Novel causes and refined phenotypes of hereditary spastic paraparesis

Studies of subgroups of hereditary spinocerebellar disorders in a Norwegian cohort

Doctoral thesis by
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Summary

Hereditary spastic paraparesis (HSP) represent a group of monogenic neurodegenerative disorders, characterized by progressive gait difficulties due to increased muscle tone in the legs. The clinical presentation can range from pure spastic paraparesis to a complex disorder with additional symptoms from the nervous system or other organ systems. There are no curative or disease-modifying treatment, hence the management focuses on relieving symptoms and on rehabilitation. Genetic variants in at least 65 different genes are known to cause subgroups of HSP. Also, spastic paraparesis can be a prominent feature in overlapping disorders, in particular hereditary cerebellar ataxias (HCA). Still, many patients remain without genetic diagnosis.

This study aimed to characterize subgroups of HSP in a Norwegian patient cohort using whole exome sequencing. We identified novel genetic causes or novel modes of inheritance in four different subgroups and, by performing thorough clinical characterization, expanded and refined the phenotypes of these disorders.

Paper I describes Norwegian patients with SPG7, the most common autosomal recessive form of HSP in Norway known today. Main findings were a Norwegian founder mutation and a core phenotype comprising spastic ataxia, progressive ophthalmoplegia and bladder disturbances. Paper II describes a Norwegian family with early-onset neurodegeneration with gait disturbances and optic atrophy, confirming biallelic *UCHL1* variants as cause of the disease (SPG79). The patients had remarkably preserved memory function. One of the *UCHL1* variants resulted in increased enzyme activity, whereas the other led to an insoluble protein, suggesting that this specific combination might protect cognitive function, thereby presenting a possible mechanism for treatment. Paper III established a heterozygous missense variant in *ERLIN2* as the cause of autosomal dominant pure HSP. Variants in this gene were previously only described to cause autosomal recessive, complex HSP. Hence, we identified a new inheritance pattern and phenotype for *ERLIN2*, which will be of importance in genetic diagnostics and counselling. In Paper IV, we identified biallelic variants in the *POLR3A* gene, and in particular a specific intronic variant, as a frequent cause of complex HSP/HCA, explaining as much as 3.1% of previously undiagnosed sporadic or recessive cases in our material. Patients had a recognizable core phenotype of early onset (<20 years) ataxia, alcohol-responsive tremor, pyramidal signs and reduced deep tendon reflexes, combined with dental abnormalities and high intensities in the superior cerebellar peduncles in MRI. The findings will be of importance in diagnostics, and the intronic variant represents a possible target for gene editing.

In total, these studies have expanded the knowledge of genetic causes of HSP, of modes of inheritance and of genetic overlap between different phenotypes and genotypes, thus providing a foundation for better diagnostics and future research towards personalized treatment.

Sammendrag (summary in Norwegian)

Arvelig spastisk paraparese (HSP) er en gruppe av tilstander som medfører økende gangvansker i form av spastisitet, økt muskelspenning i bena. Tilstanden skyldes degenerasjon av de lengste motoriske banene i sentralnervesystemet. Både symptomer og genetisk årsak varierer. Genvarianter i minst 65 gener kan føre til denne typen tilstand, i tillegg har mange andre tilstander spastisk paraparese som et fremtredende funn, slik som de arvelige ataksiene (HCA). Det er noen, relativt sett vanlige former, mens mange undergrupper kun er beskrevet i få familier. Til tross for bedret diagnostikk er mange pasienter fortsatt uten genetisk diagnose.

I denne studien har vi tatt utgangspunkt i pasienter med HSP/HCA registrert i forskningsgruppens database på Oslo Universitetssykehus, Ullevål. Vi har undersøkt pasienter med tidligere uavklart genetisk diagnose for å identifisere årsak og kartlegge sykdomsbildet. Viktigste genetiske metode var eksomsekvensering.

Først karakteriserte vi SPG7, en av de vanligste undergruppene av autosomal recessiv HSP, der hovedfunn hos våre pasienter var spastisk ataksi med blæreproblemer, hengende øyelokk og øyemuskellammelser (progressiv ekstern oftalmoplegi). Vi fant også en norsk «grunnleggermutasjon». Deretter beskrev vi en familie med kompleks spastisk paraparese som hadde to varianter i *UCHL1* genet, den andre familien i verden med denne tilstanden. UCHL1 er et av de proteinene vi har mest av i hjernen og er svært viktig for å fjerne proteiner som skal degraderes (i ubiquitin-proteasom systemet). Den ene genvarianten viste seg å gi en økt funksjon av proteinet, mens den andre førte til et uløselig protein. Vi spekulerer i om økt funksjon kan bidra til bevart kognitiv funksjon, slik at videre forskning på dette genet og denne spesifikke varianten kan føre til ny kunnskap om hukommelsesfunksjon. Videre har vi i samarbeid med tyske forskere funnet ut at HSP formen HSP-ERLIN2/SPG18 også kan arves dominant. Vi beskriver to store familier med autosomal dominant ren HSP forårsaket av en variant i *ERLIN2* genet, den første sikre beskrivelsen av dominant arvegang ved denne HSP formen som tidligere var kjent som en kompleks, tidlig startende autosomal recessiv HSP. I den fjerde studien har vi beskrevet en tilstand som var ukjent i HSP/HCA-populasjonen i Norge; POLR3A-relatert spastisk ataksi (ATX/HSP-POLR3A). Ti norske familier hadde bialleliske varianter i *POLR3A*. Sykdomsbildet var karakteristisk med hovedfunn ataksi, pyramidale tegn, alkoholresponsiv tremor (skjelving), utslukkede dype senereflekser i bena og som regel symptomstart før 20 år. De fleste hadde en spesifikk variant i ikke-kodende DNA som kan har potensial til å kunne påvirkes med behandling.

Disse studiene har utvidet kunnskapen om genetiske årsaker til HSP, type arvegang og om genetisk overlapp mellom forskjellige sykdomspresentasjoner. Dette legger grunnlag for bedre diagnostikk av pasientene og muliggjør videre forskning med funksjonelle studier med mål om å finne potensielle nye persontilpassede behandlingsmuligheter.

Abbreviations

AD	Autosomal dominant
AR	Autosomal recessive
ATX	Ataxia
CNS	Central nervous system
CNV	Copy number variation
DNA	Deoxyribonucleic acid
EA	Episodic ataxia
HCA	Hereditary cerebellar ataxia
HSD	Hereditary spinocerebellar disorders = HSP + HCA
HSP	Hereditary spastic paraparesis
HTS	High-throughput sequencing
iPSC	Induced pluripotent stem cell
LOD score	Logarithm of the odds score
MLPA	Multiple ligation-dependent probe amplification
MRI	Magnetic resonance imaging
NMD	Nonsense-mediated decay
OMIM	Online Mendelian Inheritance of Man
OUH	Oslo University Hospital
RNA	Ribonucleic acid
SARA	Scale for the Assessment and Rating of Ataxia
SCA	Spinocerebellar ataxia
SPG	Spastic gait (SPG + number = name of HSP loci)
SPRS	The Spastic Paraplegia Rating Scale
UiO	University of Oslo
UPS	Ubiquitin-proteasome system
WES	Whole exome sequencing
WGS	Whole genome sequencing

Genes and disorders described in the articles in this thesis

Paper	**Gene**	**Protein**	**Disorder name used in thesis**
I	*SPG7*	Paraplegin	SPG7
II	*UCHL1*	Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1)	SPG79
III	*ERLIN2*	Endoplasmic reticulum lipid raft-associated protein 2 (ERLIN2)	HSP-ERLIN2/ HSP-ERLIN2-AD (SPG18)
IV	*POLR3A*	RNA polymerase III, subunit A (POLR3A)	ATX/HSP-POLR3A

1 List of publications

The thesis is based on the following original articles:

Paper I

A founder mutation p.H701P identified as a major cause of SPG7 in Norway. Rydning SL*, Wedding IM*, Koht J, Chawla M, Øye AM, Sheng Y, Vigeland MD, Selmer KK[#], Tallaksen CM[#]. *European journal of neurology*. 2016;23(4):763-71.

Paper II

Novel *UCHL1* mutations reveal new insights into ubiquitin processing. Rydning SL*, Backe PH*, Sousa MML, Iqbal Z, Øye AM, Sheng Y, Yang M, Lin X, Slupphaug G, Nordenmark TH, Vigeland MD, Bjørås M, Tallaksen CM[#], Selmer KK[#]. *Human molecular genetics*. 2017;26(6):1031-40.

Erratum in: *Human molecular genetics.* 2017;26(6):1217-1218.

Paper III

A novel heterozygous variant in *ERLIN2* causes autosomal dominant pure hereditary spastic paraplegia. Rydning SL*, Dudesek A*, Rimmele F, Funke C, Krüger S, Biskup S, Vigeland MD, Hjorthaug HS, Sejersted Y, Tallaksen CM, Selmer KK, Kamm C. *European journal of neurology*. 2018;25(7):943-e71.

Paper IV

Biallelic *POLR3A* variants confirmed as a frequent cause of hereditary ataxia and spastic paraparesis. Rydning SL, Koht J, Sheng Y, Sowa P, Hjorthaug HS, Wedding IM, Erichsen AK, Hovden IA, Backe PH, Tallaksen CM, Vigeland MD, Selmer KK. *Brain : a journal of neurology*. 2019;142(4):e12.

*/[#] These authors contributed equally to this paper

2 Introduction

2.1 Preface

In 2012, when this PhD project was initiated, 52 subgroups of hereditary spastic paraparesis (HSP) were described (Finsterer et al., 2012). Knowledge of the phenotypic and genotypic characterization of the specific genetic subgroups were sparse, except for the more common forms, and the majority of patients were without molecular diagnosis. Our Norwegian patient material has been collected since 2002 by the project manager Chantal Tallaksen and her group, and registered in a research database (Erichsen et al., 2009). In 2012, 404 families and in total 602 patients with hereditary spinocerebellar disorders (HSD), comprising HSP and the related group of disorders hereditary cerebellar ataxias (HCA), were included. High-throughput sequencing (HTS) and HTS based gene panel analyses were not yet available, but all other readily available methods for clinical and molecular investigations had been applied. Even so, as much as 74% of the families in this patient material were still without a genetic diagnosis.

In this PhD project, we aimed to further characterize this genetically unsolved patient group, using novel molecular methods, to find and understand the molecular causes and clinical characteristics. The work has been performed in the time period 2012-2019, the first years combined with a clinical position as a doctor at the Department of Neurology, Oslo University Hospital (OUH), Ullevål, and from autumn 2016, combined with a position as a University teacher at the Institute of Clinical Medicine, the University of Oslo (UiO). My role, being a neurologist and clinician, has been to investigate and combine patient information from the database, novel literature providing clues of novel subgroups of HSP, to guide the specific studies, depending on what were available of methods and resources (Figure 2.1).

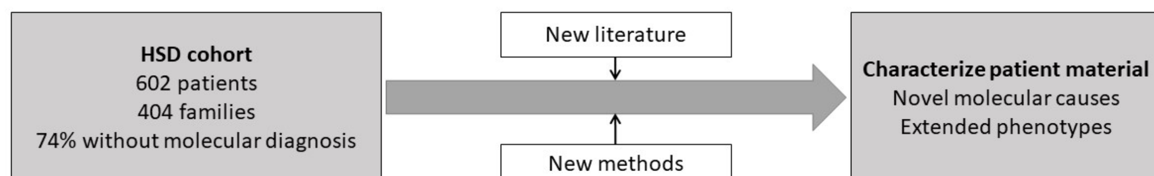

Figure 2.1. Overview of background material and goals of this PhD. HSD = hereditary spinocerebellar degenerations = HSP + HCA. Patients without molecular diagnosis were selected from the database. Further investigations were performed guided by available literature and available methods, aiming to characterize novel molecular causes and extended phenotypes of subgroups of HSP and related disorders.

The specific studies were selected based on what was considered the most promising preliminary findings. During the studies, my main role was to coordinate the studies, perform the clinical patient examinations and to participate in analyses and interpretation of molecular and supplementary clinical data, resulting in the four papers constituting the basis of this PhD thesis. None of the studies had been possible without joining forces with geneticists, molecular biologists and other collaborators, both locally, nationally and internationally.

The starting point and main theme of my project was to study HSP. However, during the time course of this project, it has become increasingly clear that there is wide overlap between the HSPs, hereditary cerebellar ataxias, and other hereditary neurodegenerative disorders, which is also reflected in the papers in this thesis.

2.2 History of HSP – clinical descriptions to DNA to synergy

The history of HSP runs from a century of clinical descriptions, to increasing molecular understanding, and onto the importance of synergy combining clinical and molecular characterization. Figure 2.2 presents an overview of milestones in the history of HSP.

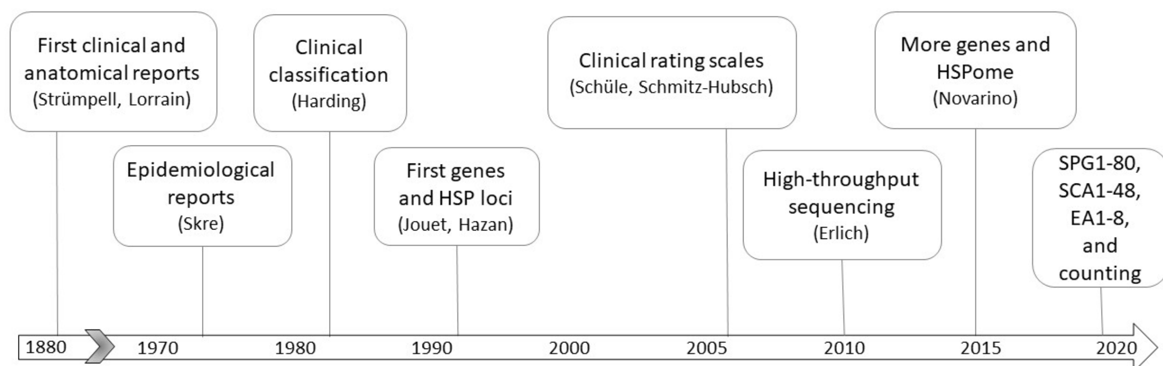

Figure 2.2. Milestones in the history of HSP. Numbers of genetic forms of HSP and HCA (box at the far right) are retrieved from Online Mendelian Inheritance of Man (OMIM), accessed Sept 17th 2019. EA = Episodic ataxia; HSP loci = chromosomal position linked to HSP; HSPome = Network of known and possible HSP genes, developed by (Novarino et al., 2014); SCA = Spinocerebellar ataxia; SPG = SPastic Gait = subgroups of HSP.

2.2.1 Clinical characterization 1880-1990

The last decades of the 19th century presented a breakthrough in the clinical description, definitions and anatomo-functional studies of several neurological symptoms and syndromes. The first clinical and anatomical studies of patients with hereditary forms of spastic paraparesis were published in the 1880s (Lorrain, 1898; Strümpell, 1880), already introducing the core anatomical findings of degeneration of the lateral corticospinal tracts, fasciculus gracilis and the spinocerebellar tracts,

which still are the main anatomical hallmarks defining the disorder (Deluca et al., 2004). Some of the first epidemiological studies of HSP were performed in Norway, led by H. Skre (Refsum & Skre, 1978; Skre, 1974). For the clinical characterization and classification of HSP, A. Harding made a considerable contribution in the 1980s. She introduced the clinical classification system dividing HSP into pure and complex forms depending on the set of clinical signs and mode of inheritance, and refined the phenotypic descriptions (Harding, 1983, 1993). Diagnostic criteria were later elaborated by J. K. Fink *et al*. (Fink et al., 1996).

2.2.2 Molecular characterization 1990-today

After a century of increasingly thorough clinical and anatomical descriptions, the discovery of genetic loci causing HSP commenced (Figure 2.3). The first loci were identified using positional cloning and linkage analyses, mapping the disorders to specific chromosomal positions (loci). A novel nomenclature system of HSP, based on genetic loci was introduced. The loci were called “SPG” for “SPastic Gait”, and numbered sequentially as novel loci were identified, starting with SPG1, independent on whether the specific causative gene was identified or not.

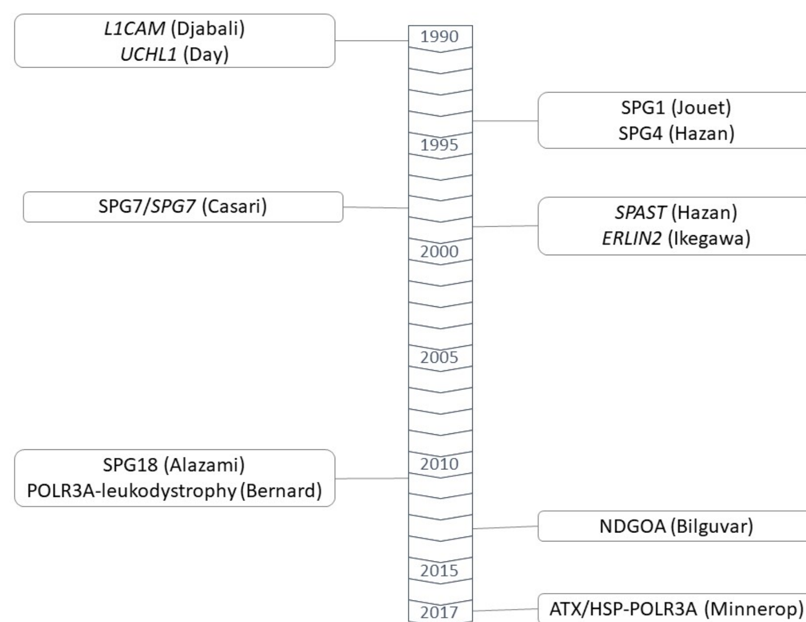

Figure 2.3. Timeline showing the identification of selected HSP genes and loci, including subgroups and loci described in the articles in the thesis. NDGOA = Childhood-onset neurodegeneration with optic atrophy.

For the X-linked SPG1 locus, the *L1CAM* gene was cloned before it was identified as the causative gene of SPG1 (Djabali et al., 1990; Jouet et al., 1994). The SPG4 locus was mapped in 1994, while the causative *SPAST* gene was identified in 1999 (Hazan et al., 1999; Hazan et al., 1994). *SPG7* (SPG7) was the first AR gene to be identified (Casari et al., 1998). Discovery of genes and disorders studied in the papers in this thesis, is elaborated in section 2.8.

From around 2010, high-throughput sequencing revolutionized the genetic sequencing capacity (Erlich et al., 2011; Ng et al., 2010; Shendure & Ji, 2008), and the number of identified disease-causing genes has since increased exponentially. Table 2.1 shows the increase in the number of SPG loci and identified HSP genes in 2008, before HTS was introduced (Stevanin et al., 2008), in 2012, when this PhD study begun (Finsterer et al., 2012), and compared to the situation today (Boutry et al., 2019).

Table 2.1. Number of HSP loci and identified HSP genes at different time points

	2008	2012	2019
SPG loci (genes)	33 (17)	51 (30)*	80 (65)
Autosomal dominant	14 (9)	19 (11)	21 (13)
Autosomal recessive	16 (6)	27 (16)	53 (48)
X-linked	3 (2)	5 (3)	5 (3)
Variable MOI	0	0	6 (6)**

Number denotes number of SPG loci, in parenthesis is the number of loci with identified causative genes. MOI = mode of inheritance. Numbers from (Stevanin et al., 2008; Finsterer et al., 2012; Boutry et al., 2019; OMIM).
*In addition Finsterer et al., 2012 included one maternally inherited HSP.
**Variable MOI includes; SPG3A (mainly AD), SPG7 (mainly AR), SPG9 (initially AD), SPG18 (initially AR), SPG20 (initially AR), SPG72 (both). To enable comparison, SPGs with variable MOI are also included in their main/original category.

2.2.3 Synergy between clinical and molecular characterization

In addition to all these new genetic causes of HSP, the considerable genetic overlap between HSPs and other neurodegenerative disorders, such as the HCAs, and the considerable genetic heterogeneity in previously well-defined clinical syndromes have been increasingly documented the later years (Boutry et al., 2019; Novarino et al., 2014; Parodi et al., 2018a; Tesson et al., 2015). This historical development from clinical characterization to molecular characterization has led to the acknowledgement of combining “old fashioned” thorough clinical characterization with modern molecular and other supplementary investigations to classify and delineate an even larger understanding of this complex field.

2.3 Clinical features of HSP

2.3.1 HSP – a neurodegenerative disorder

Our fascinating brain and nervous system control our movements, thoughts and emotions, and studies of the complexity of our 100 billion neurons and their connections continue to captivate researchers in the never-ending search to increase understanding of the nervous system in human biology and disease. As much as one out of three will at some point in life suffer from a brain-related disorder. A paradox

of our improved health and increased life span, is that the prevalence of disorders causing degeneration in all or parts of the nervous system is increasing (GBD 2016 Motor Neuron Disease Collaborators, 2018; GBD 2016 Neurology Collaborators, 2019; Prince et al., 2013).

Neurodegenerative disorders comprise a range of conditions that primarily affect the neurons, either in the central or peripheral nervous system, or both. In addition to HSPs and HCAs, other examples of neurodegenerative disorders are Parkinson's disease, dementia, amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), Huntington's disease, prion diseases, hereditary myopathies and hereditary neuropathies (Gan et al., 2018). Some of these disorders are hereditary in a monogenic manner, meaning that they are caused by a genetic change in a single gene, such as Huntington's disease and SMA. Others, such as Parkinsonism, ALS and dementia, most often have a genetically more complex aetiology, but monogenic forms exist.

2.3.2 Hereditary spinocerebellar disorders

Hereditary spastic paraparesis (HSP) comprise one group of hereditary neurodegenerative disorders, with large variation in both clinical symptoms and in the genetic causes. The main symptoms are progressive gait difficulties caused by increased muscle tone and mild loss of muscle strength, which is a result of degeneration of the long corticospinal tracts in the central nervous system. The hereditary cerebellar ataxias (HCAs) also cause gait disturbances as the main symptom, in this case due to difficulties with coordination of movements (ataxia), mainly as result of degeneration of Purkinje cells in the cerebellum. Spastic paraparesis and ataxia often coexist in these disorders and share several common pathological and genetic mechanisms, therefore HSP and HCA together can be designated hereditary spinocerebellar disorders (HSD).

2.3.3 Etymology of HSP

Hereditary spastic paraparesis/paraplegia is often abbreviated to HSP in practical use. As the "H" implies, HSP is a hereditary disorder and the causes are mainly monogenic. The prefix *para-* comes from Greek and means "side by side" and *paresis* means partial motor paralysis/weakness. *Plegia* means complete paralysis, which is seldom in HSP and only seen very late in the disease course. Hence, the term "hereditary spastic paraparesis" presents a more accurate description of the disorder. Even so, the most commonly used term in literature is "hereditary spastic paraplegia". Spasticity can be defined as velocity-dependent hypertonia with hyperreflexia that is typically accompanied by muscle weakness, and occurs as the result of injury to the myelin and/or axons of the primary motor pathways of the central nervous system, disrupting the CNS inhibitory control system (T. D. Sanger et al., 2003).

2.3.4 Clinical classification

The main and defining symptom of HSP is progressive gait difficulties due to stiffness in the legs. However, there is wide variety in the clinical presentation in both age at onset, severity and additional symptoms and signs. The age at onset of symptoms may vary from as early as a child tries to walk, to the eighth decade of life. The first symptom might be muscle spasms in the legs, often preceding the spasticity with several years. A child might show a tendency to walk on toes. The gait pattern changes towards a scissoring gait and after some time, and difficulties with running develops. Symptoms are progressive, and most patients will eventually need some form of walking aid.

The HSP diagnosis is based on the clinical findings, and confirmed genetically when possible. According to diagnostic criteria of HSP (Fink et al., 1996), a definite diagnosis of HSP can be made when (1) alternate disorders have been excluded; (2) the family history suggests a monogenic mode of inheritance; (3) the patient has progressive gait disturbance; and (4) findings of corticospinal tract deficits in the lower extremities, including hyperreflexia and extensor plantar responses.

Clinically, HSP can been classified into pure ("uncomplicated") and complex ("complicated") forms (Harding, 1983). In pure forms the patient has pure spastic paraparesis, often combined with bladder disturbances and mild proprioceptive loss (reduced sensation for vibration at ankle level). The paresis is often mild compared to the degree of spasticity. In complex forms the patient has additional symptoms and findings from the central nervous system or other organ systems. A common additional feature is cerebellar ataxia: The cerebellum controls the coordination of movements, hence dysfunction results in gait instability, dysmetria, dyssynergia and intention tremor, affected eye movements, speech, swallowing and even cognitive function (Marsden, 2018). Other frequent findings in complex HSP are neuropathy, cognitive decline, tremor, dystonia, extrapyramidal disturbances, epilepsy, amyotrophy, ophthalmological problems such as ophthalmoplegia (paresis of ocular muscles), optic atrophy and retinopathy. Non-motoric symptoms, such as bladder disturbances and cognitive difficulties, are frequent, and may be important causes of reduced quality of life (Kanavin & Fjermestad, 2018; Rattay et al., 2019). The combination of additional symptoms might provide clues for specific genetic subgroups. The clinical classification is somewhat subjective, and complex HSP may overlap clinically with several other disorders such as HCA, neuropathy, motor neuron disease, cognitive impairment or leukodystrophy. Overlapping HSP/HCA forms may be named as spastic ataxia or ataxic spastic paraparesis (Synofzik & Schule, 2017).

