amplification (MLPA)

[4] Value may be artificially low, as additional patients may have been tested for PMP22-duplication/deletion prior to referral and thus not included in the study

[5] Number has been altered compared to original article after communication with the author (G.J. Braathen)

[6] Pathogenic variants found in two different genes

most cases been diagnosed during earlier assessment in a neurology department.

There are currently two centres in Norway that test for point mutations associated with hereditary peripheral neuropathies – the University Hospital of North Norway and Telemark Hospital. We therefore cannot exclude the possibility that the proportion of point mutations detected in those genes that are «traditionally» tested is artificially low in this study (12 %). Patients who tested positive for mutations in these genes may have been excluded at an earlier stage, such that we instead received samples from those with negative results in previous genetic testing. On the other hand, the percentage of point mutations in the seven genes traditionally tested was higher (12 %) in this study than in the study from Northern Norway (8 %) (18). Both of these studies recruited patients from a clinical population (18). However, patients in the eastern Akershus study were recruited from the general population (6). The studies in which NGS was performed are therefore not directly comparable.

Genes that are typically regarded as rare causes of hereditary peripheral neuropathy and are not therefore Sanger sequenced (e.g. *SH3TC2*, *HSPB1* and *SOD1*), were relatively common in both this group of patients and those from eastern Akershus (6), as shown in Table 2. Other genes have been routinely Sanger sequenced (e.g. *EGR2* and *LITAF*), but have not yet been shown to contain pathogenic mutations – either in Norwegian (Table 2) or Spanish (19) studies. Mutations were detected in a wide range of genes in both our current and previous studies (6); as more patients are tested, this range is likely to expand further. This increases the benefits, in our view, of using NGS. For analyses performed with Sanger sequencing, the discovery rate in Norwegian studies is lower than in international studies (19–21). The reason for this is not apparent, but it is possible that currently undiscovered genes may play a larger role in Scandinavia, or that foreign patient populations are more highly selected.

The number of genes associated with heterogeneous diseases has increased rapidly following the introduction of NGS. The gene panel used in this study therefore does not contain all 90 of the genes associated with peripheral neuropathies today. A gene panel for NGS can easily be updated to a newer version, however.

The technical quality of NGS in this study, with > 99 % coverage at a depth of 30x, was almost as good as that previously reported for Sanger sequencing (accuracy > 99.9 %) (24). However, NGS is significantly faster at sequencing large numbers of genes. Another advantage in our view is that multiple variants are considered simultaneously in an overall picture, making it easier to detect pathogenic variants in other genes. In contrast to exome sequencing, there is no possibility of making incidental findings in gene-panel NGS because only disease-relevant genes are studied.

It was previously thought that around 90 % of the genetic defects in Charcot-Marie-Tooth disease are present in only four genes (*PMP22* duplication/deletion or point mutations in *GJB1*, *MPZ* and *MFN2*) (18–20). As a result, these genes were usually analysed first if no other information was provided, as recommended by Norwegian guidelines (15, 16). However, in recent Norwegian studies, including this one, this picture no longer appears correct now that more of the genes associated with peripheral neuropathy are being tested (6). Moreover, mutations in genes considered to be rare causes of Charcot-Marie-Tooth disease have been detected more frequently than mutations in some of the supposedly most common genes. Since populations and selection methods differ between studies, however, it can be difficult to compare results and draw firm conclusions.

Nevertheless, the fact that so many more mutations are discovered when more genes are tested surely suggests that there is a need to reconsider current practice. There is now a consensus among international experts on peripheral neuropathies that the most profitable strategy for genetic testing is NGS, after exclusion of *PMP22* duplication/deletion and possibly Sanger sequencing of *GJB1* (12, 22).

We wish to thank Hilde Tveitan Hilmarsen and Anne Signe Bø for verifying variants by Sanger sequencing.

Helle Høyer (born 1981)
PhD, researcher in genetics and hereditary peripheral neuropathy.
The author has completed the ICMJE form and reports no conflicts of interest.

Øyvind Løvold Busk (born 1982)
PhD, bioinformatician and responsible for bioinformatics.
The author has completed the ICMJE form and reports no conflicts of interest.