2.4 Relevant basic genetics

2.4.1 The human genetic material

The complete genetic material in a cell is called the genome. The human genome consists of a DNA sequence of approximately 3 x 10^9 base pairs, aligned on 23 chromosome pairs; 22 autosomes and the X and Y chromosomes. The exome comprises the around 2% protein-coding sequence of the genome (Lander et al., 2001; Venter et al., 2001). We also have separate DNA in the mitochondria, which is maternally inherited. Of the 20,000-25,000 genes in the human genome, variants in 3,766 genes have been associated with monogenic diseases (OMIM Gene Map Statistics, 2019).

2.4.2 High-throughput sequencing and whole exome sequencing

Sanger sequencing, the previous gold standard for DNA sequencing (F. Sanger et al., 1977), provides high accuracy, but is time-consuming and has low capacity. The development of HTS techniques, also called next-generation DNA sequencing, is based on massively parallel sequencing of millions of DNA fragments simultaneously, and hence enables sequencing of multiple genes, the entire exome, or even the entire genome in one single experiment (Bamshad et al., 2011; Singleton, 2011). Most known disease-causing variants are located within protein-coding regions (Fogel, 2018). Whole exome sequencing (WES) has proven to be a powerful and cost-effective approach to genetically map disease, and has resulted in a large increase in detection of causative genes for HSP, HCA and related disorders (Galatolo et al., 2018; Novarino et al., 2014).

The basic steps of WES are; (1) shearing of genomic DNA into fragments, (2) ligation of flanking adaptors to the fragments, (3) enrichment of sequences corresponding to exons, (4) capture of the exon fragments by hybridization to magnetic RNA or DNA baits, (5) amplification and massively parallel sequencing, and then (6) bioinformatic mapping and calling of variants (Figure 2.4) (Bamshad et al., 2011; Chen et al., 2015).

The output files are processed using various bioinformatic tools to identify possible disease-causing variants. In this project we used Agilent SureSelect enrichment strategy and Illumina sequencing, followed by bioinformatic analysis using the program FILTUS (Vigeland et al., 2016).

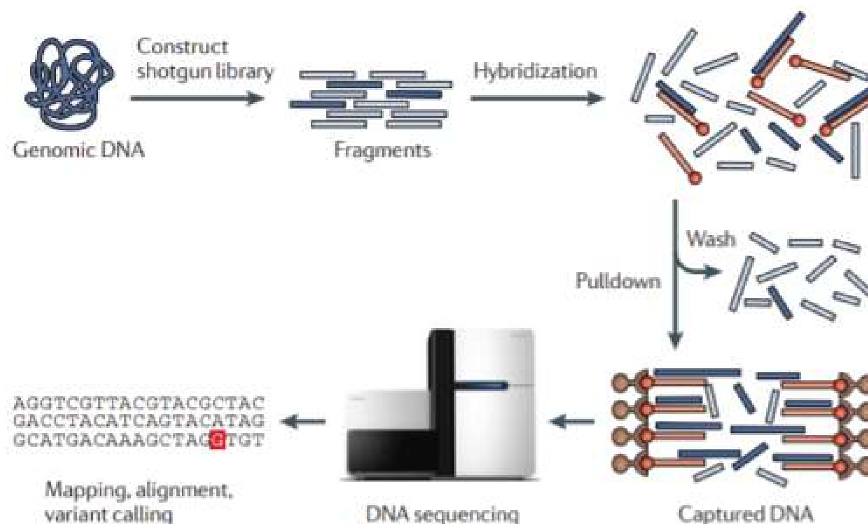

Figure 2.4. Workflow for exome sequencing. Genomic DNA is randomly shared and used to construct an *in vitro* "shot gun library". The fragments are flanked by adaptors. Fragments are enriched for sequences corresponding to exons (dark) by hybridization, and non-exonic sequence is washed out. The exon fragments are then amplified and massively parallel sequenced, followed by mapping and calling of candidate variants. Reprinted with permission from Macmillan Publishers Limited: (Bamshad et al., 2011).

2.4.3 Genetic mechanisms in HSP

The identified genetic causes of HSP are numerous and increasing (see Table 2.1). Variants have been identified in genes located throughout the genome, and all forms of monogenic inheritance have been reported; autosomal dominant (AD), autosomal recessive (AR), X-linked, even maternal/mitochondrial. A disease-causing variant can occur as a new incidence; *de novo*, also some variants may show reduced penetrance, underlining that sporadic cases may also be genetic. Most HSPs known today are caused by single nucleotide variants or small deletions/duplications (Boutry et al., 2019). However, in overlapping disorders, such as HCAs, repeat expansions are also common mechanisms, such as CAG repeats in several of the dominant spinocerebellar ataxias (SCAs) and GAA repeats in *FXN* in Friedreich ataxia.

2.5 Genetic classification

2.5.1 Genetic classification and nomenclature of HSP

The clinical classification system presented earlier, divide HSP into pure and complex forms depending on whether or not additional clinical features are present, and is still very useful and widely used (Fink et al., 1996; Harding, 1983). However, as the genetic causes of HSP have been discovered, the genetic subgroups have been named consecutively with the prefix "SPG" (SPastic Gait), and a number. The

number represents the order of which the genetic locus was identified, irrespective of clinical classification (pure/complex) or mode of inheritance. In general, AD forms more often result in pure phenotypes, while AR forms starts earlier with complex phenotypes, and X-linked disorders normally result in a more severe phenotype in males than females (Boutry et al., 2019; de Souza et al., 2017). So far the Online Mendelian Inheritance of Man (OMIM) catalogue has listed HSPs from SPG1 up to SPG80 (Figure 2.5) (www.omim.org, accessed Nov 6th 2019).

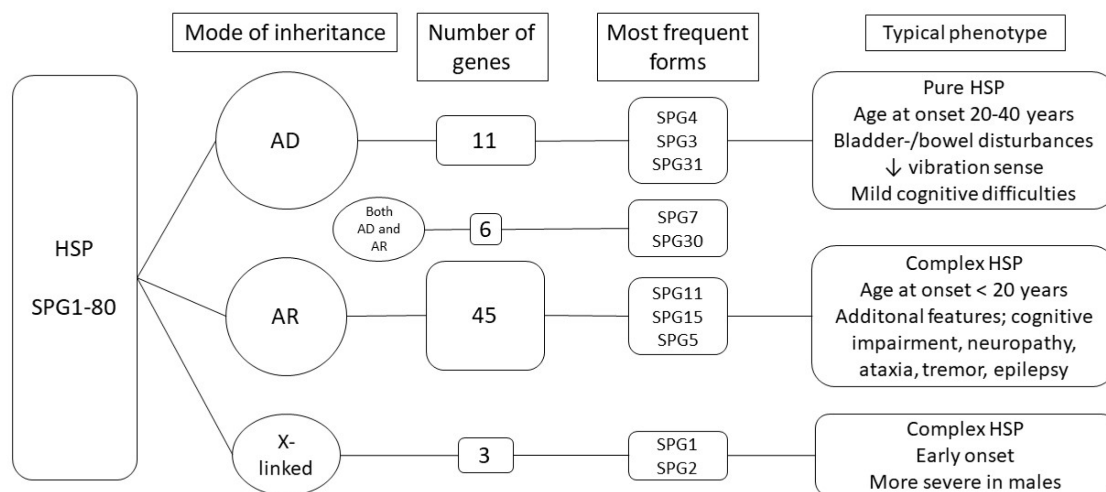

Figure 2.5. Overview of the genetic forms of HSP. The figure includes modes of inheritance, the number of identified disease genes, and the most frequently reported SPGs. Note that there are more SPG loci (80) than identified genes (65) (Boutry et al., 2019). The phenotype may differ from the listed typical phenotype.

2.5.2 Genetic versus clinical classification

The past decades, the large increase of novel disease genes and of novel or extended phenotype-genotype correlations have challenged the traditional nomenclature and classification systems. Hence, newer systems for classification and nomenclature has been suggested, including nomenclature combining the main phenotype and the involved gene (such as HSP-SPAST for SPG4 and HSP/ATX-SPG7 for SPG7) (Beaudin et al., 2019; Marras et al., 2016; Rossi et al., 2018). So far, a final consensus on nomenclature of HSDs is not reached.

2.5.3 Biochemical and imaging biomarkers

In addition to genetic testing, some forms of HSP have clinical, biochemical or imaging biomarkers that can support the diagnosis or provide clues suggestive of specific genetic forms (Beaudin et al., 2019; Parodi et al., 2017). A biomarker is any substance, structure or process that can be objectively measured in the body and

evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Strimbu & Tavel, 2010).

Some examples of available biochemical and imaging biomarkers of HSP and related disorders are listed in Table 2.2. An interesting biochemical biomarker in HSP is plasma oxysterols, which have recently proven to be a potentially useful diagnostic tool in the diagnosis of HSP type SPG5 (HSP-CYP7B1). Also, it has been shown that the level of presumed toxic metabolites is reduced by intake of cholesterol lowering drugs (Marelli et al., 2018). However, so far, there is no evidence as to whether these drugs have any effect on the neurological development/disorder. Hence, plasma oxysterols can be used as a diagnostic marker for SPG5, but it is premature to say whether it may also be a marker of degree of neurodegeneration in this disorder.

Table 2.2. A selection of biochemical and imaging biomarkers in HSP and related disorders

Biomarker	Disorder
Biochemical (plasma)	
25- and 27-oxysterols ↑	SPG5
Very long-chain fatty acids ↑	Adrenomyeloneuropathy / X-ALD
Phytanic acid ↑	Refsum's disease
Acanthocytes in blood smear	Abetalipoproteinemia
Vitamin E ↓	AVED, Abetalipoproteinemia
Alpha-fetoprotein ↑	Ataxia-telangiectasia, AOA2
Albumin ↓, cholesterol ↑	AOA1, AOA2
Immunoglobulins ↓	Ataxia-telangiectasia
Cholestanol ↑, cholesterol ↑	Cerebrotendinous xanthomatosis
Imaging	
Thinning of the corpus callosum	SPG11, SPG15
"Ear of the lynx" sign	SPG11, SPG15
Atrophy of cerebellum and brain stem	SCA (repeat expansions)
Atrophy of the cerebellum	SCA (point mutations)
High intensities in the superior cerebellar peduncles	ATX/HSP-POLR3A
High intensities in the middle cerebellar peduncles	ARSACS
AOA = ataxia with oculomotor apraxia. AVED = ataxia with vitamin E deficiency. Table made by Jeanette Koht and the author.	

Neuroimaging can be a helpful tool to guide genetic testing and interpretation (da Graca et al., 2018). The main goal of a routine MRI of the brain and spinal cord in HSP is to rule out other differential diagnoses of HSP, such as inflammatory disorders, expansive processes or other causes of pathology in the spinal cord. In HSP, structural MRI is most often normal, in particular in AD-HSP. Cross-sectional volume of the cervical and thoracic spinal cord volume may be reduced. However,

there are some typical MRI signs such as thinning of the corpus callosum and “ear of the lynx” sign in FLAIR MRI is typical of the recessive forms SPG11 and SPG15, while cerebellar atrophy would increase the possibility of an overlapping HCA or SPG7. The typical MRI finding in SCAs with repeat expansions is atrophy of both the cerebellum and the brain stem, whereas SCAs caused by point mutations typically only show cerebellar atrophy, and some subgroups have even more specific findings (Agosta et al., 2015; Pascual et al., 2019; Schulz et al., 2010).

In addition, reliable biomarkers will probably be required to select the appropriate patients for clinical trials. One example of a possible new biomarker is neurofilament light chains (NfL), which are being investigated in several neurological disorders. Plasma NfL levels have been shown to correlate with disease severity and progression in Parkinson’s disease (C. H. Lin et al., 2019) and multiple sclerosis (Canto et al., 2019). For HSP, a pilot study showed increased CSF concentrations of NfL in HSP patients compared to healthy controls (Zucchi et al., 2018), while a study of three patients with SPG10 showed increased NfL only in the one patient with the longest disease duration, however, alterations in monoamine levels were observed (Andreasson et al., 2019). Thus, further studies are needed to establish whether measurements of NfL might be useful in HSP. Hence, the search for efficacy measures to be of use in clinical trials and possible markers of progression is ongoing (Trummer et al., 2018).

2.6 Pathomechanisms

2.6.1 Normal physiology

Several parts of the central nervous system (CNS) are involved in controlling voluntary movements and the coordination of movements. Voluntary muscle movements depend in particular on the pyramidal motor system and the corticospinal tracts, where the axons of upper motor neurons innervating the lower limbs can reach up to as much as one meter in length before synapsing with the lower motor neurons. Coordination of movements depend largely on the function of and connections to and from the cerebellum.

2.6.2 Neuropathology

In neurodegenerative disorders, progressive degeneration of neurons in a specific region or system, or generalized, in the brain and/or spinal cord occurs. The symptoms and signs will depend on which anatomical parts, systems and biochemical processes that are disturbed. In HSP, the main system affected is the primary motor pathways, i.e. the corticospinal tracts of the CNS, resulting in spasticity, hyperreflexia and paresis.

The main macro-anatomical findings in HSP are degeneration of the lateral corticospinal tracts, fasciculus gracilis and the spinocerebellar tracts, as described already in the first autopsy studies (Lorrain, 1898). These anatomical findings relate to the main clinical findings of spasticity, proprioceptive loss, and ataxia. A marked, symmetrical reduction in both axonal density and total axonal number of the corticospinal tracts throughout the length of the neuroaxis has been described, and the difference in axonal loss was larger than the degree of spinal cord atrophy, which imply that MRI measurements may not reflect the true extent of axonal loss in HSP (Deluca et al., 2004). Loss of myelin is also observed and considered secondary to a primary neuronal/axonal degeneration process. The main mechanism of degeneration appear to be "dying-back" in which axons degenerate progressively from their distal ends (Schwarz & Liu, 1956). This length-dependent axonopathy, affecting the longest axons first, explains why the clinical features are most prominent in the lower limbs.

Additional symptoms such as cognitive impairment, suggest involvement of other brain structures. Recent studies with advanced MRI show widespread involvement of both grey and white matter, even in clinically pure HSP forms (Aghakhanyan et al., 2014; Lindig et al., 2015; Rucco et al., 2019). This confirms that the pathological mechanisms in HSP are more complex and goes beyond only motor dysfunction.

2.6.3 Cellular pathology

Even with the vast amount of genes causing HSP, the function of most of the encoded proteins converge into a relatively small group of cellular pathways. The long motor neuron axons require a complex intracellular machinery to sort, distribute and degrade proteins and other necessary molecules, and many of the genes involved in HSP encode proteins that are involved in axon development and maintenance. Figure 2.6, reprinted from (Blackstone, 2018a), illustrates the main pathogenic cellular themes in HSP. The most frequent mechanisms are disrupted intracellular trafficking, distribution, biogenesis and shaping of membrane compartments. This includes the most common subgroups of HSP; SPG4, SPG3A, SPG31, SPG11, and SPG15. Other affected cellular functions include mitochondrial regulation (SPG7), myelination and lipid/sterol modification (SPG5, SPG2) and axon pathfinding (SPG1), nucleotide metabolism or other/hitherto unknown cellular functions (Blackstone, 2012, 2018a, 2018b).

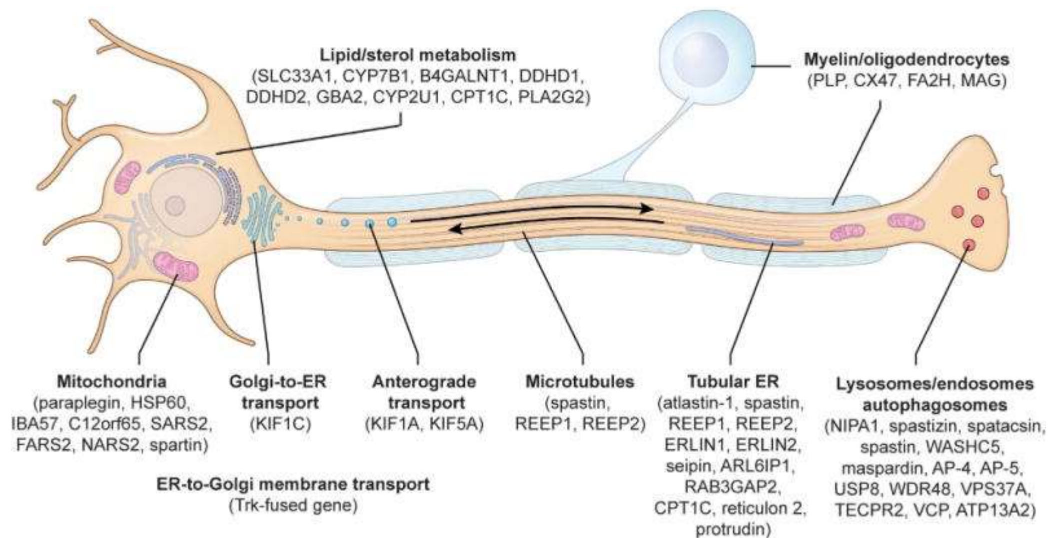

Figure 2.6. **Common pathogenic themes in HSPs, emphasizing where the listed proteins are known or believed to function.** Reprinted from (Blackstone, 2018a), with permission from Elsevier.

2.7 Epidemiology

Neurodegenerative disorders as a group are common, while HSP is a relatively rare group of disorders. In the European Union, a rare disease is defined as a disorder with a prevalence below 5:10,000. There is no consensus definition of an ultra-rare disorder, but prevalence rates < 1-2:100,000 are used. Prevalence values vary in different studies, but in total, around 1:10,000 people are affected by either HSP or HCA (Ruano et al., 2014). In Norway, early epidemiological studies of HSP and HCA estimated relatively high prevalence of HSP, in particular AD-HSP compared to later studies (Refsum & Skre, 1978; Skre, 1974). In 2009, the minimum prevalence of HSP in South-eastern Norway was estimated to 7.4:100,000 (Erichsen et al., 2009). The prevalence estimates of the meta-analysis and Norwegian prevalence studies are summarized in Table 2.3.

The distribution of HSP diagnoses is relatively similar in Norway compared to international studies, the most common HSP in both AD and sporadic cohorts being SPG4 (Erichsen et al., 2009; Ruano et al., 2014). However, in the AD-HCA group there are differences in the distribution of different subgroups as the triplet expansion disorders seem to be much less common in Norway, compared to studies from other countries (Sullivan et al., 2019). Friedreich ataxia has been reported as the most frequent AR-HCA, as in Norway (Wedding et al., 2015). The proportion of families without genetic diagnosis after systematic testing were 45-67% in AD-HSP, 71-82% in AR-HSP groups and 33-92% in HCA (Ruano et al., 2014). This confirms that a large proportion of molecularly unsolved patients is not unique to the Norwegian patient material presenting the basis for this PhD study.

Table 2.3. Prevalence estimates of HSP and HCA

	Skre, 1974 (Western Norway)	Erichsen et al., 2009 (South-East Norway)	Ruano et al., 2014 (Meta-analysis)
AD-HSP	12	5.5	1.8 (0.5-5.5)
AR-HSP	2	0.6	1.8 (0.0-5.3)
AD-HCA	3.2	4.2	2.7 (0.0-5.6)
AR-HCA	3	2.85	3.3 (0.0-7.2)

Estimated prevalence is indicated per 100,000. For the meta-analyses both mean prevalence and range is given. Isolated (sporadic) subjects are not included in this table. The study by Erichsen et al. is included in the meta-analysis by Ruano et al.

2.8 Subgroups studied in this thesis

Among the large number of genetic forms of HSP and related disorders, some are more common, while many are described in only a handful or even fewer patients (see Figure 2.5). Table 2.4 provides an overview of the genetic subgroups which will be further discussed in this thesis. For comparison, also selected frequent HSDs are included in the table.

2.8.1 Nomenclature in this thesis

Based on the various nomenclature systems, this thesis will use the traditional disorder names (OMIM nomenclature) for historically well-known or uniform phenotypes, such as **SPG7** (Paper I), **SPG79** (Paper II), and for the traditional AR SPG18 caused by biallelic *ERLIN2* variants. However, the nomenclature suggested by the The International Parkinson and Movement Disorder Society (MDS) Task Force, combining the main phenotype and the gene (Marras et al., 2016), will be used for novel phenotypes; such as **HSP-ERLIN2(-AD)** (Paper III) and **ATX/HSP-POLR3A** (Paper IV), to differentiate the phenotype and/or genotypes of the patients in the papers from the assigned classifications in OMIM; SPG18 and HLD7.

2.8.2 SPG7

After mapping the SPG7 locus in a family with pure AR HSP to chromosome position 16q24.3 (De Michele et al., 1998), the same group identified the affected gene which encoded the protein paraplegin (Casari et al., 1998). The paraplegin protein is located at the inner mitochondrial membrane, and is involved in processing of mitochondrial proteins (Karlberg et al., 2009) and assembly of the mitochondrial ribosome (Nolden et al., 2005). Mutated paraplegin has been shown to result in multiple mitochondrial DNA deletions in respiratory deficient muscle fibres, accumulation of mitochondrial DNA damage and multiple respiratory chain deficiencies (Pfeffer et al., 2014; Wedding et al., 2014).

Table 2.4. Genetic forms of HSP described in Papers I-IV, and selected frequent HSDs

Disorder (alternative name)	MOI	Gene/ Protein	Main protein function	Frequency	Age at onset, years	Main pheno-type	Rarer or additional findings
Genetic forms described in Papers I-IV							
SPG 7 (HSP/ATX-SPG7)	AR, AD	*SPG7* paraplegin	Mito. ATPase	7% of AR	11-42	cHSP, CPEO	pHSP, pATX, optic atrophy
SPG79 HSP/ATX-UCHL1	AR	*UCHL1* UCHL1	DNA damage response	3 families	Child-hood	cHSP, optic atrophy	ATX, PN
HSP-ERLIN2 SPG 18	AR, AD	*ERLIN2* ERLIN2	ERAD, lipid metabolism	5 AR families, 3 AD families	1-19	cHSP (AR)	pHSP (AD)
ATX/HSP-POLR3A POLR3A-leukodystrophy[b]	AR	*POLR3A* POLR3A	RNA-transcription	3.1% of AR/spo HSP/HCA (estimate)	child-early adult	cATX, cHSP, tremor	4H, hypodontia, MRI findings
Other, frequent HSDs							
SPG4 HSP-SPAST	AD	*SPAST* spastin	Microtubule-severing, secretory pathway, BMP signalling	40% of AD, 9-18 of sporadic	0-74	pHSP	-
SPG3 HSP-ATL1	AD, AR[a]	*ATL1* atlastin-1	GTPase, ER-Golgi transfer, spastin partner, BMP-signalling	10%, 1 AR family	<10	pHSP	PN
SPG31 HSP-REEP1	AD	*REEP1* REEP1	ER-shaping, ER-microtubule interaction, mito. function	4.5% of AD	variable	pHSP	Amyotrophy, PN
SPG11 HSP-SPG11	AR	*SPG11* spatacsin	Lysosome shaping, autophagy	21% of AR, 59% of AR with TCC	child-adult	cHSP, TCC	pHSP, PN
SPG5 HSP-CYP7B1	AR	*CYP7B1* CYP7B1	Cholesterol and neurosteroid metabolism	7% of AR	0-74	pHSP	ATX, cognitive impairment, nystagmus
Friedreich ataxia ATX-FXN	AR	*FXN* frataxin	Mito. function, iron-sulphur cluster biogenesis	Most common AR-HCA, 1:176.000[c]	<25, variable	cATX, sensory ataxia	Retained reflexes, late onset

4H = 4H syndrome; Hypomyelination, hypodontia and hypogonadotropic hypogonadism; BMP = bone morphogenetic protein; cHSP/ATX = complex HSP/ataxia; CPEO = chronic progressive external ophthalmoplegia; ER = endoplasmic reticulum; ERAD = ER associated degradation; mito. = mitochondrial; MOI = mode of inheritance; pHSP/ATX = pure HSP/ATX; PN = polyneuropathy; TCC = thin corpus callosum. a) One report of AR SPG3 (Kahn et al, 2014). b) Full OMIM title: Leukodystrophy, hypomyelination, 7, with or without oligodontia and/or hypogonadotropic hypogonadism; HLD7. c) Prevalence of Friedreich in Norway from (Wedding et al., 2015). The table is modified from (Bounty et al, 2019).

SPG7 is, together with SPG11 and SPG5, the most frequent AR-HSPs (Boutry et al., 2019). A large study of a Dutch cohort identified *SPG7* mutations in 60 out of 791 patients (van Gassen et al., 2012). The clinical features vary, and patients may present with pure HSP, complex HSP, spastic ataxia or pure ataxia. Common additional findings are bladder disturbances, chronic progressive external ophthalmoplegia, spastic dysarthria, dysphagia, optic neuropathy, or polyneuropathy. Cervical dystonia, intellectual disability and upper limb involvement may also be observed (Casari & Marconi, 1993, updated 2018 Oct 25; van Gassen et al., 2012). MRI may show cerebellar atrophy, or hyperintensities in the dentate nucleus in T2-weighted images (Hewamadduma et al., 2018). Isolated optic neuropathy without cerebellar or pyramidal findings have also been reported (Klebe et al., 2012).

All types of mutations have been reported in *SPG7*. Genotype-phenotype correlation has been described for a specific and frequent missense variant, c.1529C>T (p.Ala510Val), in particular resulting in late-onset pure ataxia, while loss of function variants more often results in predominant spastic paraparesis (Mancini et al., 2019; Roxburgh et al., 2013). Also, some heterozygous *SPG7* variants may be disease-causing, illustrating the possibility of mixed inheritance patterns in HSP (Coarelli et al., 2019; Klebe et al., 2012; Sanchez-Ferrero et al., 2013). At the initiation of this PhD study in 2012, SPG7 was known as a frequent pure or complex AR-HSP (Fink, 2013), but not as a cause of pure ataxia or of AD transmission.

2.8.3 *UCHL1* and SPG79

The *UCHL1* gene (ubiquitin C-terminal hydrolase L1) (Day et al., 1990; Day & Thompson, 1987) encode the enzyme UCHL1 (initially called PGP9.5), one of the most abundant soluble proteins in the human brain. UCHL1 has been estimated to represent 1-2% of the total cytoplasmic protein within nerve cells (Doran et al., 1983; Jackson & Thompson, 1981). UCHL1 is important in the homeostasis of degradation of proteins, in particular in the nervous system. The enzyme is involved in regulating the level of free ubiquitin in the ubiquitin-proteasome system (UPS). The UPS serves as the cell's quality control (Hochstrasser, 2009). UCHL1 has mainly hydrolase, but may also have ligase activity, and can thereby regulate the level of free ubiquitin (Liu et al., 2002).

Dysfunction of the *UCHL1* gene or the UCHL1 protein has been implicated in several neurodegenerative disorders; Parkinson's disease (Leroy et al., 1998) and Alzheimer's disease (Tramutola et al., 2016; Zhang et al., 2014), and also in a

variety of cancers (Fang & Shen, 2017; Finnerty et al., 2019; Gu et al., 2018; Nakao et al., 2018).

An association between UCHL1 and monogenic neurodegeneration with a complex phenotype was established by Bilguvar et al. in 2013, who described a family with early onset neurodegeneration with optic atrophy, ataxia and pyramidal signs, and a homozygous variant in *UCHL1* (Bilguvar et al., 2013). The disorder was named "Childhood-onset neurodegeneration with optic atrophy" (NDGOA) (OMIM 615491). The SPG-number SPG79 has been assigned later, as a consequence of our findings in Paper II.

2.8.4 *ERLIN2* and HSP-ERLIN2

The *ERLIN2* gene (Endoplasmic reticulum lipid raft-associated protein 2, previously C8orf2) was identified in 1999 (Ikegawa et al., 1999). The product ERLIN2 form heterodimers with the highly homologous ERLIN1, and both are lipid raft-associated proteins localized to the endoplasmic reticulum (ER). ERLIN2 is part of the ER-associated degradation (ERAD) pathway, a degradative pathway encompassing ubiquitin-proteasome-mediated degradation of ER proteins (Blackstone, 2012). Biallalic variants causing a complex AR-HSP named SPG18 was reported in 2011 (Alazami et al., 2011). A few additional families with SPG18 has been described (Novarino et al., 2014; Wakil et al., 2013; Yildirim et al., 2011), and also one family where the phenotype was classified as AR juvenile ALS (Al-Saif et al., 2012). Most patients had a complex phenotype, with intellectual disability, motor dysfunction and contractures, and very early age at onset. Also, there were two reports SPG18 with a pure HSP prior to paper III, however also with AR mode of inheritance (Morais et al., 2017; Tian et al., 2016).

2.8.5 *POLR3A* and ATX/HSP-POLR3A

The *POLR3A* gene (RNA polymerase III, subunit A) encodes POLR3A, the largest subunit of RNA polymerase III (Pol III). Pol III is a large nuclear enzyme complex, consisting of 17 subunits, responsible for transcribing non-coding RNAs, in particular transfer RNA (tRNA), but also ribosomal 5S RNA, small nuclear RNA, short interspersed nuclear elements (SINEs) and other RNAs (Sepehri & Hernandez, 1997).

Geneviève Bernard and colleagues identified biallelic variants in *POLR3A* as the cause of several cases of leukodystrophies (Bernard et al., 2011). Leukodystrophy literary means "degeneration of white", i.e. destruction of the CNS white matter. Leukodystrophies affect the myelin, the white substance coating the nerves like an insulating sheath and increases nerve conduction velocity (Kohler et al., 2018; Waldman, 2018). A unifying criterion for POLR3-leukodystrophy was diffuse of localized hypomyelinating leukodystrophy in MRI. The clinical phenotype was

wide; tremor-ataxia with central hypomyelination (TACH), leukodystrophy with oligodontia (LO) or hypomyelination, hypodontia and hypogonadotropic hypogonadism (4H) syndrome. Biallelic variants in *POLR3A* and also *POLR3B* are now well-established causes of these leukodystrophy syndromes. Although the typical hypomyelination pattern was not always present, MRI findings supporting the classification as leukodystrophies were reported in all cases (Cayami et al., 2018; La Piana et al., 2014; Wolf et al., 2014).

Then, in 2017, Martina Minnerop et al. identified biallelic variants in *POLR3A*, and in particular an intronic variant resulting in an aberrant splicing, in much as 3.1% of a large cohort of previously undiagnosed patients with HSP or HCA (Minnerop et al., 2017). These patients did not exhibit the typical MRI findings previously described, but instead high intensities in the superior cerebellar peduncles. In addition, biallelic variants in the gene has been established as the cause of an AR premature ageing disorder; Wiedemann-Rautenstrauch syndrome (Paolacci et al., 2018).

2.9 Management of HSP

The huge unsolved problem of neurodegeneration is that we have not yet figured out how to reverse or stop the processes, and, for most cases, not even how to slow it down. Treatment is therefore symptomatic, and based on the individual needs of each patient.

The main motoric symptom of HSP is spasticity of the legs, and treatment aims to reduce spasticity, pain and muscle spasms and to prevent contractures. Physiotherapy is paramount, and active stretching of the spastic muscles at least at a weekly basis is a general recommendation. Spasmolytic drugs such as baclofen is often indicated (Bellofatto et al., 2019). Botulinum toxin injections may have effect, in particular when combined with subsequent stretching (van Lith et al., 2019). In selected patients, intrathecal baclofen may be tried (Margetis et al., 2014). Current treatment is largely based on experience and case reports (Bellofatto et al., 2019), hence better documentation is warranted for existing symptomatic treatment. It is also important to address additional difficulties, which may be cause major disabilities for the patient, such as bladder and bowel disturbances, sexual problems, cognitive impairment, fatigue, and psychological and social difficulties.

HSPs are chronic, life-long and progressive disorders that require long-term multidisciplinary health care services. Multidisciplinary collaboration between the primary health care system, the rehabilitations centres and several medical specialities is necessary. Studies on targeted treatment are so far on a preclinical stage (Shribman et al., 2019), and treatment directed at the causal gene or

mechanism in specific HSPs will be a major focus for future research. To move on towards personalized treatment, we still need to delineate the clinical phenotypes, molecular causes and pathological mechanisms.

3 Aims of the study

The overall aim of the study was to characterize molecular causes and the resulting clinical phenotypes of patients with HSP in Norway, based on a cohort of HSD patients without molecular diagnosis.

3.1.1 Paper I

To identify and characterize patients with SPG7.

3.1.2 Paper II

To characterize a family with biallelic variants in *UCHL1*, and the functional consequences on the UCHL1 protein.

3.1.3 Paper III

To characterize families with a heterozygous variant in *ERLIN2* and autosomal dominant pure HSP.

3.1.4 Paper IV

To identify patients with biallelic variants in *POLR3A* in our cohort and to characterize this patient group.

4 Summary of results

Paper I. A founder mutation p.H701P identified as a major cause of SPG7 in Norway

SPG7 is among the most common AR-HSPs world-wide and may cause a wide range of clinical features within the spastic-ataxic spectrum. In Paper I we identified and characterized Norwegian patients with SPG7. With either Sanger sequencing of SPG7 or WES, six families, in total 11 patients, with biallelic variants in *SPG7* were identified. Four of these families shared the same novel missense variant, c.2102A>C (p.His701Pro), homozygous in one family and compound heterozygous in three families. This variant was found to reside on a shared haplotype, supporting that this is a Norwegian founder mutation in *SPG7*. The patients shared a core clinical phenotype comprising spastic paraparesis with ataxia, urge incontinence and chronic progressive external ophthalmoplegia (CPEO). MRI showed mild cerebellar atrophy, of both the vermis and the hemispheres.

In conclusion, SPG7 was found to be a frequent cause of HSD in our Norwegian cohort, and a Norwegian founder variant was frequently observed. The phenotype confirms the heterogeneous presentation of SPG7, but a core clinical phenotype was observed. Of diagnostic importance, SPG7 should be considered in HSP combined with mild cerebellar atrophy, and in HCA combined with urge incontinence, and also any HSD combined with CPEO.

Paper II. Novel *UCHL1* mutations reveal new insights into ubiquitin processing

In Paper II we studied two monozygotic twin brothers and a sibling with complex HSD. The main phenotype in the twins was childhood onset optic atrophy and progressive spastic paraparesis with additional findings of ataxia, myoclonus and neuropathy. The third affected sibling had optic atrophy, more prominent cerebellar findings and less pyramidal findings. By WES, we identified two compound heterozygous variants in the *UCHL1* gene. The combination of a complex neurodegenerative phenotype and variants in this gene had only been reported once before (Bilguvar et al., 2013). The reported phenotype was comparable to our adult patients, apart from the difference in cognitive abilities. Interestingly, the Norwegian twins had working memory and memory functions 0.5-2.67 standard deviations above norm.

One *UCHL1* variant, c.533G>A (p.Arg178Gln), was located in the active site of the UCHL1 protein and, surprisingly, resulted in a 4-fold *increased* hydrolytic activity of the enzyme. The other variant, c.647C>A (p.Ala216Asp), located in the

hydrophobic core of the protein, was predicted to destabilize protein folding, and resulted in an insoluble protein. Mass spectrometry-based proteomics showed that the total level of UCHL1 in patient fibroblasts was about 25-35% compared to the level in controls. Hence, this specific combination of variants led to an increased hydrolytic enzyme activity, combined with a reduced total amount of the protein.

In conclusion, this study describes the second family in the world with UCHL1-related childhood-onset neurodegeneration with optic atrophy, previously known as NDGOA. As a consequence of our publication, the disorder was assigned the OMIM-name SPG79. Based on the particularly high memory functions we hypothesise that the contradictory effect of the two *UCHL1* variants may somehow protect cognitive function, warranting further studies.

Paper III. A novel heterozygous variant in *ERLIN2* causes autosomal dominant pure hereditary spastic paraparesis

In Paper III we studied one Norwegian and one German family with pure AD-HSP with variable age at onset (9-46 years). WES was performed separately of the two families, identifying the exact same missense variant, c.386G>C (p.Ser129Thr), in the *ERLIN2* gene. Biallelic variants in *ERLIN2* may cause SPG18, a complex, early-onset AR-HSP with contractures. The variant was not present in public databases, all bioinformatic tools predicted pathogenicity and indicated a very high interspecies sequence conservation. The variant segregated with disease in the families. The combined single point logarithm of the odds (LOD) score was as much as 4.34, supporting that the variant is the cause of the disease. A possible mechanism could be a site-specific dominant negative effect, causing dysfunction of the ERLIN2/ERLIN1 complex. The variant was concluded to be recurrent as a putative shared haplotype was limited to a maximum common region of 99.7 kb, making recent common ancestors unlikely.

In conclusion, we describe the first two families with a heterozygous *ERLIN2* variant as the cause of a pure HSP phenotype, thereby adding *ERLIN2* to the list of AD-HSP genes, and establishing *ERLIN2* as one of the HSP genes with mixed inheritance forms.

Paper IV. Biallelic *POLR3A* variants confirmed as a frequent cause of hereditary ataxia and spastic paraparesis

Biallelic variants in *POLR3A* were a known cause of 4H syndrome (hypomyelination, hypogonadotropic hypogonadism and hypodontia), and similar leukodystrophy syndromes. In 2017, Minnerop et al. proposed the gene as a

frequent disease-cause also in the HSD population (Minnerop et al., 2017). In Paper IV, we scrutinized WES data of 95 molecularly undiagnosed HSD probands (families/index cases) from the Norwegian patient cohort, and identified biallelic variants in the *POLR3A* gene in 10 probands, and in total 13 affected individuals. This represented as much as 3.1% of the undiagnosed AR or sporadic HSD probands in our cohort. Nine of the ten probands carried the specific intronic variant, c.1909+22G>A (p.Tyr637Cysfs*14), also found in 80% of the patients reported by Minnerop et al. Thorough clinical and supplementary investigations were performed to characterize the Norwegian patient group.

The patients presented with a strikingly similar and recognizable phenotype, comprising cerebellar ataxia, tremor, spasticity in the lower limbs and bilateral extensor plantar responses, combined with reduced lower limb tendon reflexes and loss of proprioception. Age at onset were typically below 20 years. Other neurological findings were muscle atrophy, reduced superficial sensation in the lower limbs, dystonia and urinary urgency. In seven out of the 13 patients, the main phenotype had changed over the years, in some from almost pure spastic paraparesis to severe tremor and ataxia, while pyramidal signs had become less prominent. Interestingly, patients with postural tremor reported a pronounced tremor-reducing effect of small amounts of alcohol. Most patients also had dental problems, either hypodontia, short dental roots or early periodontal disease. One patient even reported to have developed several extra permanent teeth. Hypogonadism requiring medical treatment was identified in one patient.

MRI of all available patients showed high intensities in the superior cerebellar peduncles in FLAIR and T2-weighted sequences. Except in one patient, these findings were not reported in the initial radiological report. Also, subtle signs of dysmyelination was found in other brain areas, in particularly the pyramidal tracts in the posterior limb of the internal capsule.

We confirmed that the c.1909+22G>A variant results in aberrant splicing of RNA and subsequent nonsense-mediated decay (NMD). A novel variant, c.3655G>T (p.Gly1219Ter), present in six of the families, produced a premature stop codon and also resulted in NMD. Haplotype analyses implied that both these variants were recurrent, and not founder variants.

In conclusion, we established biallelic variants in *POLR3A* as a frequent cause of a characteristic HSD phenotype and thereby underline the genetic heterogeneity of this gene. Also, there were typical additional extra-neurological findings and MRI findings, warranting the attention of the clinician. In addition, a specific intronic variant is frequent in patients with POLR3A-related disease clinically classified as HSP or HCA.

5 Methods and methodological considerations

All patients in the project originated from the same overarching project, and this original overarching project, including the HSD database and the current patient cohort will first be presented. Next, the phases and methods of the current PhD project will be outlined and discussed. An outline of the material and methods are provided in Figure 5.1.

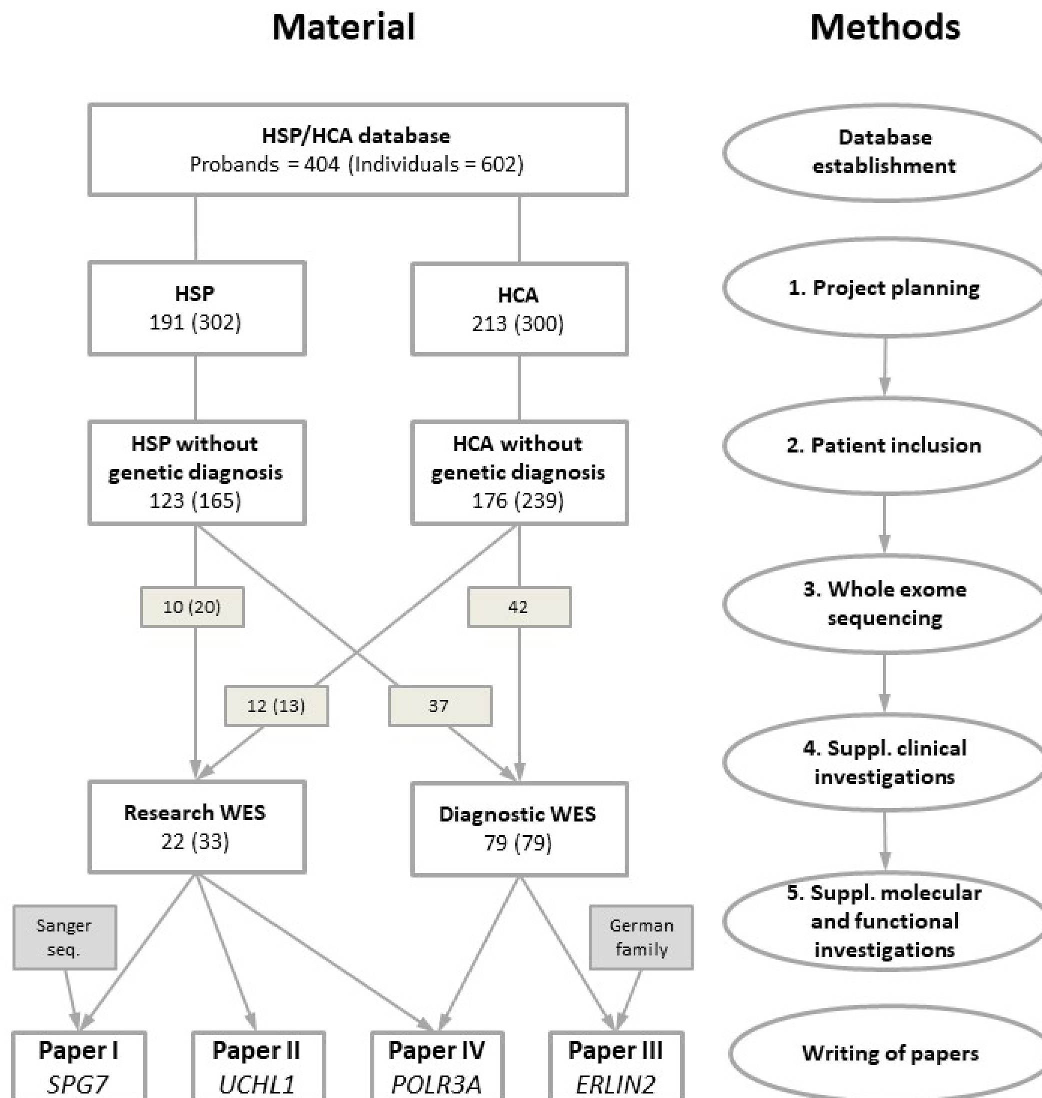

Figure 5.1. An overview of the patient material at the start of this PhD project, and of the methods applied. The number of included families/probands has increased from 404 to 522 over the time course of this PhD project. In parentheses are the number of individual patients. From the patients without molecular diagnosis, 22 probands (33 individuals) were selected for WES research studies ("Research WES"). In addition, diagnostic WES were performed in parallel, as this became available from 2014 ("Diagnostic WES"). Diagnostic WES were used in Papers III and IV. In Paper I, three of the six probands were diagnosed by Sanger sequencing of *SPG7*. In Paper III, the study combined the findings in the family from our cohort with findings in a German family. Suppl. = Supplementary.

5.1 Hereditary spinocerebellar disorders in Norway

The patient material for this PhD project originated from the overarching research project “Hereditary spinocerebellar disorders in Norway”. This overarching project was initiated in 2002 by professor Chantal Tallaksen, who initially identified seven patients with the diagnosis HSP in the diagnostic medical record system at Ullevål University Hospital. Now, as much as 767 HSD patients are registered in the database (June, 2019).

5.1.1 Patient inclusion to the database

All patients referred to the Department of Neurology, Oslo University Hospital (OUH), fulfilling a definite or probable HSP or HCA diagnosis were asked for consent to be included in the project. From 2005-2007 the research group performed a large epidemiological study aimed to establish the prevalence rates of HSDs in South-eastern Norway. Patient records in all hospitals in the region were scrutinized, and all consenting patients with a HSP or HCA diagnosis were examined and included, hence the patient material at that time represented an epidemiological cohort representing the HSD population in South-eastern Norway (Erichsen et al., 2009). Thereafter, the inclusion of patients has not been as systematic, but depended on the referral practice from other Neurological departments, and on ongoing studies of specific families or genetic subgroups. OUH is the local hospital for Neurology for most of the county of Oslo, and the regional hospital for South-eastern Norway, and patients with suspected HSP or HCA have continuously been referred to our clinic for first or second opinion, mainly from South-eastern Norway, but also occasionally from other parts of Norway. A more active inclusion initiated by the research group has been applied in family studies or studies of a specific genetic form, such as for Friedreich ataxia (Wedding et al., 2015).

Patients with a definite diagnosis fulfilling the clinical diagnostic criteria for HSP or HCA were included (Fink et al., 1996; Harding, 1983), and patients not fulfilling all diagnostic criteria were included as probable or possible HSP/HCA. Also, sporadic cases, patients without known family history of the disease, were included when the medical history and clinical features were compatible with HSP/HCA, and other causes were ruled out to the best of our abilities.

The project mainly included adult patients (≥18 years). Even though we have tried not to limit inclusions to the traditional original phenotypic descriptions, patients with atypical presentations may have been missed. Also, in particular for sporadic cases, patients with non-monogenic causes might have been included.

5.1.2 Standard examinations

All study subjects were examined by a project neurologist and extensively investigated according to a European protocol (SPATAX) (Tallaksen & Dürr, 2003). Demographic information, clinical findings and results of supplementary investigations have been systematically registered, based on standardized registration forms (Clinical chart SPATAX-EUROSPA-2011, https://spatax.wordpress.com/downloads/). The same protocol is used by many research groups internationally, enabling easy comparing of studies.

DNA was stored in a biobank, and all frequent genetic causes of HSD were systematically excluded based on available methods, guided by clinical presentation and ongoing research projects (Erichsen et al., 2009). The selection of methods for genetic testing, both in research and diagnostics have evolved over the years, and now include WES and large gene panels (Norsk portal for medisinsk-genetiske analyser, 2018), however this was not available before the start of this PhD project. Complementing genetic analyses, biochemical markers such as vitamin E and very long chain fatty acids were measured. Additional standard supplementary investigations were performed when diagnostically relevant, such as MRI of the brain and spinal cord and electroneurography/electromyography.

At the start of this PhD project in 2012, a total of 404 probands (meaning the first included family member with the phenotype, also called index case) and 602 individuals (meaning total number of patients with HSD) were included in the project, relatively equally distributed between the main classifications HSP and HCA (see Figure 5.1). The 299 probands that were without molecular diagnosis served as the core material to select patients for the current PhD studies.

5.1.3 Overview of the database and the current patient cohort

Over the years, patients with HSDs have been continuously recruited to the project database. Due to the overlap in genetic diagnoses between the main clinical categories HSP and HCA, both HSDs are presented. As of June 11th 2019, there were 767 individual patients included in the database, 392 classified as HSP, and 375 as HCA. There were in total 522 probands (families or sporadic cases), of these 41% had a genetic diagnosis (Table 5.1).

Table 5.1. Probands and proportion with molecular diagnosis in the Norwegian cohort

Probands	2012	2019
HSP	191 (68, 36%)	238 (120, 50%)
HCA	213 (37, 17%)	284 (94, 33%)
Total HSD	404 (105, 26%)	522 (214, 41%)

The table denotes the total number of probands/families included in the database at the start of the PhD study, compared to June 2019. In parenthesis is the number and percentage of probands with a molecular diagnosis

In total, 206 probands were registered with a probable AD mode of inheritance, and 115 with presumed AR inheritance. As much as 80% of AR probands, and 55% of AD probands had a genetic diagnosis. The most common genetic forms are listed in Figure 5.2.

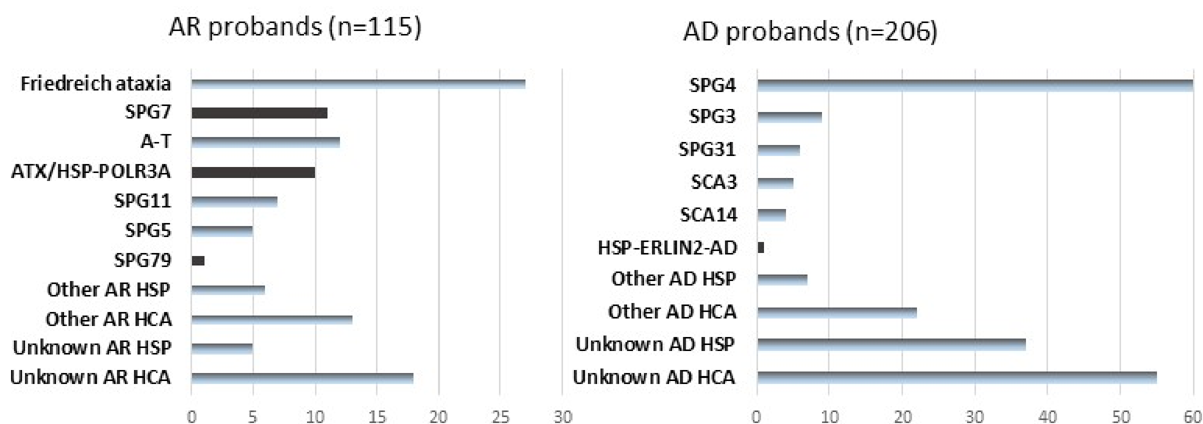

Figure 5.2. Number of probands with genetic diagnoses in database. The genetic forms described in Papers I-IV are marked in black. Of note, the figure does not include the 184 sporadic probands without genetic diagnosis. A-T = Ataxia-telangiectasia; Other HSP or HCA = other molecular diagnoses found in less than four probands.