Øystein Lunde Holla (born 1978)
PhD, responsible for next-generation sequencing.
The author has completed the ICMJE form and reports no conflicts of interest.

Linda Strand (born 1968)
PhD, responsible for development and quality assurance. She is an inspector for Norwegian Accreditation.
The author has completed the ICMJE form and reports no conflicts of interest.

Michael Bjørn Russell (born 1961)
MD PhD, DSc, professor, senior consultant in neurology and head of the Head and Neck Research Group.
The author has completed the ICMJE form and reports no conflicts of interest.

Camilla Furu Skjelbred (born 1967)
PhD, head of section.
The author has completed the ICMJE form and reports no conflicts of interest.

Geir Julius Braathen (born 1958)
PhD, specialist in neurology and medical genetics, and senior consultant.
The author has completed the ICMJE form and reports no conflicts of interest.

References

1. International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. Nature 2004; 431: 931–45.
2. Koboldt DC, Steinberg KM, Larson DE et al. The next-generation sequencing revolution and its impact on genomics. Cell 2013; 155: 27–38.
3. Singleton AB. Exome sequencing: a transformative technology. Lancet Neurol 2011; 10: 942–6.
4. Sikkema-Raddatz B, Johansson LF, de Boer EN et al. Targeted next-generation sequencing can replace Sanger sequencing in clinical diagnostics. Hum Mutat 2013; 34: 1035–42.
5. Lemke JR, Riesch E, Scheurenbrand T et al. Targeted next generation sequencing as a diagnostic tool in epileptic disorders. Epilepsia 2012; 53: 1387–98.
6. Høyer H, Braathen GJ, Busk ØL et al. Genetic Diagnosis of Charcot-Marie-Tooth Disease in a Population by Next-Generation Sequencing. BioMed Research International 2014; 2014: 13.
7. Braathen GJ, Sand JC, Lobato A et al. Genetic epidemiology of Charcot-Marie-Tooth in the general population. Eur J Neurol 2011; 18: 39–48.
8. Skre H. Genetic and clinical aspects of Charcot-Marie-Tooth's disease. Clin Genet 1974; 6: 98–118.
9. Pareyson D, Marchesi C. Diagnosis, natural history, and management of Charcot-Marie-Tooth disease. Lancet Neurol 2009; 8: 654–67.
10. Harding AE, Thomas PK. The clinical features of hereditary motor and sensory neuropathy types I and II. Brain 1980; 103: 259–80.
11. Davis CJ, Bradley WG, Madrid R. The peroneal muscular atrophy syndrome: clinical, genetic, electrophysiological and nerve biopsy studies. I. Clinical, genetic and electrophysiological findings and classification. J Genet Hum 1978; 26: 311–49.
12. Rossor AM, Polke JM, Houlden H et al. Clinical implications of genetic advances in Charcot-Marie-Tooth disease. Nat Rev Neurol 2013; 9: 562–71.
13. Timmerman V, Strickland AV, Züchner S. Genetics of Charcot-Marie-Tooth (CMT) Disease within the Frame of the Human Genome Project Success. Genes (Basel) 2014; 5: 13–32.
14. Kaplan JC, Hamroun D. The 2014 version of the gene table of monogenic neuromuscular disorders (nuclear genome). Neuromuscul Disord 2013; 23: 1081–111.