The most common AD-HSPs were SPG4 (60 probands, 125 individuals), followed by SPG3 (9/18) and SPG31 (6/24), and the most common AR-HSDs SPG7 (11/18), ATX/HSP-POLR3A (10/13), SPG11 (7/9), SPG5 (5/5), and Friedreich ataxia (26/29). Twelve probands had ataxia-telangiectasia, these were included as children as part of the previously performed epidemiological study (Erichsen et al., 2009). In total, there were as much as 49 different genetic diagnoses present. The distribution of HSP diagnoses were similar to global distributions of the subtypes while the repeat expansion SCAs were less frequent in our cohort than reported globally (Sullivan et al., 2019).

These numbers also include all patients diagnosed through the work with this thesis, and the only discrepancy between the number of patients reported in the papers and these updated numbers, are for SPG7. As might be expected from the increased awareness of the broad phenotype, the easier access to gene panel sequencing, and the high frequency of the disorder, the number of families with SPG7 has increased from the six (11 individuals) published in 2016, to 11 (18 individuals) today.

5.2 Project planning and design

This PhD project was based on the existing database, with the aim to characterize the vast proportion of undiagnosed patients. A detailed overview of the project's phases and methods, including an indication of the candidate's contribution is given in Figure 5.3.

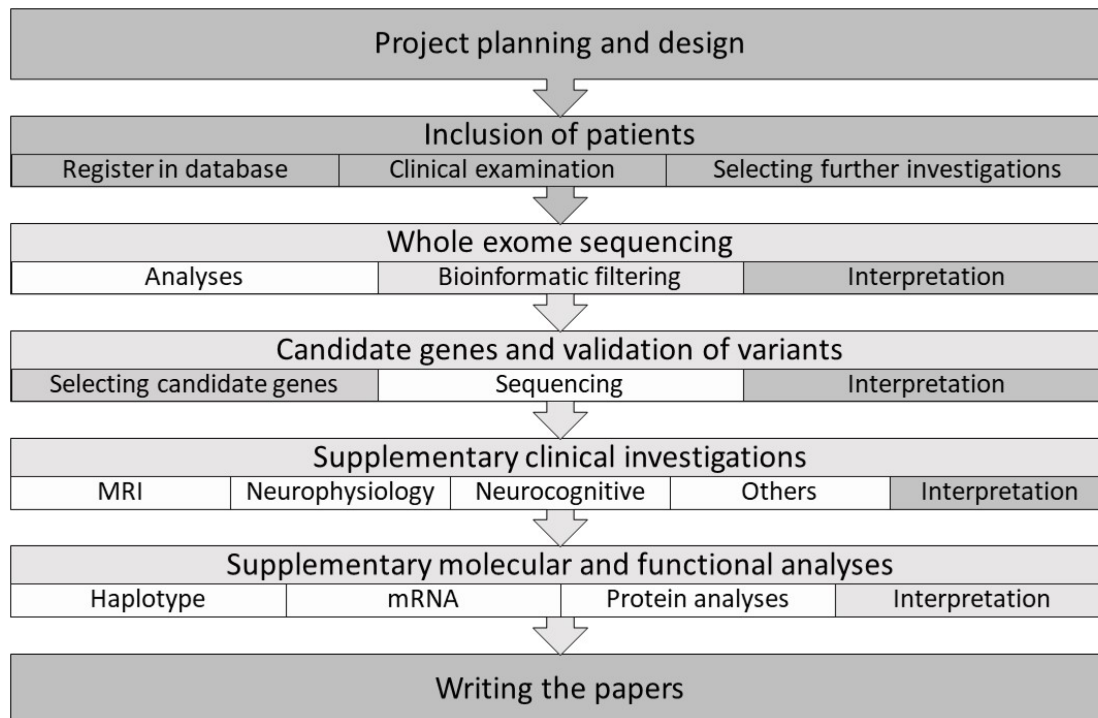

Figure 5.3. Overview of the PhD project and the candidate's contribution. Dark grey: Major contribution by the candidate. Light grey: Contribution by the candidate. White: Performed by collaborators, minor contribution by the candidate.

5.2.1 Design

The overall design was to select well-suited patients from the pool of molecularly undiagnosed patients in the database. We aimed to select patients with a high probability to find the molecular cause. Hence, we selected patients with presumed autosomal recessive (AR) mode of inheritance, and when possible, two affected members from each family. As patients were already investigated and found negative for the common forms of HSP, using all available diagnostic methods at the time, we needed a broader approach. By 2012, the new and promising method whole exome sequencing (WES) had become available for research projects at the Department of Medical Genetics, OUH, and was chosen as the main molecular approach in this PhD study. WES was performed, analysing known HSP genes first. Once a presumed molecular cause was identified, the candidate performed thorough clinical characterization of the patients. Also, the database and WES data of as many patients as possible were scrutinized for additional patients possibly sharing the same diagnosis. The design is further elaborated in the following sections.

5.3 Inclusion of patients

5.3.1 Patient selection

Molecularly undiagnosed patients and families with well-defined phenotypes were selected from our cohort of patients with HSD for WES. Over time, we had two sources of genetic data: exome data sequenced as part of the research project, "Research WES" (22 probands), and exome data obtained from diagnostic gene panels, "Diagnostic WES" (79 probands) (see Figure 5.1). A more detailed overview of the selection process of patients to the different projects is presented in Figure 5.4.

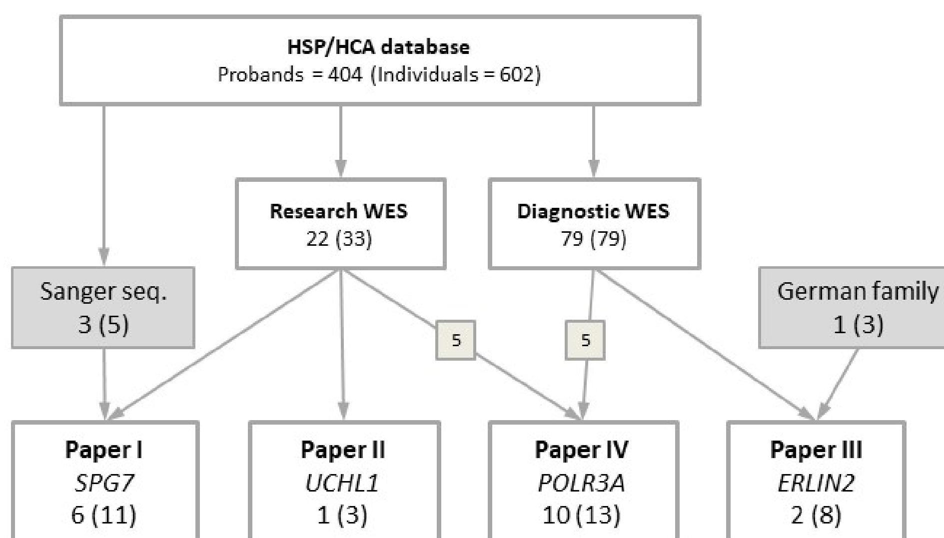

Figure 5.4. Overview of the patients selected for the four papers. Number of probands, and in parenthesis the number of affected individuals, included in each paper. Paper I also included three families where the SPG7 diagnosis was established by Sanger sequencing of *SPG7*. All patients in the papers were included the Norwegian database, except one German family included from a separate German research project, and described together with the Norwegian family in Paper III.

Research WES

From the 299 probands in the database without molecular diagnosis, 22 probands with AR-HSP (10) or HCA (12) were carefully selected. The selection was based on; a verified family history suggesting a recessive mode of inheritance and a well characterized clinical phenotype. In most cases, this comprised patients with a complex form of disease with onset in childhood or early adult age. Also the availability of probands and family members for additional examinations was considered. When possible, more than one affected individual from each family was included, to possibly reduce the number of putative disease causing variants in the bioinformatic analysis. WES was performed of two family members in the 10

families classified as HSP, and one of the HCA families (9 sibling pairs and two healthy first degree relatives). These research exomes, identified three of the families with SPG7 in Paper I (the remaining identified by Sanger sequencing), the family described in Paper II, and five of the families described in Paper IV.

Diagnostic WES

Gene panel sequencing for HSP and HCA, based on WES, has since 2014 been performed in the HSD patients as a diagnostic method, when indicated based on clinical phenotype and prior genetic investigations. The number of included genes in the diagnostic gene panels have increased over the years. The current gene panel for movement disorders at the Department of Medical Genetics, OUH include 412 genes (Norsk portal for medisinsk-genetiske analyser, 2018). The *ERLIN2* variant in the Norwegian family described in Paper III was identified in the diagnostic gene panel, while the other family in Paper III originated from a separate German cohort. For Paper IV, diagnostic gene panels were transferred to our research platform for re-analyses of the entire exome, and in particular for variants in the *POLR3A* gene. Five of the families described in Paper IV were identified through diagnostic exome data, including one where WES was performed at another hospital (Telemark Hospital).

Inclusion of family members

When the respective genetic variants were identified in the probands, available family members, both affected and not affected were contacted through the patients and asked for consent to join the project. As many family members as possible were examined by a project neurologist and gave blood samples for DNA analyses. Reasons to include family members were to identify other family members with the disease and thereby increase the impact of the clinical characterization, to establish whether the variants were *in trans* (on both alleles) in AR cases, and to test for segregation of the variant(s), meaning that the presumed disease-causing variant(s) were only present in the affected individuals.

5.3.2 Clinical investigations

As mentioned in section 5.1.2 describing the database, we used a standardized investigation protocol (SPATAX network, 2011). The protocol included the Spastic Paraplegia Rating Scale (SPRS) for patients with spasticity (Schule et al., 2006) and the Scale for the Assessment and Rating of Ataxia (SARA) for patients with ataxia (Schmitz-Hubsch et al., 2006). The patients were examined by a project neurologist at least once, to ensure an evaluation as standardized as possible. In Paper IV, one patient only consented to the use of genetic material and previous medical record. When a genetic diagnosis was made, available patients were examined once more. This included examining for specific features known to be part of the diagnosis,

such as dental problems and endocrinal signs in the patients reported in Paper IV with ATX/HSP-POLR3A.

Clinical considerations
Thorough clinical investigations remain important both in diagnostics, the follow-up, and as baseline characteristics for clinical trials (Trummer et al., 2018). The use of validated clinical rating scales makes the clinical results comparable to other studies. In the collaborative project in Paper III, the SPRS scoring system were used by both the German and our Norwegian group (see Table 1 in Paper III). There is a possible bias in the scoring system as it will depend on the rating from the individual investigator. Also, the score will only show the function of the patient on that particular day. The degree of spasticity will depend on treatment (such as time since botulinum toxin injections), the amount of physical activity or other stressors at the day of the investigation. However, the SPRS and SARA scoring systems have shown high interrater consistency (Schmitz-Hubsch et al., 2006; Schule et al., 2006). Also, these rating systems are relatively comprehensive and time-efficient.

In addition to the clinical examination, the patient history itself proved important in several of our patients. In particular, this was illustrated in Paper IV, where, when asked specifically, almost all the patients reported dental problems. Also, one patient reported signs of gonadal dysfunction, and subsequent biochemical analyses confirmed hypogonadism requiring medical treatment.

5.4 Whole exome sequencing

At the beginning of this project, WES was a relatively new method, while today it is a well-established approach used both in research and in diagnostics. A brief overview of the method is presented in the Introduction chapter and further details can be read in reviews of the topic (Shendure & Ji, 2008; Singleton, 2011; Voelkerding et al., 2009).

The HTS method was essentially the same in the Research WES and the Diagnostic WES approach, and is described in the articles. The sequencing was performed at the Department of Medical Genetics by The Norwegian Sequencing Centre (www.sequencing.uio.no), using the newest available exome enrichment kits (Agilent SureSelectXT Human All Exon versions 4 or 5), and sequencers (Illumina HiSeq2000 before 2014, thereafter Illumina HiSeq 2500).

5.4.1 Variant ranking and bioinformatic filtering

After sequencing and variant calling, the next step is bioinformatic filtering and analysis to identify candidate pathogenic variants. For this, the program FILTUS was used (Vigeland et al., 2016). FILTUS is an openly available HTS bioinformatic

analysis tool, developed in-house by Magnus Dehli Vigeland (http://folk.uio.no/magnusv/filtus.html). Only variants of good quality (PASS) were considered. We excluded common variants with frequency >0.01 in 1000Genomes (2010) or EPS5400 or with >2 hits in an in-house database consisting of 280 exomes (comprising patients with Addison disorder, neurologic disorder other than HSP/HCA and some healthy controls, registered at the Department of Medical Genetics, OUH). Also synonymous single nucleotide variants (SNVs), variants that do not alter the amino acid or amino acid sequence, were excluded. Various tools, including PolyPhen2, SIFT, PhyloP, MutationTaster and Gerp score, were used to predict the variants' effect on the protein or the protein function.

Genes that were known to cause other monogenic disorders with phenotypes not comprising spasticity, ataxia or other movement disorders, were removed from the analyses. This included a list of 1718 genes. This choice was made from ethical considerations, to minimize the risk of incidental findings (see chapter 6). It cannot be excluded that variants in these genes may also cause a HSP phenotype; however, avoiding incidental findings was considered more important than the possible gain of novel relevant findings.

5.4.2 Bioinformatic interpretation – candidate genes and validation

Variants that remained after filtering, were manually inspected, looking for a possible connection between the gene and the patient's phenotype. The exome data was re-analysed for each study, using the newest versions of analysis tools, and candidate gene lists. Once identified, the variants were validated by Sanger sequencing, confirming that the variants present in the WES data were actually present in the patient's DNA. Testing for segregation of the variants in the families was also performed by Sanger sequencing.

In an AR disorder, there is a pathogenic variant on both copies/alleles of the gene, either homozygous, with an identical variant on both alleles, or compound heterozygous, where different variants are located on each of the alleles. When finding two different putative pathogenic variants in a gene in an AR disorder, it is necessary to establish whether these variants reside on the same allele (*in cis*), and thereby not causing an AR disorder, or on separate alleles (*in trans*), thereby possibly causing an AR disorder. In most cases, Sanger sequencing of family members were also used to rule out that the variants (in AR cases) resided on the same allele (*in cis*).

5.5 Supplementary clinical investigations

Supplementary clinical investigations were mainly performed as part of the diagnostic work-up. This included standard MRI of the brain and spinal cord, and

electromyography/electroneurography in patients with sensory involvement, in addition to standard biochemical tests. Many patients also underwent detailed ophthalmological assessment (Papers I, II, IV), and/or investigation of visual, brain stem and somatosensory evoked potentials (Papers II and IV). Non-motor symptoms, such as cognitive difficulties, are common in HSDs, but still needs to be better characterized in the separate subgroups. For this reason, neurocognitive evaluation was performed in selected patients for Papers I, II and IV, with tests specifically selected to minimize motor skill interference (Wedding et al., 2013). It would be interesting to perform in particular ophthalmological and neurocognitive evaluation of all study patients, however this was not practically possible within this PhD project.

In Paper II, only the two twins underwent thorough neurocognitive evaluation, which showed remarkably high memory functions. However, to enlighten whether this could be explained by the aberrant UCHL1 function, formal neurocognitive evaluation of the third affected sibling, and also of the unaffected siblings would have been warranted. Subjectively, all siblings were considered to have good memory capacities, while the twins very obviously had memory capacity beyond normal (for instance they remember the date and name of doctors at every hospital visit back to the 1970s).

MRI

MRI of the brain and spinal cord was considered an important part of the clinical characterization in all patients. For all papers, specialists in neuroradiology were consulted in varying degrees, and the images were re-examined once the genetic diagnosis was made, to search for any specific related findings.

In Paper IV, the role of the neuroradiologist were of particular importance. Seven of the patients underwent renewed MRI on a 3 tesla MRI scanner. Interestingly, the specific MRI finding of high intensities in the superior cerebellar peduncles present in all patients was only reported in the first radiological description of the images in one out of the ten patients. This illustrates the need for experienced and interested neuroradiological eyes, and the necessity to re-examine images described as "normal" or with "unspecific white matter lesions".

Another methodological challenge, in particular for MRI, was the technical difficulties related to the patients' movement disorder. In particular tremor, contractures and severe difficulties with moving, rendered some investigations inconclusive or not possible to perform. In studies of such rare disorders every patient's contribution is extremely valuable to increase the strength of the results. In future studies the need to reduce artefacts and avoid inconclusive investigations,

such as measures to reduce tremor with medications or head support (Vrij-van den Bos et al., 2017), need to be addressed in the protocol.

The last years, advanced MRI techniques have been applied to some subgroups of HSP and HCA (Adanyeguh et al., 2018; da Graca et al., 2018; Mascalchi & Vella, 2018; Rezende et al., 2015; Sadeghi et al., 2018), and present promising methods to explore in future studies.

5.6 Supplementary molecular and functional analyses

Further studies of the variants were performed to verify pathogenicity, and to explore the pathological consequences. The methods were chosen depending on the specific variants and genes, and feasibility. In Paper I, bioinformatics were supplemented with haplotype analysis of the novel His701Pro variant. In Paper II, both *UCHL1* variants were novel, and functional studies were performed, led by Magnar Bjørås. Paul Hoff Backe performed the analyses of protein structure and enzymatic activity, while targeted mass spectrometry was performed at the Norwegian University of Science and Technology. Paper III included haplotype analyses to establish the variant as recurrent rather than a founder variant, and computing of the LOD score. In Paper IV, analyses of haplotypes, RNA transcripts and effects on protein structure were performed. All supplementary analyses were performed by collaborators and the candidate's role were to participate in the interpretation of the results, hence a detailed discussion of these methods lies beyond the scope of this thesis.

5.6.1 Haplotype analysis – establishing the origin of variants

In Papers I, III and IV, efforts were made to establish whether the variants represented founder variants or recurrent variants. A genetic variant observed in a group of individuals is called a *founder variant* if the observed alleles are inherited from some common ancestor of the carriers. Conversely, it is *recurrent* if the observations are the result of multiple independent mutation events. Carriers of a founder variant will in general also share a chromosomal region surrounding the variant, and may therefore be identified by *haplotype analysis*. A haplotype is a genetic sequence of closely linked alleles present on the same chromosome which tend to be inherited together. Different methods were used to narrow down the length of shared haplotypes, based on the available sequencing data. In Papers I and III, the WES was supplemented with Sanger sequencing of additional nearby SNPs (single nucleotide polymorphisms) or microsatellites for this purpose.

The origin of a pathogenic variant is important for genetic clinical work. For example, a disease-causing founder variant, such as the *SPG7* variant c.2102A>C described in Paper I, may explain a higher prevalence of a certain disorder in a

geographically limited area, and deserve high priority when performing genetic testing in this region. On the other hand, recurrent variants. such as the *ERLIN2* variant in Paper III, and the two most frequently observed *POLR3A* variants (c.1909+22G>A and c.3655G>T) presented in Paper IV, must be considered in every population.

5.6.2 LOD score

In Paper III, a logarithm of the odds (LOD) score was used as evidence that the variant was linked to the disease. Statistically, the LOD score is computed on the bases of the observed genotypes within a family, and is used to test the null hypothesis that a variant and the disease locus are independently inherited. A LOD score > 3 is normally considered enough to reject the null hypothesis and conclude that the loci are situated close to each other. An important mathematical fact about LOD scores is that scores from unrelated families may be added. We applied this in Paper III, where haplotype analysis showed that the *ERLIN2* variant was most likely recurrent and that the families were not related. The LOD scores from the two families gave a total LOD as high as 4.34, thus providing strong statistical evidence that the *ERLIN2* gene is involved in the phenotype.

5.6.3 Nonsense-mediated decay

Nonsense-mediated mRNA decay (NMD) is a mRNA quality-control mechanism, degrading mRNA that contains a premature stop codon, thereby preventing production of shortened protein products which could result in disease (Kurosaki & Maquat, 2016). Whether an aberrant transcript is subject to NMD or not depends on the location of the variant. Premature stop codons located more than approximately 50-55 nucleotides upstream of an exon-exon junction generally trigger NMD (Nagy & Maquat, 1998). In Paper IV, sequencing by RT-PCR (reverse transcription polymerase chain reaction) with and without NMD inhibitor showed that the transcripts of both *POLR3A* variants c.1909+22G>A and c.3665G>T were subject to NMD, and thereby resulted in a reduced protein level.

5.6.4 RNA to reveal compound heterozygosity

In Paper IV, in the patients with the *POLR3A* variants c.3655G>A and c.1909+22G>A, the aberrantly spliced transcript produced by the c.1909+22A allele was sequenced. The c.3655G>A variant was not to be found in this transcript, thereby confirming compound heterozygosity as the variants must be located *in trans*. This method, using reverse transcription and sequencing of the transcript, neatly allowed us to avoid the need of DNA from unaffected family members.

5.6.5 Protein structure analyses

In Papers II and IV, available protein structure models were used to estimate the consequences of the variants on the three-dimensional protein structure, either based on crystal structure of human protein (Paper II), or modelled using templates (Paper

IV). In Paper II, protein structure analyses predicted both that the c.533G>A variant in *UCHL1* might lead to altered catalytic activity, and that the c.647C>A variant might destabilize folding and cause aggregation, in line with the enzymatic activity and protein quantification analyses that were also performed.

In Paper IV, the structure analyses also proved a useful addition to bioinformatic analyses, to strengthen the evidence of pathogenicity of the c.1682G>A and c.1378_1380del variants in *POLR3A*. The c.1682G>A missense variant, changing the amino acid in position 586Arg may disturb the interaction of the POLR3-subunits and thereby affect the enzymatic activity of Pol III, while the c.1378_1380del variant was predicted to delete a conserved amino acid, thereby probably destabilizing the POLR3A subunit.

For the *ERLIN2* variant in Paper III however, the available template for the homology model had a low sequence identity, and the exact structure and subsequent consequence of the *ERLIN2* variant could not be established with certainty.

This underlines that protein structure analyses can be a helpful and very illustrative tool, in the search of evidence of pathogenicity of missense (or other) variants causing single amino acid changes. However, the utility depends on available models of the respective proteins.

5.6.6 Enzymatic activity and mass spectrometry

Paper II describes the rarest and least known of the four studied disorders. We describe one family, where neither of the identified *UCHL1* variants were previously reported. Without the possibility of validating our findings in other patients or families, we needed strong evidence that the variants were indeed causing the disease. We therefore performed enzymatic activity assays to measure the hydrolytic activity of UCHL1 and mass spectrometry-based proteomics to identify, characterize and quantify UCHL1 mutant proteins.

By mass spectrometry we found reduced levels of UCHL1 in patients' fibroblasts, compared to controls. This was based on fibroblasts from skin biopsies of the monozygotic twins, and from a single unrelated healthy control, which was not matched for age or gender. It would however have been a strength to obtain skin biopsies and perform the same analyses of the other affected, or of unaffected sibling, and also in more and matched control samples.

The surprising findings of increased hydrolase activity, did not fit with what we predicted, nor with findings the previous article (Bilguvar et al., 2013). The hydrolase activity assays were carefully performed and repeated in three independent experiments with different batches of enzyme. The data consistently showed that the Arg178Gln mutant displayed a higher turnover (catalytic

efficiency) than the wild type enzyme. Still, this observation cannot be fully explained, and further experiments to follow up this finding would be of great interest, such as UCHL1 activity in neuronal tissue or cells. In lack of brain tissue, reprogramming of patient fibroblasts to induced pluripotent stem cells and subsequent neuronal differentiation would be a suitable model system to study the impact of the variants on neuronal development.

5.7 Collaboration

The close collaboration with other group members and collaborators is reflected in the many joint first and last authorships (Papers I-III). All papers are the result of intensive team-work with contribution from collaborators both locally at OUH (all papers), nationally (Papers I, II and IV) and internationally (Paper III). Departments of Neurology and Medical Genetics in several parts of Norway has aided with information on patients. In short, the clinical work and DNA analyses have been performed by the core research group, whereas functional analyses such as protein structure, enzymatic activity assays and mass spectrometry were performed by collaborators from other research groups.

A single centre study provided a robust study material, as all patients were investigated and examined in the same way, by a limited number of project neurologists, and supplementary investigations were performed by the same protocols in all study patients. Also, having experts in the respective fields to perform and analyse the supplementary investigations and functional analyses, further ensured the quality of the data presented in the papers.

However, HSDs are rare, even ultra-rare, disorders, and the number of individuals with any of these rare genetic forms of HSP will be limited. This calls for collaborative studies and for sharing of data, both nationally and internationally. The importance of international collaboration and sharing of results is illustrated by the history resulting in Paper III. The *ERLIN2* variant identified in a diagnostic gene panel was initially considered a variant of uncertain significance, however an attentive bioengineer at the Department of medical genetics, OUH, discovered an abstract from a German conference in which the exact same variant was described in a family with AD pure HSP. This led to the fruitful collaboration with prof. Christoph Kamm and his colleagues in Germany. The importance of data sharing and publication to guide future studies was also shown by the article by Minnerop et al. and the discussion that followed (Gauquelin et al., 2018; Minnerop et al., 2018; Minnerop et al., 2017), which proved useful when designing our own study (Paper IV).

5.8 Statistical considerations

5.8.1 Descriptive and not epidemiological studies

As sample sizes were small in each patient group, only descriptive measures of symptoms and findings were used in the studies. The studies were not designed to establish prevalence estimates, but rather to characterize the patients in the Norwegian cohort.

5.8.2 Power assessment

In all studies, we aimed to identify the genetic cause of the patients' disorder. As monogenic diseases are rare, and often genetically heterogeneous, the chance of achieving such an aim depends on two central factors: the availability of a multiple independent patients/families with the same molecular cause, and functional evidence of the mutation's pathogenic consequences.

In this project we had a fairly large cohort. Also, a major strength was the national and international collaborative network of our project group. This provided the possibility of additional patients, and to perform functional testing (see section 5.7 Collaboration). In Paper III, statistical estimation of the LOD score provided strong support of the involvement of the *ERLIN2* gene in the phenotype (see section 5.6.2). In all our studies, more than one family with the combination of variants in the same gene and the HSD phenotype were identified, either from prior publications (Papers I, III and IV), or presented in our paper (Paper III). In general, all novel genetic findings will need to be considered with caution. In the four studies presented in this thesis, we regarded the evidence of the phenotype-genotype correlations as strong. Later on, our results have been confirmed in subsequent studies, thus increasing the reliability of our results as well.

5.9 Why WES?

WES was the main molecular method in this PhD thesis, and some methodological considerations regarding WES will be discussed. At the time of the start of the project, most available genetic analyses had been performed on the patients in our cohort, and we needed new methods to gain progress. WES had proven a cost-efficient and effective strategy to identify the molecular causes in heterogeneous monogenic disorders (Bamshad et al., 2011; Erlich et al., 2011; Ng et al., 2010). Alternatives to WES could be to investigate *less* of the genetic material, such as subsequent single genes or selected subsets of genes, or *more*, as in whole genome sequencing.

5.9.1 Single genes or selected groups of genes

When it comes to diagnostics, one could argue that for subtypes with very characteristic phenotypic features, single gene testing could precede a broad genetic work-up. Examples from our papers would be the combination of CPEO, bladder disturbances and spastic ataxia suggestive of SPG7, and tremor, spastic ataxia and dental abnormalities suggestive of ATX/HSP-POLR3A. However, this would be inefficient for research purposes. WES offers hypothesis-free analysis, and opens up for identification of both new disease-causing variants and extended phenotypes of known genetic variants, such as in POLR3A-related disorders (Minnerop et al., 2017).

Importantly, WES will not effectively detect copy number variations, or repeated sequences such as triplet expansions. Therefore, additional genetic analyses needs to be considered, which will depend on the clinical phenotype. In the papers presented in Papers I-IV, relevant additional analyses, such as GAA repeats in *FXN*, (Friedreich ataxia) and CGG expansions in *FMR1* (Fragile X tremor-ataxia syndrome) had, when relevant, been performed prior to WES.

Targeted HTS, capturing only a selected panel of genes, not the entire exome, was performed in a parallel study of molecularly undiagnosed patients from the same cohort (Iqbal et al., 2017). That study identified a definite or probable genetic diagnosis in 19% of the patients (12/105). The targeted HTS approach proved time- and cost-efficient with low risk of incidental findings. However, considering the steady increase in the number of established disease genes, it would be a disadvantage not to have the entire exome sequence available for re-analysis.

5.9.2 Whole genome sequencing versus WES

Extending the search from the protein coding regions to the entire genome is an intriguing next step in future studies, however this was not a feasible option during this PhD project. The obvious advantage of whole genome sequencing (WGS) compared to WES is more evenly distributed coverage, giving the potential to detect variants both in coding and non-coding DNA, and also improved calling of copy number variations (CNVs) and repeat expansions (Belkadi et al., 2015). Costs are continuously reduced, data storage capacity increases and bioinformatic pipelines are improved, making WGS an increasingly available option. At the same time, with the increased information obtained by WGS also comes the ethical difficulty of increased risk of incidental or uncertain findings.

So far, most of the known disease-causing variants are single nucleotide changes or small duplications or deletions in the protein-coding exons likely to be identified by WES, and most known disease-causing variants are located within the protein-coding sequence (Fogel, 2018). Statistics from the HGMD database show that 88%

of the mutations reported are missense, nonsense or splicing variants, or small (<20 bp) deletions or insertions (indels) (HGMD, 2017), biased by the fact that the exome is more extensively investigated and understood than non-coding DNA, at the moment.

In conclusion, WES is still considered the most cost-efficient and effective strategy to identify the molecular causes in heterogeneous monogenic disorders (Bamshad et al., 2011; Galatolo et al., 2018; Posey et al., 2019; Rexach et al., 2019; Suwinski et al., 2019). However, should a similar study be designed today, WGS would be included as a method of choice, also because most of the "low hanging fruits" will already have been picked up by WES.

5.10 Summary of methodological strengths and limitations

A major strength in this project was the extensive and well characterized research database, containing detailed clinical information of more than 700 patients. The patients were included at a single centre by a small team of project neurologists and investigated using well defined clinical scales and standardized examinations, thus making an excellent starting point to select patients for the different studies. As exome data became available in an increased amount of these patients, in the last article, as much as 95 patients could be retrospectively investigated for the *POLR3A* variants. WES with the applied bioinformatic and subsequent supplementary molecular analyses, proved to be an efficient approach to identify the molecular diagnosis in patients with HSP and HCA. In our "Research WES" group, comprising 22 very carefully selected families with "unsolved" AR-HSP/HCA, as much as 55% (12 probands) has now received a molecular diagnosis.

Our results cannot be used directly for epidemiological purposes. Also, supplementary investigations, such as neurocognitive assessment, MRI and neurophysiological evaluation were not possible to perform in all patients in all four studies. Additional functional studies could provide further evidence of the pathogenicity of the variants identified in the papers, and of the underlying pathomechanisms. In a novel study, WGS would also be included. In total, the methods used were considered appropriate and efficient to reach the research aim of the papers, namely to identify the molecular cause in selected subgroups of HSP and to characterize these disorders.

5.10.1 An "ideal" methodological approach

Removing all financial and other obstacles, an outline of an "ideal" study protocol aimed to identify and characterize the genetic subgroups of HSP in Norway, could be to:

- Include *all* patients in Norway with HSP/HCA (or even broader phenotypic inclusion)
- Standardized, thorough clinical characterization
- Biobank storage of DNA and fibroblasts
- Whole genome sequencing (and other -omics) of all patients
- Bioinformatic analyses, filtering for known disease genes, including repeat expansions and CNVs
- Functional studies depending on the gene/variant, including *in vitro* and/or *in vivo* model systems
- International and multidisciplinary collaboration in studies of genetic subgroups

6 Ethical considerations

6.1 Formalities

The project, including the patient database and the storage of DNA in a biobank was approved by the Regional Committee for Medical and Health Research Ethics, South East Norway, ethical agreement number 2010/1579A. An amendment was approved in 2012 for the use of high-throughput sequencing data. Also, an amendment including an updated consent form was approved in 2017, further specifying the use of supplementary investigations and the possibility of sending DNA to international collaborators. All patients and family controls were informed to the best of our abilities, both orally and in the written consent forms.

6.2 Gene-ethics

The use of "real" individuals affected with a disease and their DNA in research, represent a gold standard "model system" to study human disease. However, this also raises ethical considerations of which a few will be mentioned.

6.1.1 Mixing of roles. Patient - doctor versus research subject - scientist

Most patients in our study were included as part of an outpatient visit, where the including neurologist had a role both as therapist and as researcher. Hence, the patient becomes both a patient seeking help, and a study subject contributing to research at the same time. HSDs are rare disorders, and the neurologists with the most knowledge of their disorder are often also the most eager researchers. This mixing of roles may present ethical difficulties to both the doctor/researcher and the patient/research subject. Patients might feel obliged to consent to studies and participate in whatever investigations we may suggest, when asked by their follow-up neurologist. In addition, it might obscure the line between diagnostics and research. Despite our best intentions, a constant search for genetic diagnosis might not be what gives every patient the best quality of life. On the other hand, patients registered in our research database might get easier access to new diagnostic options and to medical follow-up. To us, the researchers, it was important to be aware of these issues and to separate the two roles to the best of our abilities. Also, the information given to the patients was important. Both orally and in the written consent form it was clearly stated that the inclusion was voluntary and that withdrawal was possible, without affecting any follow-up, and that any results identified as part of the research project would only be reported back to the patients if considered relevant for the diagnosis or medical follow-up.

6.1.2 Anonymity

Special care was taken to inform all patients who were to be described in the articles about the risk of backwards identification through the published articles. The patients were informed that, even though their patient information was anonymized

as much as possible, the disorders are very rare and the clinical presentations are unique. Hence, the patients and their close relations would be able to recognize their patient histories in the article.

6.1.3 Variants of uncertain significance and incidental findings

The approach of broad genetic investigations with WES or gene panels will help us to expand the phenotypic spectrum of disease, but perhaps in some cases with the cost of more uncertainty for the patients. When analysing large amounts of genetic material as in WES, there is a risk of identifying variants of uncertain significance (VUS) (Richards et al., 2015) or incidental findings. Finding a VUS in a gene that could be relevant for the phenotype will require further investigations before concluding on relevance to the patient's disease, which might lead to unnecessary uncertainties and extra examinations of both the patients and their families.

Incidental findings are potential risk variants in genes not related to the investigated phenotype. To minimize this risk we excluded all genes known to cause monogenic disease without movement disorders, in the analyses of exome data. However, WES does produce the entire protein coding part of a patient's genome, and one could argue that *not* assessing potential risk variants is also ethically problematic. Some guidelines even suggest a list of genes where pathogenic findings should always be reported (Kalia et al., 2017). This will require even larger capacity for bioinformatic work-up, and for genetic counselling and information both in advance and in case of any findings. In our study, we chose to omit all genes known to cause monogenic disease other than movement disorders from the bioinformatic analysis, to ensure that we only analysed the phenotype of the study. This approach was chosen to reduce the risk of incidental findings, and was in accordance with the information given in the consent forms. Also, only variants relevant to the patient's medical follow-up were reported back to the patient. In cases where the study identified a possible or certain disease-causing mutation, the patients and family were offered genetic counselling.

7 General discussion

7.1 Clinical and scientific significance of our findings

The overall aim of this study was to characterize molecular causes and clinical phenotypes of previously genetically undiagnosed patients with HSP. Analyses of exome data led to the specific studies of four different genetic forms presented in this thesis. Main findings are summarized in Table 7.1. Important findings included the high frequency and characteristic clinical and molecular features of SPG7 and ATX/HSP-POLR3A, the latter previously an unknown cause of disease in our patient material. We also expanded the range of rare causes of HSP; SPG79 (*UCHL1*) causing a complex early onset HSD phenotype, and the novel AD mode of inheritance of *ERLIN2*, causing pure HSP. Investigation of underlying mechanisms of the two biallelic *UCHL1* variants, surprisingly identified two "opposing" effects on enzyme function, with potential importance to future studies of cognition and even cancer.

Table 7.1. Main findings in the papers this thesis

Paper	Disorder (Gene)	Novel molecular findings	Refined phenotypes
I	SPG7 *(SPG7)*	• Norwegian founder variant in *SPG7*	• Frequent AR-HSP with a core phenotype in our patients
II	SPG79 *(UCHL1)*	• Confirming disease-causing effect of biallelic variants in *UCHL1* • Paradoxical effect on UCHL1 enzyme activity • Possible protective effect on cognition	• New OMIM name: SPG79 • 2nd report of NDGOA, • 1st report of SPG79
III	HSP-ERLIN2 *(ERLIN2)*	• Novel mode of inheritance of *ERLIN2* • Novel heterozygous missense variant	• 1st report of AD pure HSP-ERLIN2
IV	ATX/HSP-POLR3A *(POLR3A)*	• Confirming biallelic *POLR3A* variants as cause of HSD • Novel understanding of *POLR3A* variants and non-coding DNA • Frequent cause of disease in our HSD cohort	• Extensive delineation of characteristic phenotype • 2nd report of ATX/HSP-POLR3A in a HSD cohort

The papers contribute to the steadily increased understanding of genetic causes, phenotypic variability and variable modes of inheritance in HSP. Also, the papers confirm the overlap between classical HSPs and other disorders, which has emerged in particular after the introduction of HTS (Boutry et al., 2019; Novarino et al., 2014; Parodi et al., 2018a; Synofzik & Schule, 2017). Hence, our studies shed light on several aspects that will be of importance, both for better diagnostic approaches and follow-up, and to future research on mechanisms and potential treatment targets.

7.1.1 *SPG7* has a Norwegian founder variant and SPG7 is a frequent HSD with a wide phenotypic spectrum (Paper I)

SPG7 is one of the most common forms of AR HSD, both in cohorts of patients HSP, spastic ataxia and late onset ataxia. In large cohorts, SPG7 has been found to explain around 7% of AR HSP, while SPG11 in general is reported as the most common (21% of AR HSP) (Boutry et al., 2019; Choquet et al., 2016; Mancini et al., 2019; Orsucci et al., 2014). To date, adding to the six families described in Paper I in 2016, additional five unrelated patients in the Norwegian cohort have been diagnosed with SPG7. In comparison, there are seven families with the SPG11 diagnosis (Sjaastad et al., 2018). This makes SPG7 the most frequent AR HSP in our cohort, explaining as much as 26% (11/43) of AR HSPs, and 10% (11/115) when also including AR HCA probands.

A founder variant may exert a founder effect, resulting in a higher prevalence of a given disorder in the specific population. Hence, founder variants may be of importance in genetic analyses, interpretation and genetic counselling. The study was not designed to assess prevalence of SPG7, hence no conclusion can be drawn on whether the Norwegian founder variant (p.His701Pro) present in four of the six families in Paper I, contributes to the relatively large number of SPG7 patients in our cohort. Notably, none of the five newly diagnosed patients carried the founder variant, however, two of these patients were not of Norwegian origin.

In Paper I, the patients shared a characteristic core phenotype comprising spastic ataxia, CPEO, bladder disturbances and subtle cerebellar atrophy. The five more recently diagnosed patients represent the entire span of phenotypes now known in SPG7, from the core phenotype as described in Paper I, to pure HSP, and on to late onset ataxia. The variety in phenotype may in part be explained by the site and type of the specific mutations, however, patients with identical mutations may have different clinical presentation, suggesting that other genetic and/or environmental factors must play a part in the pathogenesis (Roxburgh et al., 2013; Sanchez-Ferrero et al., 2013; van Gassen et al., 2012).

7.1.2 SPG79 is a rare form of complex HSD (Paper II)

In Paper II, we described the second family in the world with early onset neurodegeneration with optic atrophy caused by biallelic *UCHL1* variants, providing thorough clinical characterization of three siblings with this disorder. A genetic finding described only in a single family (Bilguvar et al., 2013) will be encumbered with uncertainly. Our replication thereby confirms a causal role of variants in this gene and the phenotype. Based on our article, the disorder was included in the HSP-classification in OMIM, and was assigned the name SPG79.

Additional evidence was provided more recently, when a third family was described (Das Bhowmik et al., 2018). Das Bhowmik et al. described two siblings of Indian origin, with consanguinity in the family, carrying a homozygous splice-site variant (c.459+2G>C). Clinical features were comparable to the Norwegian family, comprising complex neurological deficits, in addition to optic atrophy, thereby confirming the main phenotype of SPG79. Interestingly, even though the neurological progression seemed more severe, cognitive function appeared normal. However, the patients were young (aged 10 and 7) and the future cognitive profile is difficult to predict. Even so, this might point to that the typical phenotype of SPG79 is in fact normal cognition, and that the patients reported by Bilguvar et al., where there were also consanguinity in the family, might have had cognitive impairment due to other causes than the *UCHL1* variants. Unfortunately, additional functional investigations on effects on the protein function were not performed in the study by Das Bhowmik et al., and the role of these variants in UCHL1 function compared to the investigations in the Norwegian study cannot yet be made.

7.1.3 UCHL1 dysfunction cause complex effects in the CNS (Paper II)

As previously introduced, UCHL1 is described as one of the most abundant proteins in the brain, comprising as much as 1-2% of the total soluble brain protein (Jackson & Thompson, 1981; Wilkinson et al., 1989). UCHL1 is mainly a deubiquitinase, but may also have ligase activity, and is important in maintaining stability of protein degradation in the ubiquitin-proteasome system (UPS) in neurons (Bishop et al., 2016). A proper function of UCHL1 is important in a very broad field of human biology and pathology, including cognition, movement disorders and cancer.

UCHL1 and UPS in monogenic movement disorders

The *UCHL1* gene has been suggested to play a role in Parkinson's disease. A single family with AD Parkinson's disease and a heterozygous rare variant in the *UCHL1* gene (p.Ile93Met), resulting in decreased catalytic activity of UCHL1 has been reported (Leroy et al., 1998). The patients had typical clinical features of Parkinson's disease (resting tremor, bradykinesia and muscle rigidity). However, some carriers of the variant in the original family did not develop disease, and so far, no additional patients with this genetic variant have been described. Hence, this

finding must be interpreted with caution. The role of the UPS in Parkinsonism have since been elaborated. Another polymorphism (p.Ser18Tyr) has in different studies been considered to either decrease or increase the risk of Parkinson's disease, respectively (Lee & Hsu, 2017; Liu et al., 2015). Recently, a heterozygous deletion in a patient with early onset Parkinson's disease was reported. An asymptomatic sibling also carried the variant, and the authors suggest a possible susceptibility effect (Y. Lin et al., 2019). Hence, the exact role of variants in UCHL1 in Parkinson's disease remain elusive.

Even so, dysfunction of parts in the UPS has been robustly documented in several other monogenic disorders. In the AR juvenile Parkinson's disease-2 (PARK2), a parkinsonian disorder with early onset, intact cognitive functions, and marked dystonia, the affected protein PARK2 (also known as Parkin) is a ubiquitin ligase, showing that the UPS is indeed involved in parkinsonian syndromes (Kitada et al., 1998). Also, the UPS is affected in several ways in polyglutamine SCAs, where ubiquitin-positive protein aggregates are found in neuropathological studies (Silva et al., 2018). In addition, other genes involved in ubiquitination may cause AR HCA with hypogonadism (Gordon Holmes Syndrome); *STUB1* (SCAR16) and *RNF216* (Heimdal et al., 2014).

Paradoxical enzymatic effects - protecting cognitive function?

Of particular interest in our paper was the well preserved cognition in the twins who had severe motoric disabilities, and the paradoxical finding that one of the variants (c.533G>A) caused *increased* hydrolytic activity of UCHL1, while the other (c.647C>A) resulted in an insoluble protein. Could we have stumbled upon a combination of *UCHL1* variants that may somehow protect cognitive function?

As mentioned, the main function of UCHL1 is deubiquitination, however the complete range of functions remains enigmatic and a wide array of alternative functions has been proposed (Bishop et al., 2016). UCHL1 is indeed involved in cognition, and overexpression of UCHL1 has been shown to delay progression of Alzheimer's disease *in vivo*, probably through regulating the degradation of amyloid precursor protein (APP) in a long-term fashion (Tramutola et al., 2016; Zhang et al., 2014). How the variants in our patients may affect only parts of UCHL1 function, preserving cognitive function, while at the same time causing severe focal neurological deficits, remains to be explored.

Neurodegeneration versus cancer

Both reduced and increased expression of UCHL1 has been identified in various forms of cancer, and *UCHL1* is a potent oncogene in mice (Fang & Shen, 2017; Finnerty et al., 2019; Gu et al., 2018; Moore et al., 2018; Nakao et al., 2018; Sanchez-Diaz et al., 2017). Hence, in future studies of the potential neuroprotective

effect of UCHL1 overexpression, it will be important to bear in mind a possible concurrent increased risk of cancer.

Monogenic disorders as model systems
The involvement of the same protein in several forms of neurodegeneration, cancer and normal cognition is exciting and presents a hot topic for future studies. Hence, the results in Paper II highlight how studies of monogenic disorders, no matter how rare, may serve as model systems and shed light onto a much broader field of human physiology and pathology, and even be of use in the search of potential treatment targets for more common disorders.

7.1.4 Novel mode of inheritance of HSP-ERLIN2 (Paper III)

In Paper III we identified a novel heterozygous missense variant in the *ERLIN2* gene in two large families with AD pure HSP. Haplotype analyses did not show a founder effect, encouraging that this was a recurrent variant. The finding of the identical variant segregating with disease in these two large and unrelated families with identical phenotypes with a LOD score of as much as 4.34, strongly suggest that the variant is the cause of the disease. Hence, we establish a novel mode of inheritance of the *ERLIN2* gene, causing AD-HSP, opposed to the previously acknowledged complex AR form SPG18. This has large implications in interpretation of diagnostic genetic analyses and genetic counselling, as heterozygous *ERLIN2* variants now need to be considered as possible pathogenic. Very recently, our findings were replicated when another heterozygous missense variant (c.194C>T) in *ERLIN2* was identified as the disease cause of AD pure HSP in a large family (personal communication (Stevanin et al., 2019)). Our findings are in line with other recent studies, as both AD and AR mode of inheritance is now known for several forms of HSP, including SPG7, SPG3 (*ATL1*, one AR family), SPG9 (*ALDH18A1*), SPG30 (*KIF1A*) and SPG72 (*REEP2*) (Boutry et al., 2019; Sanchez-Ferrero et al., 2013).

Mechanisms of dominant variants
The patients in the two families in our study all had pure HSP, while the disease course was more severe in the previously described patients with AR SPG18. This suggests that the heterozygous variants exhibit less deleterious effect on protein function than biallelic variants. While recessive disorders most often result from loss of function (LOF) variants, the mechanism of variants causing an autosomal dominant disorder is more frequently through dominant negative mechanisms, haploinsufficiency (partial LOF) or a toxic gain of function. The mechanism in patients with AR SPG18 carrying biallelic *ERLIN2* variants is assumed to be complete loss of function (Wakil et al., 2013). Heterozygous carriers of these previously reported variants did not develop neurological signs, and a high tolerance for LOF variants were predicted in ExAC (exac.broadinstitute.org/). In

Paper III, this led us to assume a site specific dominant negative effect, probably affecting the ERLIN1/ERLIN2 heterodimer complex. Our assumptions were recently supported by functional investigations by Stevanin et al., where they showed that the c.194C>T variant had a dominant negative effect on the IP3 (inositol 1,4,5-trisphosphate) receptor degradation in vitro, resulting in reduced degradation capacity of IP3 receptors (personal communication, (Stevanin et al., 2019)).

7.1.5 ATX/HSP-POLR3A – a novel, frequent and characteristic HSD, illustrating the importance of non-coding DNA

Surprisingly frequent disorder

ATX/HSP-POLR3A has, after the study resulting in Paper IV, risen as one of the top three genetic causes of AR HSD in our patient database. We found assumed causal *POLR3A* variants in as much 3.1% (10/322) of all genetically undiagnosed sporadic and recessive probands with HSP/HCA. The only other HSD with a higher frequency in our database is Friedreich ataxia (26 families), followed by SPG7 (11 families). For Friedreich ataxia however, genetic testing has been available for decades, and as the result of a nationwide epidemiological study, most adult patients with molecularly confirmed Friedreich ataxia in Norway have been included into our database (Wedding et al., 2015). Given the novelty of our findings, there are likely to be more individuals in Norway with ATX/HSP-POLR3A who have not yet been genetically investigated. The frequency was similar to the findings by Minnerop et al., and underlines the importance of including this gene in the work-up of patients with possible AR-HSD.

Surprisingly frequent non-coding variant

To date, most of the known monogenic disorders are caused by variants located in exons. Intronic regions remain to be completely understood, as there are challenges in both finding and interpreting intronic variants. The identified intronic c.1909+22G>A variant in *POLR3A* affects splicing and the aberrant mRNA transcript is subject to nonsense-mediated decay (NMD). In total, as much as 9 out of 10 of families in Paper IV, and 19 out of 23 of families with *POLR3A* variants in the paper by Minnerop et al., carried this recurrent variant. The specific variant seems to drive the phenotype towards the HSD spectrum rather that into the more severe classical phenotype with leukodystrophy. However, the variant has also been reported in cohorts of patients classified with leukodystrophies. La Piana et al. reported one patient (out of six with biallelic *POLR3A* variants), who was compound heterozygous for this variant and had a phenotype of ataxia and dental abnormalities, combined with atypical MRI features without diffuse hypomyelination (La Piana et al., 2016), thereby very similar the patients identified in the HSD cohorts, further illustrating the phenotypic range. The high frequency of

this specific intronic variant is important knowledge for genetic analyses of patients classified with either a HSD or leukodystrophy.

Recognizable phenotype with treatable additional findings
The core phenotype comprised onset of neurological signs before 20 years of age, cerebellar ataxia, alcohol-responsive tremor, pyramidal signs including extensor plantar responses, combined with absent/reduced lower limb deep tendon reflexes and loss of proprioception. Interestingly, in some of our patients the phenotype had changed over the years, mainly from a HSP phenotype with some cerebellar features, to pronounced tremor, increased cerebellar findings and loss of deep tendon reflexes.

Neurological signs were accompanied by different dental problems in most patients, and hormonal dysfunction is some patients. Also, all patients had hyperintensities in the superior cerebellar peduncles in T2 and FLAIR-sequences in MRI. This is in line with, and elaborate the findings by Minnerop et al. (Minnerop et al., 2017). Thus, these robust imaging markers need to be included in the neuroradiological evaluation in patients with complex movement disorders.