>>>

15. Senter for medisinsk genetikk og molekylærmedisin, Haukeland universitetssykehus. Norsk portal for medisinsk-genetiske analyser. www.genetikkportalen.no/default.asp?act=tilst&TgID=1&katID=19&TilID=527 (1.9.2014).
16. Norsk Elektronisk Legehåndbok. Norsk nevrologisk forening. Nevrologiske prosedyrer. http://nevro.legehandboka.no/nevromuskulere-sykdommer/tester/teststrategi-ved-cmt-41917.html (1.10.2015).
17. Reilly MM, Shy ME. Diagnosis and new treatments in genetic neuropathies. J Neurol Neurosurg Psychiatry 2009; 80: 1304–14.
18. Østern R, Fagerheim T, Hjellnes H et al. Diagnostic laboratory testing for Charcot Marie Tooth disease (CMT): the spectrum of gene defects in Norwegian patients with CMT and its implications for future genetic test strategies. BMC Med Genet 2013; 14: 94.
19. Saporta AS, Sottile SL, Miller LJ et al. Charcot-Marie-Tooth disease subtypes and genetic testing strategies. Ann Neurol 2011; 69: 22–33.
20. Murphy SM, Laura M, Fawcett K et al. Charcot-Marie-Tooth disease: frequency of genetic subtypes and guidelines for genetic testing. J Neurol Neurosurg Psychiatry 2012; 83: 706–10.
21. Sivera R, Sevilla T, Vílchez JJ et al. Charcot-Marie-Tooth disease: genetic and clinical spectrum in a Spanish clinical series. Neurology 2013; 81: 1617–25.
22. Harel T, Lupski JR. Charcot-Marie-Tooth disease and pathways to molecular based therapies. Clin Genet 2014; 86: 422–31.
23. Wallis J, Payne S, McAnulty C et al. Practice Guidelines for the Evaluation of Pathogenicity and the Reporting of Sequence Variants in Clinical Molecular Genetics, 2013. www.acgs.uk.com/media/774853/evaluation_and_reporting_of_sequence_variants_bpgs_june_2013_-_finalpdf.pdf (1.10.2015).
24. Shendure J, Ji H. Next-generation DNA sequencing. Nat Biotechnol 2008; 26: 1135–45.

Received 5 September 2014, first revision submitted 17 March 2015, accepted 1 October 2015. Editor: Are Brean.

Table 1 Recommended criteria for classification of variants. If a variant satisfies criteria belonging to different classes, it is generally assigned to the lowest class. It is important to emphasise that these criteria are for guidance only, and that interpreting genetic variants involves a considerable degree of judgement. None of the criteria in the table below should therefore be considered absolute requirements. Table adapted from Høyer et al. (6)

Class	Conclusion	Criteria
1	Clearly neutral variant	1. Present in ≥ 1 % of dbSNP137, 1000 genomes and/or Exome Sequencing Project (ESP) 2. and/or present in ≥ 4 internal controls[1] 3. Described as benign in multiple high-quality published reports
2	Likely neutral variant	1. Present in 0.1 – 1.0 % of dbSNP137, 1000 genomes and/or ESP 2. and/or present in 2 – 3 unrelated internal controls 3. and/or described as benign in the literature 4. and/or found in other phenotypes in multiple patients at the Department of Medical Genetics, Telemark Hospital 5. and/or no loss/gain of splice sites predicted by 5/5 predictors (Splice-SiteFinder-Like, MaxEntScan, NNSPLICE, GeneSplicer and Human Splicing Finder) (applies only to variants in introns)
3	Variant of unknown significance (VUS)	1. Present in ≤ 0.1 % of dbSNP137, 1000 genomes and/or ESP 2. and/or present in ≤ 1 internal controls 3. Divergent predictions from variant prediction databases/software: SIFT, Polyphen, Align GVGD and Mutation Taster (applies only to non-synonymous variants in coding regions)
4	Likely pathogenic variant	1. Reported as pathogenic in one study, with the same genotype and phenotype 2. and/or functional studies available showing a physical effect of the mutation 3. and/or proximity to other reported pathogenic mutations with similar degree of amino acid conservation that correlates with phenotype and zygosity. Also, predicted pathogen in 2 of 4 variant prediction databases/software: SIFT, Polyphen, Align GVGD and Mutation Taster (applies only to non-synonymous variants in coding regions) 4. and/or stop codon mutations ≥ 50 bp upstream of the last exon-intron boundary in coding regions 5. and/or frameshift insertions/deletions 6. and/or loss/gain of splice sites predicted by multiple predictors (Splice-SiteFinder-Like, MaxEntScan, NNSPLICE, GeneSplicer and Human Splicing Finder) 7. and/or found in multiple patients with similar phenotypes by Section of Medical Genetics
5	Clearly pathogenic variant	1. Reported as pathogenic in multiple studies, and with an appropriate phenotype and the same genotype as that reported previously. Established causal relationship with phenotype 2. Multiple functional studies available showing a physical effect of the mutation. Disease may be due to loss or gain of function

[1] The internal controls used in this analysis undergo neurological examination to exclude peripheral neuropathy, and they are not related to individuals with peripheral neuropathy.