Alcohol-responsive tremor in POLR3A-related disorder was first described in our paper, and was also confirmed in the German patient cohort (Minnerop et al., 2019). Such a pronounced tremor-reducing effect of alcohol is uncommon, apart from in essential tremor and the rare disorder *SGCE* Myoclonus-Dystonia (DYT11) (Raymond et al., 1993). Given the overlapping features, ATX/HSP-POLR3A should also be considered in patients in the "essential tremor plus" patient group.

Further proof of that *POLR3A* is a frequent cause of a spastic ataxic phenotype, in particular driven by the specific intronic variant, was recently provided in a study from Spain. They identified biallelic variants in *POLR3A* in ten patients, from six families, where all patients carried the c.1909+22G>A variant. Also, the patients presented with identical neurological and MRI findings as the Norwegian and German patients (Infante et al., 2019).

In conclusion, these three recent studies on ATX/HSP-POLR3A in HSD cohorts prove that this is a frequent HSD with specific clinical and imaging findings providing clues for the diagnosis. Also, the relevance of obtaining a molecular diagnosis, is underlined, as treatable additional findings may be retrospectively investigated. In addition, the intronic splice site variant involved in most of the patients, presents a promising area of further research towards treatment specifically targeting this variant.

7.2 A guide to the diagnostic approach

7.2.1 Diagnostic clues based on the results of Papers I-IV

Two of the articles describe relatively frequent forms of HSD; SPG7 and ATX/HSP-POLR3A, the latter previously unknown in the Norwegian HSD cohort before this study. While SPG7 is now well characterized in many cohort, our publication still represents the most extensive and thorough clinical description of ATX/HSP-POLR3A, supported by (Infante et al., 2019; Minnerop et al., 2017). Both SPG7 and ATX/HSP-POLR3A were shown to result in a core set of symptoms and findings, including specific MRI findings and extra-neurological features in ATX/HSP-POLR3A. The findings are so characteristic that targeted single gene sequencing approach of *SPG7* or *POLR3A* could be suggested as the primary genetic investigation. We also identified two ultra-rare causes of HSP; the complex HSP SPG79, and the pure HSP HSP-ERLIN2-AD. Because these forms are so rare, and also that the clinical features are not very specific to the respective genotypes, genetic investigations of these patients require HTS technology and broad gene panels.

Additional findings providing clues for the diagnosis – and vice versa

Another important point illustrated in the papers, is the necessity and value of thorough clinical assessment, including examining for symptoms and signs outside your main speciality. In addition to providing clues for the specific diagnosis, related and possibly treatable additional findings may also be revealed. In Paper IV, several patients had specific accompanying features both clinically (dental problems and hypogonadism) and in MRI (high signal intensities in the superior cerebellar peduncles), which are now established as strong clues for the ATX/HSP-POLR3A diagnosis. However, in most cases these findings had not been previously recognized, and obtaining the molecular diagnosis thus aided to the subsequent identification of these additional features. This subsequently led to proper medical treatment of hypogonadism in one patient (Paper IV).

7.2.2 How to find a genetic diagnosis in the clinical setting?

The diagnosis of HSP is based on the clinical findings, and confirmed genetically when possible. The cornerstone of diagnostics is a thorough phenotypic description of the symptoms, the family history and the clinical signs at examination. Also, as illustrated by our papers, including examination for extra-neurological symptoms and signs is useful and necessary, especially in complex cases. The clinical phenotype, together with supplementary investigations, may indicate specific genetic subgroups. HSP is rare, and differential diagnostics is paramount, in particular in sporadic cases. To differentiate between HDS and disorders such as multiple system atrophy or non-monogenic primary lateral sclerosis can be difficult. Of particular importance is not to overlook other treatable conditions, such as

structural causes, metabolic disorders, neuroimmunological disorders and paraneoplastic syndromes.

A suggestion for the work-flow in obtaining a genetic diagnosis of patients with HSP is presented in Figure 7.1.

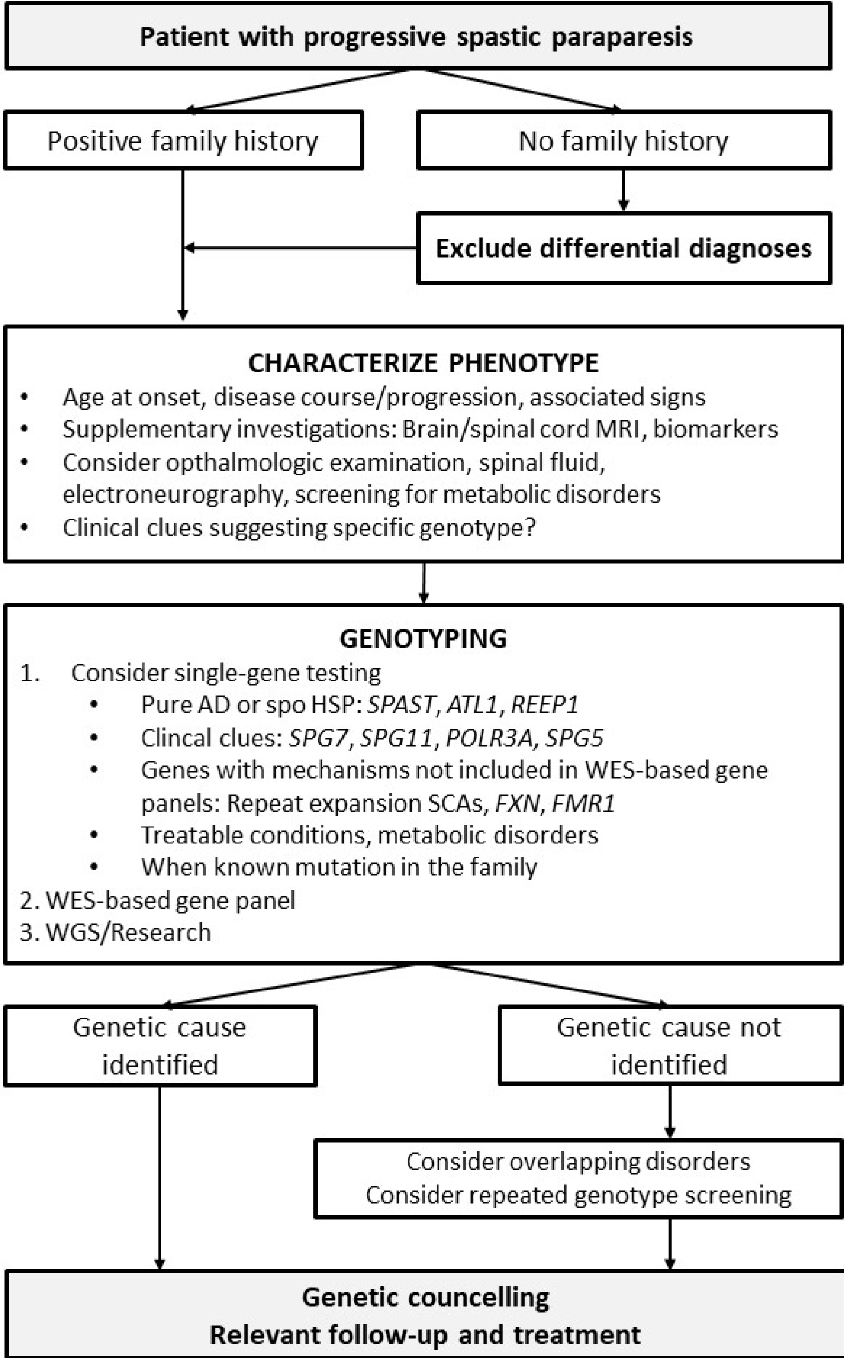

Figure 7.1. A suggested work-flow in the diagnostic process of patients with progressive spastic paraparesis. Made by the author and Jeanette Koht.

The choice of genetic test will depend on what is locally available and cost-efficient guided by the clinical presentation and additional findings. Before ordering a genetic test, in particular a broad gene panel, other causes must be ruled out. Genetic counselling, and the potential benefit for the patient, should be considered prior to genetic testing.

In general, genetic work-up will need to be broad due to the genetic heterogeneity and pleiotropy, however there may be clinical clues suggestive of specific genetic causes. In AD or sporadic pure HSP, sequencing and MLPA of *SPAST* (SPG4) may proceed gene panel testing, as this is by far the most common hereditary cause, and because up to 20% of *SPAST* variants are small deletions, which may not be identified in gene panels based on exome sequencing (Parodi et al., 1993, updated 2019 June 19). Single gene sequencing can proceed HTS based gene panels in patients with well-defined clinical syndromes. In these cases, the testing needs to be directed by clinical clues combined with relevant supplementary findings in MRI, ophthalmological examinations, or biochemical analyses (examples in Table 2.2). Also, a gene panel based on WES does *not* efficiently detect copy number variation, nor repeated sequences such as triplet expansions, hence other methods are needed for repeat expansion disorders such as polyglutamine SCAs (SCA1, 2, 3, 6, 7, DRPLA), Friedreich ataxia (*FXN*) and Fragile X-tremor-ataxia syndrome (*FMR1*). Lastly, when a genetic diagnosis is not reached, it is advisable with regular follow-up to consider re-evaluation of the diagnosis and also to perform new or repeated genetic analyses, as knowledge of new variants, genes and biomarkers are continuously improved.

7.2.3 What is the use of a genetic diagnosis for a patient?

Often, a genetic HSD diagnosis will not change the follow-up considerably or reveal new treatment options for the individual patient. So why continue to struggle our way through symptoms, findings and exhaustive additional examinations and experiments to identify new causes of HSDs and to give a specific diagnosis to each patient?

Firstly, obtaining the *clinical* diagnosis is important, to delineate the patient's individual challenges and disabilities, and thereby establish a personalized treatment plan. However, identifying the genetic cause will confirm the clinical diagnosis, thereby ending an, often very long, diagnostic odyssey. This is of importance to the patient both providing a definite cause, a better assessment of prognosis and more exact genetic counselling for both the patient and family.

The genetic diagnosis might lead to identification of related features not previously recognized, such as dental abnormalities and gonadal dysfunction in the case of ATX/HSP-POLR3A (Paper IV). Another example of the importance of gaining a

diagnosis to assess risk of extra-neurological disease, is ataxia-telangiectasia, where both immunodeficiency and increased cancer risk is part of the syndrome (Gatti & Perlman, 1993, updated 2016 Oct 27). Also, it is of course of major importance to identify monogenic disorders where targeted treatment exist, such as in many metabolic disorders (Ramirez-Zamora et al., 2015) and ataxia with vitamin E deficiency or coenzyme Q10 deficiency. In such cases, treatment should be initiated as early as possible to hopefully halt neurological deterioration, underlining the importance of early diagnosis.

Encouragingly, treatment targeting molecular mechanisms in neurodegenerative disorders are emerging (Bennett et al., 2019). While no such treatment is available for any HSD today, treatment with antisense oligonucleotides (ASO) is now approved and in clinical use in the monogenic neurological disorders spinal muscular atrophy (Darras et al., 2019) and Duchenne muscular dystrophy (Lim et al., 2017). Should clinical trials on a HSD be initiated, the patients need to be ready for inclusion, which will require a molecular diagnosis and a well characterized phenotype. For instance, the intronic *POLR3A* variant (c.1909+22G>A) represents a possible target for ASO modification, which warrants future studies.

7.3 Still many without genetic diagnosis

This PhD study, parallel studies and improved diagnostics have increased the proportion of probands with molecular diagnosis in our HSD cohort considerably, from 26% in 2012, to 41% today. At the same time, this shows that even after all our efforts, nearly three out of five probands are still without genetic diagnosis. However discouraging, the proportion is comparable to other patient materials, different selection criteria taken into account (Morais et al., 2017; Novarino et al., 2014; Schule et al., 2016). The highest diagnostic yield in our material was in patients with assumed AR mode of inheritance (80% solved), and the rate was higher in patients classified with HSP (50%) than HCA (33%). The high proportion of solved AR cases may reflect that these patients had more characteristic phenotypes, suggestive of specific diagnoses and a higher probability of genetic findings with our available methods. The HCA group most likely comprise more heterogeneous disease causes, perhaps also reflecting the vast amount of differential aetiologies that may be difficult to separate from HCA, such as autoimmune causes and multiple system atrophy.

There are probably several reasons why this diagnostic gap persists. WES might not have covered the relevant DNA sequence, and variants may be located in non-coding DNA regions or consist of repeat expansions or CNVs. Some of the sporadic cases may not be monogenic after all. Variants in a potential novel HSP-gene in a

single family may be overlooked, as extensive additional functional studies, beyond the capacity of small research groups, would be required to provide evidence that the variant in a gene not previously related to the phenotype is the cause of the disease. There may also be other mechanisms involved, such as di- or oligogenic inheritance or involvement of other genetic modifying factors (Bis-Brewer & Zuchner, 2018). To increase the proportion of patients with a defined diagnosis, a broad network-based approach, both in clinical and molecular methods and in collaborative networks will be necessary.

In the studies in this thesis, in particular the role of non-coding DNA has been underlined (Paper IV).

7.3.1 Unexplored monogenic causes – the role of non-coding DNA variants

Non-coding DNA are not yet as extensively investigated as the exome, and challenges remain on how to interpret variation in these DNA regions, but the importance of scrutinizing non-coding regions in the search of molecular diagnosis is increasingly appreciated. Notably, as have been described, a specific intronic variant is frequently involved in ATX/HSP-POLR3A. There are several other examples of disease-causing non-coding variants in neurological disorders. Friedreich ataxia is caused by a GAA repeat expansion in intron 1 of the *FXN* gene, and non-coding repeat expansions are implicated in several SCAs, including SCA8, 10, 12 , 31, 36 and 37 (Sullivan et al., 2019). Intronic variants have also been reported in disorders such as in neurofibromatosis, Duchenne muscular dystrophy, Fabry disease and ataxia-telangiectasia (Vaz-Drago et al., 2017).

Most recent studies have used WES, which is efficient to identify sequence variation within the protein coding sequence. However, repeat expansions and CNVs are not readily picked up, hence whole genome sequencing (WGS) hold a great potential for future studies. A recent example is the identification of the genetic cause of the recessive disorder CANVAS (cerebellar ataxia, neuropathy, vestibular areflexia syndrome) (Cortese et al., 2019), where an intronic AAGGG expansion in the *RFC1* gene was identified by non-parametric linkage analyses and WGS. Once the genetic cause was discovered, it turned out to be a frequent cause in sporadic cases with late-onset ataxia, explaining as much as 22% of sporadic late-onset ataxia cases (n=150), and as much as 92% only considering patients with the typical clinical features of CANVAS. In line with the ATX/HSP-POLR3A story, this highlights the value of well-defined clinical features. So far, analyses for this expansion has not been possible in our patient material, but it is very likely that this will solve some of our “missing diagnoses”.

7.4 Blurred lines

The overlap of clinical features and genetic causes between HSP and HCAs, and even towards other monogenic disorders, has become increasingly evident over the timespan of this PhD project. This opens for exploring common pathways, mechanisms and possibly treatment targets across traditional disease borders. At the same time, this overlap challenges the existing nomenclature systems for HSDs.

7.4.1 Genetic heterogeneity and pleiotropy

The HSDs are disorders showing both major genetic *heterogeneity*; as a similar phenotype can be caused by mutations in several genes, and genetic *pleiotropy*; as variants in *one* gene can cause multiple phenotypes. Studies of modifiers and variable expressivity in the most common HSP and SCAs are emerging, challenging our dichotomous classification of disorders into either monogenic of complex disorders.

Genotype-phenotype correlations

The type and mechanism of the specific genetic variant may affect the phenotype. For instance, a missense variant in *SPG7*, c.1529C>T (p.Ala510Val), typically result in late-onset pure ataxia, while loss of function variants more often results in a phenotype with predominant spastic paraparesis (Mancini et al., 2019; Roxburgh et al., 2013). Also, missense variants in *SPAST* have been associated with an earlier age of onset of SPG4 than truncating variants (Parodi et al., 2018b), and in the polyglutamine SCAs, the length of expansion is shown to modulate the age at onset and severity of symptoms (Silva et al., 2018).

Modifying factors

Also, *other* alleles may modify the expressivity. In SPG4, having a specific second variant *in trans* with a pathogenic variant in *SPAST*, tend to reduce the age at onset (Parodi et al., 2018b). In the polyglutamine ataxias, the number of repeat expansions in the *other* SCA genes affected age of onset in several SCAs (Tezenas du Montcel et al., 2014). In addition, variants in DNA repair genes were found to modulate onset in both SCAs and Huntington's disease (Bettencourt et al., 2016). However, much is still not understood, such as the role of environmental factors, and little is known of modifiers of the rarer or newly described HSDs, presenting possible aims for future studies.

Overlapping phenotypes and genotypes

Only a few HSPs and HCAs almost exclusively cause pure phenotypes, either pure HSP (the most common being SPG4) or pure HCA (the most common being SCA5 and SCA6), while most forms are complex and may present with a variable combination of symptoms in the spastic-ataxia axis. SPG7 is one example of a "traditional" HSP, shown to have a wide phenotypic range, from pure HSP to near pure ataxia. Other forms where broad, "hypothesis-free" WES-studies have recently revealed wide phenotypic spectra, some examples include SCAR8/ARCA1

(*SYNE1*), SPG39 (*PNPLA6*), SCAR16 (*STUB1*), SPG46 (*GBA2*) and SPAX2 (*KIF1C*) (Synofzik & Schule, 2017). Also, in the case of HSP-ERLIN2, biallelic variants cause a complex phenotype, while certain heterozygous variants may cause pure HSP.

The recent years it has also become increasingly clear that the pleiotropy goes beyond the HSDs, and that these traditionally separately classified disorders share many common mechanisms and pathways. In this thesis, this is in particular proven for *POLR3A*, as biallelic *POLR3A* variants may cause phenotypes classified as either HSP, HCA, hypomyelinating leukodystrophy (HLD7) or even, a disease of premature ageing (Wiedemann-Rautenstrauch syndrome) (Paolacci et al., 2018). Figure 7.2 shows an overview of disorders with overlapping phenotypes and genotypes to the traditional HSDs.

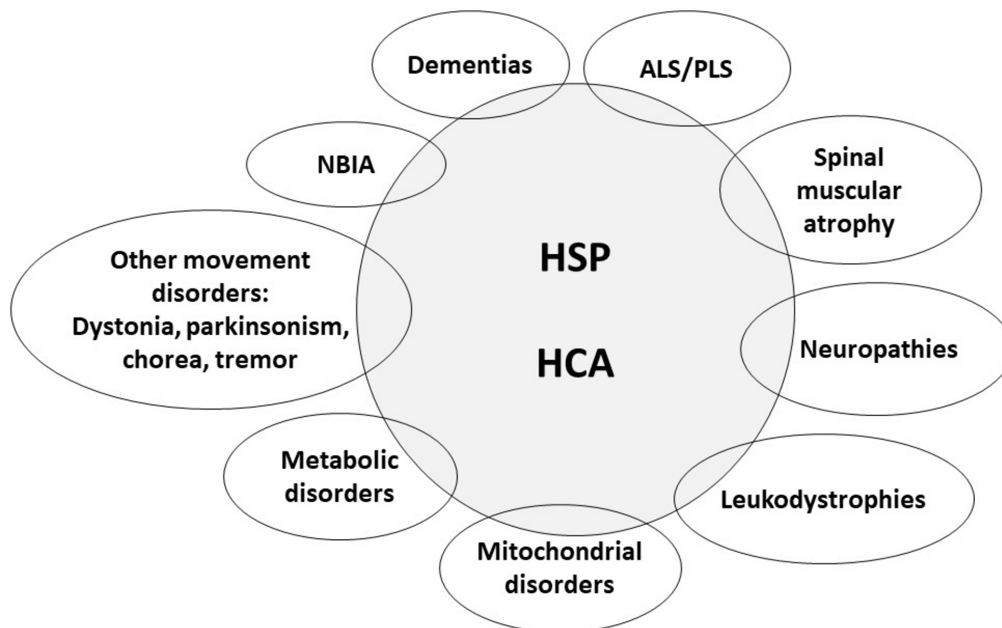

Figure 7.2. Disease entities melting together. The figure shows examples of hereditary disorders with overlapping clinical features and genetic causes to HSP and HCA. ALS = amyotrophic lateral sclerosis, NBIA = Neurodegeneration with brain iron accumulation, PLS = primary lateral sclerosis.

7.4.2 Nomenclature – what's in a name?

With all this overlap involving genotypes, phenotypes, mechanisms and inheritance patterns, how can we keep the overview and obtain an understandable nomenclature?

While the nomenclature of HSP traditionally has been kept relatively simple, by sequential numbering of SPGs, not all SPGs have been confirmed, some are duplicated and some are still only defined by a locus, and not a gene. For HCA, in particular the naming of the recessive ataxias has been creative. We have descriptive names based on clinical findings (e.g. ataxia with oculomotor apraxia/AOA, and ataxia with vitamin E deficiency/AVED), names based on surnames of people first describing the disease (e.g. Friedreich ataxia) or on geographical regions where the disease is prevalent or first identified (e.g. autosomal recessive spastic ataxia of Charlevoix-Saguenay/ARSACS). Efforts of simplification has ended up with even more names for a single disorder, such as "ARCA", "SCAR " and/or "SPAX" combined with a number. Clinicians often prefer the traditional and historical name, while the geneticist prefers the gene name, complicating interdisciplinary communication.

With the aim to unify the nomenclature of hereditary movement disorders, the consensus group "The International Parkinson and Movement Disorder Society (MDS) Task Force" proposed a more comprehensive nomenclature system (Marras et al., 2016; Rossi et al., 2018). This system combine a prefix presenting an abbreviation of the typical phenotype(s), combined with the gene name approved by HGMD (The Human Gene Mutation Database), such as HSP/ATX-SPG7, HSP-ERLIN2, HSP/ATX-UCHL1 and ATX/HSP-POLR3A. However, in literature, different nomenclature system are still in use, and a final consensus is not achieved.

All studies in this PhD thesis has challenges with regards to nomenclature (see Table 7.2). SPG7 was first classified as HSP, but as knowledge of the phenotypic spectrum evolved, SPG7 could also have been classified as a HCA. SPG79 was given this OMIM name as a consequence of the publication of Paper II, replacing the descriptive name "Childhood-onset neurodegeneration with optic atrophy" (NDGOA) (Bilguvar et al., 2013). Considering the overlap and the emerging new classification systems, one could argue whether it is advisable to continue adding new SPGs. Perhaps it is now time to stop and rather start using the MDS Task force recommendations or similar classification, at least for newly identified genotypes.

Recently, Stevanin et al. replicated the results in Paper III, confirming AD mode of inheritance of *ERLIN2* variants in one family (personal communication (Stevanin et al., 2019)). Interestingly, the very same family was the origin of the SPG37 locus (Hanein et al., 2007), presenting us with another dilemma of nomenclature: Do these patients now have SPG18 or SPG37? Here, a name based on the MDS task force recommendations, combined with the mode of inheritance will be useful to avoid confusion, therefore the name HSP-ERLIN2-AD was used in this thesis text.

Also, biallelic variants in *POLR3A* has emerged as a frequent cause in patients classified with HSP or HCA (Paper IV, Minnerop et al., 2017). The current OMIM nomenclature of this disorder; "Hypomyelinating leukodystrophy-7 with or without oligodontia and/or hypogonadotropic hypogonadism" (HLD7), does not comprehensively include the phenotype of these HSP/HCA-patients, and none of these patients had signs of leukodystrophy in initial MRIs. Therefore, the name suggested by the MDS task force, ATX/HSP-POLR3A is better suited in this patient group. The fact that this gene has been so strongly related to leukodystrophy, was most likely part of the reason to why this frequent cause had not been uncovered in any HSD population at an earlier stage. As this disorder has been included in the HSD classifications, it might ease the diagnostic process for future patients.

Table 7.2. Disorder names used in this thesis and alternative names

Gene	Chosen name in the thesis	Other names in use (*and allelic disorders*)
SPG7	SPG7	HSP/ATX-SPG7
UCHL1	SPG79	HSP/ATX-UCHL1, Childhood-onset neurodegeneration with optic atrophy (NDGOA)
ERLIN2	HSP-ERLIN2-AD	HSP-ERLIN2, SPG18, SPG37
POLR3A	ATX/HSP-POLR3A	POLR3-related disorder, POLR3A-leukodystrophy, 4H syndrome; Hypomyelinating leukodystrophy-7 with or without oligodontia and/or hypogonadotropic hypogonadism (HLD-7) (*Wiedemann-Rautenstrauch syndrome*)

A shortcoming of the MDS-classification is the use of fixed prefixes. In my opinion, the name of a disorder should both describe (1) the main phenotype(s) of the specific patient, (2) the gene, and (3) also the inheritance form, when more modes of inheritance are possible. For the patients with "AD SPG18" or SPG37 this would be specified to; HSP-ERLIN2-AD. For the patients in Paper IV the prefix would be different for the different patients, with the relevant combination of "HSP", "ATX" (ataxia) and "TRE" (tremor), followed by POLR3A. This will provide a more flexible system adjustable to the continuous new discoveries in the field as knowledge of new phenotypes and inheritance forms emerge. The abbreviations of the phenotype need to be self-explanatory and flexible enough to cover all forms of movement disorders.

However; will a patient (or any clinician not specialized in genetics or movement disorders) understand the meaning of a diagnosis called ATX/HSP-POLR3A? Or

for instance HSP/ATX-BSCL2, which is the same as SPG17 and Silver syndrome? A patient with prominent ataxia diagnosed with SPG7 might be confused by a disease name related to “another” disorder. Also, family members with the same molecular cause but different expressivity may end up with different names for their disorder. Even though our group suggested the disease name SPG79, the previous OMIM name “childhood-onset neurodegeneration with optic atrophy” sound more understandable to a general neurologist and a patient than SPG79 or HSP/ATX-UCHL1. Taken this into account, descriptive disease names will still be useful. Also, many disease names are thoroughly established and relatively well known, and perhaps a more pragmatic approach to nomenclature is also needed, keeping the “old” disease names of the most frequent HSPs, SCAs and recessive ataxias, while all newly discovered or very rare genotypes, are assigned novel, standardized names.

7.5 Future perspectives

7.5.1 Continued clinical, molecular and functional characterization

Even with the remarkably increased understanding of genetic mechanisms in HSDs over the last decade, still near 60% of the HSD patients in our Norwegian cohort remain without a molecular diagnosis. These patients represent a material for further molecular characterization. Better characterized phenotypes, including studies on non-motoric symptoms, will also prove useful to future diagnostic approaches, assessment of prognosis and treatment.

Imaging

Advanced imaging is a promising new method to evaluate subtle brain changes, brain networks and even brain ageing, and present an interesting field to elaborate knowledge of HSDs (da Graca et al., 2018; Hogestol et al., 2019). A small study of one family with SPG7 has been performed, finding diffusion tensor imaging (DTI) disturbances of white matter integrity in the frontal lobe, corticospinal tract and brainstem (Warnecke et al., 2010). Otherwise, this field remain to be explored in the genetic forms described in this thesis. From seven of the patients in Paper IV we have also obtained additional advanced MRI sequences. These await analyses in a future project, possibly revealing a deeper understanding of the involved structural pathways in the brain.

Novel genes

One approach to identify novel molecular causes in our patient material would be studies directed at specific newly described subtypes, such as analyses of the repeat-expansion in the *RFC1* gene causing CANVAS (Cortese et al., 2019) in patients with compatible phenotypes. To identify novel genes, WGS will now be the main

genetic method of choice. However, in our patient material, the existing WES data negative for analyses of known disease-genes, remain to be explored for possible novel genetic findings.

Functional studies in disease models

Several possibilities remain for functional studies of the pathomechanistic consequences of the variants identified in these PhD studies, and for other HSDs. Network-based analyses, integrating multiple -omic technologies (transcriptomics, lipidomics, proteomics, metabolomics) are interesting novel approaches (Bis-Brewer et al., 2019; Santiago et al., 2017). For specific variants, induced pluripotent stem cell (iPSC) models have been established for some forms of HSP and HCA (Bird et al., 2014; Hauser et al., 2016; Ishida et al., 2016; Nakazeki et al., 2019; Xia et al., 2013). Newer model systems are development of organoids, an *in vitro* model of a culture of cells similar to a human organ, even neuronal structures (Lancaster et al., 2013; Perez-Branguli et al., 2019). Also, several animal models have proven efficient in studies of neurodegenerative disorders. For studies of HSP and HCA both fruit fly (Monnier et al., 2018), c. elegans (Ma et al., 2018), zebrafish (Babin et al., 2014; Novarino et al., 2014) and mouse models (Genc et al., 2019; Scoles et al., 2017; Xu et al., 2007) have been developed. For the *UCHL1* variants identified in Paper II, the group of our collaborator prof. Bjørås, is currently establishing iPSC models and differentiating neuronal cells, to study the pathogenic effect during stages of neurodevelopment.

Collaboration

All subgroups of HSDs are rare, most even ultra-rare. To gain power in future studies, including clinical trials and replication of novel genetic findings, it will be necessary to join forces internationally to recruit as many patients as possible. Enabling such collaboration are freely accessible web sites, such as GeneMatcher, and international research networks, such as the SPATAX network, and the newly established platforms TreatHSP, ARCA Global and SCA global.

7.5.2 Evidence-based and novel treatments

The ultimate goal for future studies would be to find a cure for neurodegeneration. However, in our search of novel treatments, we must not forget the importance of optimizing the treatment with measures that are already available. Merely obtaining a diagnosis will improve patient care, providing better assessment of prognosis, multidisciplinary follow-up and even treatment. Specialized treatment exist for several metabolic disorders, and for concurrent signs, such as hypogonadism in ATX/HSP-POLR3A. Currently, even the well-established treatments of HSP lack robust evidence (Bellofatto et al., 2019), hence systematic studies of symptomatic treatment, in particular physiotherapy and treatment of bladder disturbances, are warranted.

One important next step will be development of treatment targeting specific genes, molecular mechanisms or cellular pathways. In similar disorders, such as spinal muscular atrophy, treatment targeting genetic mechanisms is already in clinical use (Bennett et al., 2019). Results from both Paper II and Paper IV could pave way towards targeted treatment studies. The intronic variant frequent in ATX/HSP-POLR3A (Paper IV, Minnerop et al., 2017) activates a cryptic splice site causing an aberrant transcript which is degraded by NMD. This mechanism could be a potential site for targeted treatment, for instance with antisense oligonucleotides (ASO). The ASOs could be designed to target and block the aberrant splicing and thereby possibly result in an alleviated phenotype. In Paper II, we explored the role of the *UCHL1* variants by enzyme analyses and mass spectrometry of fibroblasts. The results implied a possible role in cognition. Also, the specific combination of variants introduced a possibility to increase enzyme activity without increasing the total level of UCHL1. Given the potential benefit on cognitive function of increased enzyme activity, combined with the increased risk of cancer related to high levels of UCHL1, this might present an intriguing mechanism for treatment, which also warrants further studies. Hence, our studies provide examples of possible mechanistic treatment targets, again underlining the importance of studying the molecular mechanisms of HSDs.

7.6 Concluding remarks

HSP is a group of rare neurodegenerative disorders, severely affecting the quality of life of patients and their families, with a vast amount of genetic and clinical subgroups and wide overlap with other hereditary disorders. Still, there are no available curative or disease-modifying treatment.

This PhD project aimed to elucidate the clinical and molecular characteristics of genetic forms of HSP in a Norwegian patient cohort, and has added a few pieces to the large puzzle on how to understand neurodegeneration. We have increased the knowledge on the genetic causes of HSDs in Norway, delineated the clinical features of subgroups of these disorders and underlined the complexity and overlap within HSP, HCA and other disorders. The studies shed light on several aspects that are important for future diagnostic approaches, and also for further research on disease mechanisms and possible treatment targets.

Our understanding of the aetiology of HSP is still not complete, and evidence-based treatment is lacking. Future studies will need to combine international and multidisciplinary forces to search for further insights into clinical characteristics, molecular causes, pathogenic mechanisms and pathways. This is likely to pave way for better patient care and new therapeutic strategies in the future.

The continuously increasing knowledge on how genes, pathways and structures interact within our nervous system, and even beyond, represent a paradigm shift in the way of thinking of HSDs. We need to move out of the separated and well-organized disease boxes, over to network-based and comprehensive approaches, combining individual symptoms, signs, supplementary investigations, and genetic findings, to provide personalized treatment for each patient – no matter the name of their disorder.

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9 Papers I-IV

The four publications are attached, including the corrigendum to Paper II, and the supporting information to Paper III and Paper IV.

IV

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BRAIN
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LETTER TO THE EDITOR

Biallelic *POLR3A* variants confirmed as a frequent cause of hereditary ataxia and spastic paraparesis

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Sir,

We read with great interest the original article 'Hypomorphic mutations in *POLR3A* are a frequent cause of sporadic and recessive spastic ataxia' (Minnerop *et al.*, 2017). In a large cohort of sporadic and recessive ataxia and spastic paraparesis, Minnerop *et al.* identified biallelic *POLR3A* variants in 3.1% (19/618) of the patients. Of these, 18 carried the intronic c.1909+22G>A variant, which was shown to activate a cryptic splice site, leading to a premature stop codon resulting in nonsense-mediated decay (NMD). On MRI, high intensities in the superior cerebellar peduncles were the main finding. The article prompted an interesting discussion regarding several aspects of this specific phenotype and genotype (Gauquelin *et al.*, 2018; Minnerop *et al.*, 2018), illustrating that confirmatory studies are warranted.

In this letter, we report the results of an independent study of patients with hereditary ataxia and spastic paraparesis (HSP) from Norway, confirming and elaborating the findings reported by Minnerop *et al.* In exome data from 95 Norwegian patients with a clinical diagnosis of ataxia or HSP without molecular diagnosis, we identified 10 probands and 13 patients with biallelic and presumed disease-causing *POLR3A* variants (Fig. 1A). The intronic c.1909+22G>A variant was identified in 9 of 10 families. For details on material and methods, see the online Supplementary material.

The patients presented with a remarkably recognizable phenotype combining neurological, dental and MRI findings, strikingly similar to the patients in the study by Minnerop *et al.* Detailed clinical characteristics of the patients are given in Table 1 and elaborated in Supplementary Table 1A. Mean age at onset of the first neurological symptoms was 13.7 years. In 12 of 13 patients symptoms began before the age of 21. The main neurological phenotype comprised ataxia (13/13), severe tremor of the neck/upper limbs (9/13), pyramidal signs in the lower limbs including bilateral extensor plantar responses (13/13), absent/reduced lower limb reflexes (12/13) and proprioceptive loss (12/13). Other neurological findings were lower

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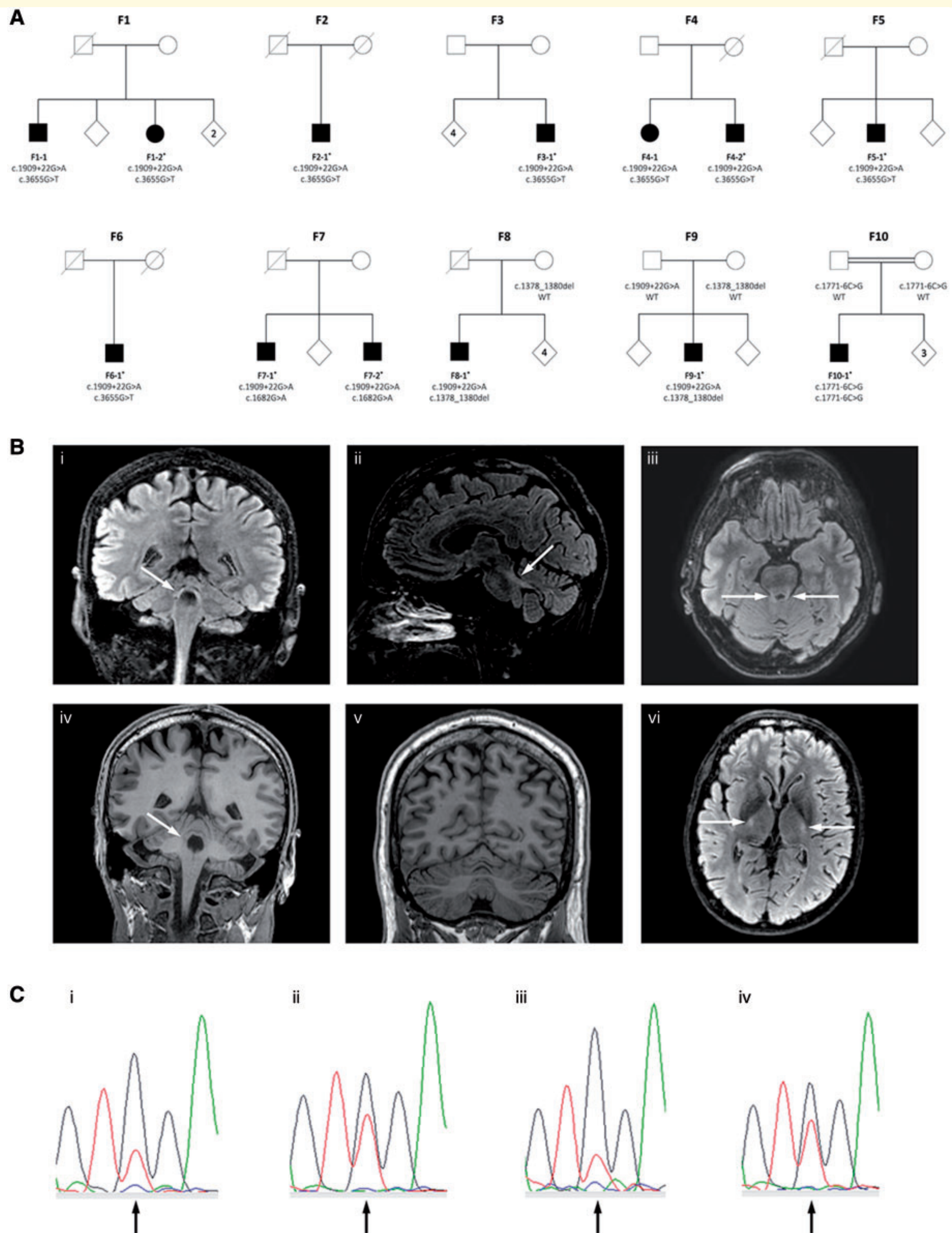

Figure 1 Pedigrees, MRIs and sequencing chromatograms. (**A**) Pedigrees of the 10 families showing co-segregation of the variants and disease. In Families F1–F6 *in trans* status was confirmed by mRNA analyses, and in Families F7-F10 by sequencing of family members. In Family 7, the parents were not available for analyses, hence adult children were sequenced and found to carry only one of the variants each (results not included in the pedigree due to anonymity restrictions). Asterisks indicate that whole exome sequencing was performed. (**B**) MRI of the brain. (**i**–**iv**) The superior cerebellar peduncles of Patient F2–1 showing hyperintense signal in FLAIR MRI in coronal (**i**), sagittal (**ii**) and axial (**iii**) views, and isointense signal in T_1-weighted coronal images (**iv**). Arrows indicate the superior cerebellar peduncles. (**v**) Coronal T_1-weighted image of Patient F7–1 showing mild atrophy of the cerebellar vermis (midline) and hemispheres. (**vi**) Axial FLAIR image of Patient F8–1 showing hyperintense signal in the corticospinal tracts at the level of the posterior limb of the internal capsule (arrows). The MRI signal in all images was assessed in relation to the reference area in the caudate nucleus (Vrij-van den Bos *et al.*, 2017). (**C**) Analysis of NMD of the c.3655G > T variant.

(continued)

limb weakness (12/13), muscle atrophy (11/12), reduced superficial sensations (7/13), dystonia (6/13) and urinary urgency (8/13). Interestingly, the tremor was alcohol-responsive. None had overt cognitive impairment. Neuropsychological evaluations in two patients showed well preserved cognitive function in one patient and a pattern compatible with mild cerebellar cognitive affective syndrome in the other (Schmahmann and Sherman, 1998).

Concerning non-neurological features, which were central in the previous debate, our findings are in line with those of Minnerop *et al.* Dental abnormalities were present in 11/13 patients, including hypodontia, retention of teeth, short dental roots, early dental loss and/or early periodontal disease. Also, one of the patients had developed several superfluous permanent teeth. Myopia was reported in 5 of 12 patients. Patient F9–1 had high myopia (< −6.00 dioptres), possibly a feature of her *POLR3A*-related syndrome. Four patients had milder myopia (> −3.00 dioptres), which is also a common finding in the general population (Williams *et al.*, 2015). Optic atrophy was not observed (0/7). Short stature and hormonal dysfunction were not prominent findings in our patient group. None had short stature (as defined by males < 166 cm, females < 153 cm) and height did not differ compared to other family members. Gonadal dysfunction was identified in one patient. Notably, in most patients, dental problems or hormonal dysfunction were not reported until specifically asked for.

Minnerop *et al.* introduced neuropathy as a clinical feature of *POLR3A*-related syndromes, describing abnormal nerve conduction studies (6/20), reduced vibration (23/24) and/or surface sensations (15/24). All patients in our study had severe sensory deficits, mainly of joint position and vibration sense, and 7/13 had markedly reduced surface sensations. Absent reflexes and muscle atrophy suggest dysfunction of peripheral nerves. Nerve conduction studies showed mild axonal sensory neuropathy in 2 of 10 investigated patients (from 2 of 10 patients), and 5 of 10 had mononeuropathies, but none had motor polyneuropathy. Similar to Minnerop *et al.*, we found abnormal somatosensory evoked responses in most patients (5/7), indicating dysfunction of 'central' sensory pathways. Brainstem auditory evoked responses were also abnormal in most patients (6/7).

MRI was available in 10 patients, seven performed on 3 T MRI. Bilateral hyperintensity along the superior cerebellar peduncles on FLAIR images (Fig. 1B) was found in all patients, consistent with the main finding reported by Minnerop *et al.*, and thus represents a convincing radiological clue for the diagnosis. To evaluate hypomyelination and atrophy, the 4H leukodystrophy brain MRI scoring system was used (Vrij-van den Bos *et al.*, 2017). Mild hypomyelination in other brain areas, represented by iso- or hyperintense T_2 signals were also identified; in particular of the pyramidal tracts at the level of the internal capsule (9/9). In addition, 9 of 10 patients showed mild cerebellar atrophy and 10 of 10 had slight thinning of the cervical spinal cord, compatible with previous reports (La Piana *et al.*, 2014, 2016), whereas none had thinning of the corpus callosum. Median total MRI score was 5 out of a possible maximum of 54 (range 4–9, mean 5.8), representing a mild degree of hypomyelination and atrophy. In comparison, Vrij-van den Bos *et al.* (2017) found a much higher score (median of 31) in patients with 4H leukodystrophy.

Five different presumed pathogenic variants in *POLR3A* were identified (Table 1). The variants were confirmed to be *in trans* in all 13 patients. The intronic c.1909+22G>A variant was found in 12, whereas one patient (Patient F10–1) was homozygous for the previously reported intronic c.1771–6C>G variant (Azmanov *et al.*, 2016). Furthermore, we identified three novel variants. In six families (Families F1–F6) we identified the variant c.3655G>T, p.Gly1219*, which leads to NMD of the mRNA transcript (Fig. 1C and Supplementary material). Another novel variant c.1682G>A, p.Arg561Gln was found in one family (Family F7). Structural analysis showed that this variant may disturb the interactions of the subunits POLR2H and the heterodimer POLR2C/POLR2J, and thereby affect the enzymatic activity of Pol III. Lastly, a deletion of three base pairs (c.1378_1380del, p.Val460del) was identified in two families (Families F8 and F9). It deletes a valine in a conserved area of the protein and is likely to destabilize the POLR3A subunit.

Haplotype analyses of exome data surrounding the *POLR3A* gene, revealed a maximal possible length of a common haplotype shared by the probands carrying c.1909+22G>A or c.3655G>T, of 1.9 Mb. The absence of longer shared haplotypes makes it unlikely that any of the two variants has a single recent founder. We could not identify any obvious genotype-phenotype correlations in our study. However, the patients carrying the c.1378_1380del had more prominent extra-neurological features.

Figure 1 Continued
Messenger RNA from lymphocytes cultured with and without inhibitor of NMD was reverse transcribed and amplified by PCR, followed by Sanger sequencing (details in the Supplementary material). The figure shows sequencing chromatograms of Patients F1–2 and F2–1. These patients carry the c.3655 G>T and c.1909+22G>A variants *in trans*, with the latter previously shown to result in a leaky splice site and partial NMD. The c.3655 position is marked by an arrow in the centre of each graph. [**C**(**i** and **ii**)] Chromatograms from analysis of Patient F1–2 without and with NMD inhibitor, respectively. [C(**iii** and **iv**)] Chromatograms from analysis of Patient F2–1 without and with NMD inhibitor, respectively. Notably, addition of NMD inhibitor led to increased amounts of product from the c.3655T allele as compared to the c.3655G allele. This result is consistent with complete NMD of the c.3655G>T variant, and partial degradation of transcripts from the c.1909+22G>A variant.

Table 1 Clinical characteristics of the patients

Patient ID	F1–1	F1–2	F2–1	F3–1	F4–1	F4–2	F5–1	F6–1	F7–1	F7–2	F8–1	F9–1	F10–1
Variant 1	c.1909+22G>A	c.1909+22G>A	c.1909+22G>A	c.1909+22G>A	c.1909+22G>A	c.1909+22G>A	c.1909+22G>A	c.1909+22G>A	c.1909+22G>A	c.1909+22G>A	c.1909+22G>A	c.1909+22G>A	c.1771–6C>G
Variant 2	c.3655G>T	c.3655G>T	c.3655G>T	c.3655G>T	c.3655G>T	c.3655G>T	c.3655G>T	c.3655G>T	c.1682G>A	c.1682G>A	c.1378_1380del	c.1378_1380del	c.1771–6C>G
Gender	M	F	M	M	F	M	M	F	M	M	M	F	M
Age at onset, y	5	14	12	17	20	13	30	10	11	20	17	5	4
Disease duration, y	62	51	35	27	28	33	15	55	46	29	28	24	41
Neurological symptoms and findings													
SARA (0–40)/SPRS (0–52)	29/34	24.5/25	28/33	22/22	np	17/18	7.5/11	27/21	17.5/28	11.5/18	16/23	5/np	np
LL spasticity/ reflexes	-/↓↓	-/↓↓	+/↓	+/↓	+/↓	+/↓↓	+/↓	+/↓	+/↓	+/↑	+/↓↓	-/↓↓	+/↓
Extensor plantar responses	+	+	+	+	+	+	+	+	+	+	+	+	+
LL weakness/ atrophy	+/+	+/+	+/+	+/+	+/np	+/+	+/+	+/+	+/+	+/+	+/+	-/-	+/+
Dysarthria	+	+	+	+	-	-	-	+	-	+	+	-	+
UL/LL ataxia	+/nt	+/+	+/+	+/+	-/+	+/+	+/-	+/+	-/+	+/+	+/+	+/+	+/+
Postural tremor UL	+	+	+	+	-	-	-	+	-	-	+	-	+
Head and neck titubation/dystonia	+/-	+/-	+/-	+/+	-/-	+/+	-/-	+/+	-/-	-/-	-/+	+/+	+/+
LL vibration/surface sensation	↓↓/↓	↓↓/↓	↓↓/↓	N/N	↓/↓	↓/N	↓↓/↓	↓↓/N	↓↓/↓	↓↓/N	↓↓/↓↓	↓/N	↓↓/N
Urinary urgency	+	+	+	-	+	-	+	-	+	+	+	-	-
Non-neurological findings													
Dental abnormalities	+	+	+	+	np	+	-	+	+	+	+	+	+
Myopia	-	-	+	-	np	-	-	+	+	-	+	+	-
Hypogonadism	np	-	-	-	np	-	-	-	-	-	+	np	-
Neurophysiology													
Polyneuropathy	np	-	nt	-	np	+	-	-	+	-	-	-	-
Abnormal SEP/BAEP/VEP	np	+/-/-	-/+/-	+/+/-	np	nt/+/nt	np	nt/nt/-	+/+/-	+/+/-	+/+/-	-/np/-	np
MRI													
SCP hyperintensity (FLAIR)	np	+	+	+	np	+	+	nt	+	+	+	+	+
Thin cervical spinal cord	np	+	+	+	np	+	+	nt	+	+	+	+	+
Thinning of corpus callosum	np	-	-	-	np	-	-	nt	-	-	-	-	-
Total white matter score[a]	np	3	6	6	np	nt	2	nt	3	5	6	1	1
Total atrophy score[a]	np	2	3	1	np	3	2	2	2	0	3	3	3

+ = present; - = absent; ↑ = increased; ↓ = reduced; ↓↓ = absent; LL = lower limbs; N = normal; np = not performed/not available; nt = not testable due to tremor/other co-morbidity; SARA = Scale for Assessment and Rating of Ataxia (Schmitz-Hubsch *et al.*, 2006); SCP = superior cerebellar peduncles; SEP/BAEP/VEP = somatosensory/brain stem auditory/visual evoked potentials; SNAP = sensory nerve action potentials; SPRS = Spastic Paraplegia Rating Scale (Schule *et al.*, 2006); UL = upper limbs.

[a]Following the 4H Leukodystrophy Brain MRI Scoring System. Total white matter score = 0–44; Total atrophy score = 0–10 (Vrij-van den Bos *et al.*, 2017).

Importantly, biallelic variants in *POLR3A* were found to be the second most common cause of recessive ataxia or HSP in our Norwegian cohort of 521 probands, second only to Friedreich's ataxia (Wedding *et al.*, 2015). In this cohort, 322 probands were classified with autosomal recessive or sporadic ataxia or HSP. Even if less than a third of these probands had exome data available for *POLR3A* analysis in this study, the 10 identified individuals with biallelic variants in *POLR3A* represent a frequency of 3.1%, similar to the frequency found by Minnerop *et al.* (2017). No additional carriers of the c.1909+22G>A variant were identified in the 95 exomes. However, our sample size is small and could be prone to several aspects of selection bias, and we thus regard it unsuitable for extrapolating any association (or lack of association) of this variant with ataxia/HSP to a general population of ataxia/HSP. Hence, a properly designed association study would be required to replicate the association results previously reported (Minnerop *et al.*, 2017; Gauqelin *et al.*, 2018).

In summary, our study confirms that biallelic variants in *POLR3A* are indeed a frequent cause of disease in hereditary ataxia/HSP patients. In particular, the c.1909+22G>A variant is prevalent, illustrating the importance and complexity of variants in non-coding regions. Furthermore, we delineate a highly characteristic and consistent clinical picture, comprising early onset ataxia with or without tremor, combined with pyramidal and posterior column findings, dental abnormalities and the key MRI finding of high intensities along the superior cerebellar peduncles. The expanding phenotypic spectrum of *POLR3A*-related syndromes, now including leukodystrophies, a disease of premature ageing—Wiedemann-Rautenstrauch syndrome (Paolacci *et al.*, 2018)—and hereditary ataxia/HSP, calls for future disease classification which includes both clinical and genetic information (Rossi *et al.*, 2018), and for increased awareness of also atypical non-neurological symptoms in complex neurological disorders.

Data availability

The authors confirm that the data supporting the findings of this study are available within the letter and its Supplementary material.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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Supplementary materials to
Letter to the editor: Biallelic *POLR3A* variants confirmed as a frequent cause of hereditary ataxia and spastic paraparesis

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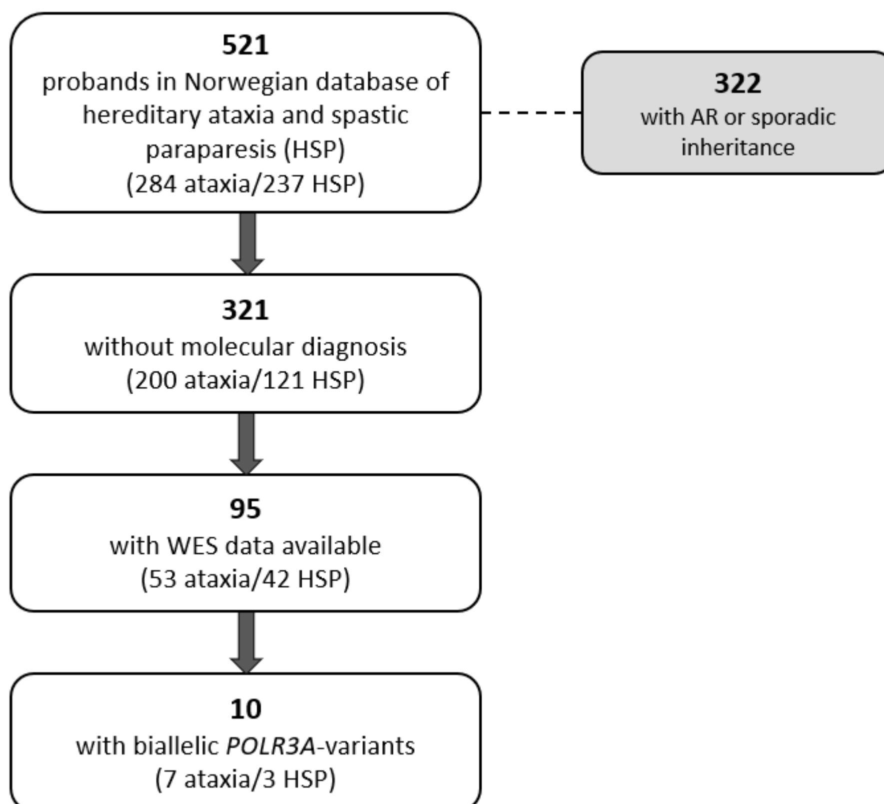
1 Patients and clinical investigations

1.1 Selection of patients

The patients were recruited from the Norwegian database "Hereditary ataxia and spastic paraparesis in Norway", a study approved by the Regional Ethical Committee of South Eastern Norway, approval number 2010/1579 (Erichsen *et al.*, 2009). All patients have given written informed consent and all probands have undergone standardized diagnostic investigations, including clinical examination (Schmitz-Hubsch *et al.*, 2006; Schule *et al.*, 2006) and relevant molecular and other supplementary investigations.

This project was prompted by the publication by Minnerop *et al*. (Minnerop *et al.*, 2017). The aims were to investigate whether biallelic variants in *POLR3A* could be a frequent cause of disease also in Norwegian patients with hereditary ataxia and/or spastic paraparesis (HSP) and if so, to characterize the clinical phenotype. Prior to Minnerop *et al*.'s paper in April 2017, the intronic c.1909+22G>A variant was not considered as pathogenic, and no patients in our cohort were previously diagnosed with *POLR3A*-related disease. The selection of patients is illustrated in Supplementary Figure 1.

Supplementary Figure 1: Selection of probands from the Norwegian database of hereditary ataxia and spastic paraparesis



From the 521 probands (index cases) included in the Norwegian database, 321 were without molecular diagnosis (200 registered as main clinical phenotype hereditary ataxia; 121 as hereditary spastic paraparesis). Of these, whole exome data was obtainable from a total of 95 probands, of which a total of 10 were found to carry presumed disease-causing biallelic variants in the *POLR3A* gene.

1.2 Clinical examination

1.2.1 Updated examinations

Updated examinations were performed when possible, including clinical neurological examination (10/13), laboratory test for endocrine dysfunction (8/13), 3 Tesla MRI of the brain (7/13)/spinal cord (5/13), neurography (8/13), evoked potentials (8/13), ophthalmological examination (6/13) and neuropsychological examinations (2/13). Height and weight were based on patient information. Dental status was based on patient information, supported by information from dentists when available. In patient F4-1, only information from prior medical journal and DNA was available.

1.2.2 Clinical demographics

Mean age at onset of the first neurological symptoms of the 13 patients (4 female/9 male) was 13.7 years (range 4-30, median 13 years), of which 12 had symptoms before the age of 21. The first symptom was unsteadiness (10/13), stiff legs (2/13) or clumsiness (1/13). Disease duration at last examination varied from 15 to 62 years. Four patients were ambulant without walking aids, two needed unilateral support, three bilateral support and four were completely wheelchair bound.

1.2.3 Clinical findings and disease course

Elaborated details of clinical characteristics are given in Supplementary Table 1A and summarized in Supplementary Table 1B. The major phenotypic feature was the combination of severe tremor and ataxia (8/13), complex ataxia (2/13) or complex spastic paraparesis (3/13). Interestingly, the tremor had a pronounced response to alcohol, transiently nearly completely suppressing the tremor.

Supplementary Table 1A. Detailed clinical characteristics of the patients

Patient ID	F1-1	F1-2	F2-1	F3-1	F4-1	F4-2	F5-1	F6-1	F7-1	F7-2	F8-1	F9-1	F10-1
Variant 1	c.1909+22 G>A	c.1909+22 G>A	c.1909+22 G>A	c.1909+22 G>A	c.1909+22 G>A	c.1909+22 G>A	c.1909+22 G>A	c.1909+22 G>A	c.1909+22 G>A	c.1909+22 G>A	c.1909+22 G>A	c.1909+22 G>A	c.1771-6 C>G
Variant 2	c.3655G>T	c.3655G>T	c.3655G>T	c.3655G>T	c.3655G>T	c.3655G>T	c.3655G>T	c.3655G>T	c.1682G>A	c.1682G>A	c.1378_ 1380del	c.1378_ 1380del	c.1771-6 C>G
Clinical demographics													
Inheritance pattern	AR	AR	spo	spo	AR	AR	spo	spo	AR	AR	spo	spo	AR
Gender	M	F	M	M	F	M	M	F	M	M	M	F	M
Age at onset (y)	5	14	12	17	20	13	30	10	11	20	17	5	4
First symptom	unsteady	unsteady	unsteady	stiff legs	unsteady	unsteady	unsteady	clumsy	stiff legs	unsteady	unsteady	unsteady	unsteady
Main clinical phenotype	TRE-ATX	TRE-ATX	TRE-ATX	TRE-ATX	cHSP	cATX	TRE-ATX	TRE-ATX	cHSP	cATX	TRE-ATX	TRE-ATX	cHSP
Age at last examination (y)	67	65	47	44	48	46	45	65	57	49	45	29	45
Disease duration (y)	62	51	35	27	28	33	15	55	46	29	28	24	41
Neurological symptoms and findings													
Disability stage (0-7)[a]	6	6	6	5	3	4	3	5	6	3	4	3	5
SARA (0-40)	29	24.5	28	22	np	17	7.5	27	17.5	11.5	16	5	np
SPRS (0-52)	34	25	33	22	np	18	11	21	28	18	23	np	np
UL/LL spasticity	-/-	-/-	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/-	-/+
UL/LL tendon reflexes	↓/↓↓	↓↓/↓↓	N/↓	↓↓/↓	↓/↓	↓↓/↓↓	↓/↓	N/↓	N/↓	↓/↑	↓↓/↓↓	↓↓/↓↓	N/↓
Extensor plantar responses	+	+	+	+	+	+	+	+	+	+	+	+	+
UL/LL weakness	+/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/-	-/+
UL/LL muscle atrophy	+/+	+/+	+/+	-/+	np	-/+	-/+	-/+	-/+	-/+	+/+	-/-	-/+
Gaze-evoked nystagmus	-	-	+	+	-	-	-	-	-	-	-	-	-
Abnormal smooth pursuits	+	+	+	+	np	+	-	+	-	+	+	-	-
Vertical gaze limitation	-	-	-	-	-	-	-	-	-	-	-	-	-
Dysarthria	+	+	+	+	-	-	-	+	-	+	+	-	+
UL/LL ataxia	+/nt	+/+	+/+	+/+	-/+	+/+	+/-	+/+	-/+	+/+	+/+	+/+	+/+
Postural tremor UL	+	+	+	+	-	-	-	+	-	-	+	-	+
Head/neck titubation	+	+	+	+	-	+	-	+	-	-	-	+	+
Dystonia, neck/UL	-	-	-	+	-	+	-	+	-	-	+	+	+
Vibration sense (ankles)	↓↓	↓↓	↓↓	N	↓	↓	↓↓	↓↓	↓↓	↓↓	↓↓	↓	↓↓
Surface sensation LL	↓	↓	↓	N	↓	N	↓	N	↓	N	↓↓	N	N
Urinary urgency	+	+	+	-	+	-	+	-	+	+	+	-	-
Cognitive affection[b]	-	-	+	-	-	+	-	-	-	-	-	-	+

Seizures	-	-	-	-	-	-	-	-	-	-	-	-	-
Non-neurological findings													
Hypo-/hyperdontia	+	+	-	-	np	-	-	-	-	-	+	-	-
Other dental abnormalities	+	+	+	+	np	+	-	+	+	+	+	+	+
Myopia[c]	-	-	+	-	np	-	-	+	+	-	+	+	-
Optic atrophy	np	-	-	-	np	np	np	-	-	-	np	np	-
Hypogon. hypogonadism	np	-	-	-	np	-	-	-	-	-	+	np	-
Height (cm)	176	172	183	185	np	170	169	160	173	172	180	164	186
Body mass index (= kg/m²)	20.3	15.5	19.4	21.9	np	26.0	27.0	27.0	31.7	23.0	17.9	21.6	18.8
Scoliosis (mild)	+	-	-	-	np	-	-	-	+	-	+	-	+
Foot deformities[d]	+	+	+	-	np	+	+	-	-	-	-	+	-
Neurophysiology													
Polyneuropathy (↓ SNAP)	np	-	nt	-	np	+	-	-	+	-	-	-	-
Abnormal SEP	np	+	-	+	np	nt	np	nt	+	+	+	-	np
Abnormal BAEP	np	-	+	+	np	+	np	nt	+	+	+	np	np
Abnormal VEP	np	-	-	-	np	nt	np	-	-	-	-	-	np
MRI													
SCP hyperintensity (FLAIR)	np	+	+	+	np	+	+	nt	+	+	+	+	+
Thin cervical spinal cord	np	+	+	+	np	+	+	nt	+	+	+	+	+
Thinning of corpus callosum	np	-	-	-	np	-	-	nt	-	-	-	-	-
Cerebellar atrophy	np	V, H	V, H	V	np	V	V	nt	V, H	-	V	V	V
Total white matter score[e]	np	3	6	6	np	nt	2	nt	3	5	6	1	1
Total atrophy score[e]	np	2	3	1	np	3	2	2	2	0	3	3	3

+ = clinical sign present; - = clinical sign absent; ↑ = increased; ↓ = reduced; ↓↓ = absent; AR = autosomal recessive; ATX = ataxia; BAEP = brainstem auditory evoked potentials; cATX = complex ataxia; cHSP = complex hereditary spastic paraparesis; H = hemispheres; Hypogon. = hypogonadotropic; LL = lower limbs; N = normal; np = not performed/not available; nt = not testable due to tremor/other co-morbidity; SARA = Scale for Assessment and Rating of Ataxia (Schmitz-Hubsch *et al.*, 2006); SCP = superior cerebellar peduncles; SEP = somatosensory evoked potentials; SNAP = sensory nerve action potentials; spo = sporadic; SPRS = Spastic Paraplegia Rating Scale (Schule *et al.*, 2006); TRE = tremor; UL = upper limbs; V = vermis; VEP = visual evoked potentials; y = years.

[a] Disability stage (SPATAX-EUROSPA): 0 = no functional handicap; 1 = no functional handicap, but signs at examination; 2 = able to run, walking unlimited; 3 = unable to run, limited walking without aid; 4 = walking with one stick; 5 = walking with two sticks; 6 = unable to walk, requiring wheelchair; 7 = confined to bed (https://spatax.wordpress.com/).

[b] F2-1 had signs of cerebellar cognitive affective syndrome; F4-2 and F10-1 had learning difficulties.

[c] Patient F9-1 had high myopia (-6.5/-5.5 dioptres (D)), patient F8-1 moderate myopia (-2.5/-3.0 D), the remaining mild myopia (-0.75 to -2.00 D).

[d] Pes cavus, hammer toes and/or pes equinovarus.

[e] Following the 4H Leukodystrophy Brain MRI Scoring System. Total white matter score = 0-44; Total atrophy score = 0-10. (Vrij-van den Bos *et al.*, 2017)

Supplementary Table 1B: Summary of clinical characteristics

Demographics	
Gender	9M/4F
Age at onset (y)	mean 13.7 (range 4-30)
Age at last examination (y)	mean 50.2 (range 29-67)
Disease duration (y)	mean 36.5 (15-62)
Neurological symptoms and findings	
Disability stage (0-7) [a]	mean 4.5 (range 3-6)
SARA (0-40)	mean 18.6 (range 5-29)
SPRS (0-52)	mean 23.3 (range 11-34)
UL spasticity	0/13
LL spasticity	10/13
Reduced UL tendon reflexes	9/13
Reduced LL tendon reflexes	12/13
Bilateral extensor plantar response	13/13
UL weakness	1/13
LL weakness	12/13
UL muscle atrophy	4/12
LL muscle atrophy	11/12
Gaze-evoked nystagmus	2/13
Abnormal smooth pursuits	8/12
Vertical gaze limitation	0/13
Dysarthria	8/13
UL ataxia	11/13
LL ataxia	11/12
Postural tremor UL	7/13
Head/neck titubation	8/13
Dystonia, neck and UL	6/13
Reduces vibration sense (ankles)	12/13
Reduced surface sensation	7/12
Urinary urgency	8/13
Cognitive affection	3/13
Seizures	0/13
Non-neurological findings	
Hypo-/hyperdontia	3/12
Other dental abnormalities	11/12
Myopia	5/12
Optic atrophy	0/7
Hypogonadotropic hypogonadism	1/10
Height (cm)	M: 169-186; F: 160-172
Body mass index (BMI=kg/m²)	mean 22.5 (range 15.5-31.7)
Mild scoliosis	4/12
Foot deformities	6/12
Neurophysiology	
Polyneuropathy (↓ SNAP)	2/10
Mononeuropathy in nerve conduction	5/10
Abnormal SEP	5/7
Abnormal BAEP	6/7
Abnormal VEP	0/8
MRI	
SCP hyperintensity (FLAIR)	10/10
Thin cervical spinal cord	10/10
Thinning of corpus callosum	0/10
Cerebellar atrophy	V: 9/10; H: 3/10
Total white matter score[b] **(n=9)**	mean 3.7 (range 1-6)
Total atrophy score[b] **(n=10)**	mean 2.2 (range 0-3)

BAEP = brainstem auditory evoked potentials; H = hemispheres; LL = lower limbs; SARA = Scale for Assessment and Rating of Ataxia (Schmitz-Hubsch *et al.*, 2006); SCP = superior cerebellar peduncles; SEP = somatosensory evoked potentials; SNAP = sensory nerve action potentials; SPRS = Spastic Paraplegia Rating Scale (Schule *et al.*, 2006); UL = upper limbs; V = vermis; VEP = visual evoked potentials; y = years.
[a] Disability stage (SPATAX-EUROSPA); 0 = no functional handicap; 1 = no functional handicap, but signs at examination; 2 = able to run, walking unlimited; 3 = unable to run, limited walking without aid; 4 = walking with one stick; 5 = walking with two sticks; 6 = unable to walk, requiring wheelchair; 7 = confined to bed (https://spatax.wordpress.com/).
[b] Scoring using the 4H Leukodystrophy Brain MRI Scoring System (Vrij-van den Bos *et al.*, 2017); Total white matter score = 0-44; Total atrophy score = 0-10.

In several patients the natural course of disease was characterized by a distinct evolvement of symptoms. The main clinical classification changed from complex ataxia/HSP at time of diagnosis, to a pronounced tremor-ataxia phenotype in seven patients. In particular tremor and cerebellar signs increased as disease progressed. The neurological phenotype resembled other recessive ataxias, such as Friedreich ataxia (ATX-FXN), ataxia with vitamin-E deficiency (ATX-TTPA), and autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS, ATX/HSP-SACS), the latter often show high intensities in the middle cerebellar peduncles in MRI.

1.2.4 Dental findings

Dental problems were, on direct examination, present in 11/12: Two had hypodontia of primary teeth; two had needed surgery due to retention of teeth; five had been told by their dentist that they had short dental roots; three had experienced loss of teeth after minor or no trauma; and three had early dental disease. One described a dysmorphic incisor. In addition, one of the patients (F8-1) described to have developed several extra permanent teeth. One patient had short roots, but further evaluation was difficult due to previous severe traumas to the face resulting in loss of teeth. Interestingly, only two patients had spontaneously reported their dental problems to their neurologist, illustrating the need for direct questioning of patients regarding non-neurological symptoms.

1.3 Neuropsychological testing of cognitive function

Detailed cognitive assessment were performed in individuals F1-2 and F2-1 with tests specifically selected to minimize motor skill´s interference, and a special emphasis was put on covering executive, visuospatial and verbal domains in accordance to the Cerebellar Cognitive Affective Syndrome (CCAS) (Schmahmann and Sherman, 1998). Patient F1-2 had normal results, whereas patient F2-1 showed a pattern more compatible with CCAS with executive dysfunction and impaired verbal functions.

Supplementary Table 2: Neuropsychological testing of cognitive function

Patient	F1-2		F2-1	
Total IQ	117	HA	100	A
Verbal IQ, t-score	58	HA	54	A
Executive IQ, t-score	61	HA	47	A
Word fluency - letter fluency, t-score	61	HA	37	LA
Word fluency - category fluency, t-score	81	VS	80	VS
Stroop 1 - Colour naming, Scaled Score	8	A	6	LA
Stroop 2 - Word reading	7	LA	8	A
Stroop 3 - Inhibition, Scaled Score	11	A	9	A
Stroop 4 - Inhibition/Switching, Scaled Score	12	HA	1	I
Line orientation, percentile	56	A	>86	HA
CVLT verbal learning, t-score	70	VS	37	LA
CVLT verbal long-delay free recall, standard score	11,5	A	10	A
CVMT learning, percentile	96	S	24	LA
CVMT memory, percentile	98.3	VS	97.1	S

CVLT = California Verbal Learning Test; CVMT = Continuous Visual Memory Test
VS = very superior; S = superior; HA = high average; A = average; LA = low average; I = inferior

1.4 Ophthalmological examination

In six patients (F1-2, F2-1, F3-1, F6-1, F7-1 and F7-2) updated ophthalmological examinations were performed, including standardized clinical eye examination, imaging of retina (Optomap) and optical coherence tomography (OCT) scan of macula. Three of the six patients had mild myopia, defined as -0.75 to -2.00 dioptres. In the seven patients of whom updated eye examination was not performed, myopia was reported in patients F8-1 (-2.5/-3.0 D) and F9-1 (-6.5/-5.5 D), however information on the degree of astigmatism was not available. None had optic atrophy (examined in seven patients), but in patient F7-2 OCT showed slight bilateral thinning of the ganglion cell layer in inferior macula. OCT was normal in four patients. One (F1-2) had severe hypermetropia since childhood. One (F3-1) had severe anisometropia with astigmatism, giving an amblyopic right eye.

1.5 Biochemical measurements

Hormone levels (hypophyseal, gonadal and thyroid hormones) were assessed in ten patients, measured by standard diagnostic procedures. In one patient the results were not interpretable due to concurrent cancer disease and treatment. One patient (F6-1) had the diagnosis of hypothyroidism since early adulthood and was treated with substitution therapy. In one patient (F8-1) gonadal dysfunction was identified, with low levels of s-testosterone and s-free testosterone index, and clinical signs with delayed puberty.

Interestingly, p-folate (vitamin B9) was below the reference limit of the laboratory (>7 nmol/L) in five out of nine patients (total range 5-21 nmol/L, mean 9.7). Homocysteine was not measured. Haemoglobin (available in 12/13), haematocrit and p-vitamin B12 levels

(available in 10/13) were normal. A finding of low levels of folate in more than half of the patients is interesting and might present a biomarker in *POLR3A*-related syndromes. However, as no additional work-up investigations were performed, the relevance of this finding remains to be clarified.

1.6 Neurophysiological examinations

Renewed neurography and evoked potentials were performed when possible, and previous examinations were re-evaluated when available. A summary is given in Supplementary Table 3. Due to technical artefacts, some examinations were inconclusive, and these patients are not included in the summary in the main manuscript, nor in Suppl. Table 1. Two patients had reduced sensory nerve action potentials (amplitudes) in >1 sensory nerve in the legs, representing a mild axonal sensory polyneuropathy. Five patients had findings compatible with mononeuropathies; carpal tunnel syndrome (F3-1, F4-2, F6-1 and F7-1); and peroneal neuropathy in (F3-1 and F10-1). The patients are considered prone to pressure mononeuropathies due to use of wheel chairs/crutches and paresis of the legs. Somatosensory and brain stem evoked potentials were abnormal in most of our patients. These findings indicate dysfunction of central sensory systems, which could be an explanation for the clinical deficits even in absence of peripheral neuropathy.

Supplementary Table 3: Neurophysiological examinations

	n	Normal results (n)	Incon-clusive[a] (n)	Patho-logical results (n)	Description of pathological results
Nerve conduction studies	11	5	1	5	↓SNAP consistent with sensory polyneuropathy (2) Carpal tunnel syndrome (4) Peroneal neuropathy knee level (2)
Median nerve somatosensory evoked potentials (SEP)	9	2	2	5	Absent or delayed cortical responses (5)
Pattern visual evoked potentials (VEP) (reversed checkerboard)	8	8	0	0	
Brainstem auditory evoked potentials (BAEP)	8	1	1	6	Prolonged interpeak latency I-V (6) Prolonged interpeak latency I-III (1) Prolonged absolute latency of waves I, III and V (2) Prolonged absolute latency of waves III and V (2)
EEG	2	2	0	0	

n = number of patients. SNAP = sensory nerve action potentials.
[a]Due to tremor/co-morbidity. These patients are not included in the summaries given in the main manuscript, nor in Suppl. Table 1.

2 Magnetic Resonance Imaging

MRI of the brain and spinal cord was available in ten patients. Seven brain MRIs were performed on 3 Tesla Discovery MR750 scanner, three were performed on different 1.5 Tesla scanners, with standard sequences. Axial, coronal and sagittal T1- and T2-weighted and FLAIR images were available in all patients. In patient F6-1, 3T MRI was unsuccessful due to severe tremor, in patient F1-1 and F4-1 MRI images were not available. In patient F4-2 hypomyelination was not possible to assess due to movement artefacts.

Different patterns of hypomyelination have been linked to *POLR3A*-related syndromes (La Piana *et al.*, 2014; Takanashi *et al.*, 2014; Cayami *et al.*, 2015; Azmanov *et al.*, 2016; La Piana *et al.*, 2016; Travaglini *et al.*, 2018). Hypomyelination and atrophy in brain MRIs were evaluated using the 4H Leukodystrophy MRI scoring system (Vrij-van den Bos *et al.*, 2017), in addition the superior cerebellar peduncles were examined specifically (T1-weighted, T2-weighted and FLAIR). Spinal cord atrophy was assessed by measurement of the anteroposterior diameter of the spinal cord at level of C5 (Frostell *et al.*, 2016).

Hyperintense signals on T2 FLAIR sequences in the superior cerebellar peduncles (SCP) were observed in *all* patients. These high intensities in SCP were only described in the original radiological report in one out of these ten patients, illustrating that targeted analysis might be valuable for both the clinician and the radiologist. For other signs of hypomyelination and atrophy, see Supplementary Table 4. The anteroposterior diameter of the cervical spinal cord at the level of C5 was 5-6 mm in *all* patients (10/10), presenting a slight thinning of the cervical spinal cord (Frostell *et al.*, 2016).

Supplementary Table 4: MRI scoring of hypomyelination and atrophy

Patient ID	F1-2	F2-1	F3-1	F4-2	F5-1	F7-1	F7-2	F8-1	F9-1	F10-1
MRI	3 T	3 T	3 T	3 T	1,5 T	3 T	3 T	3 T	1,5 T	1,5 T
Age at MRI	65	47	44	46	38	57	49	45	27	45
DD at MRI	51	35	27	33	8	46	29	28	22	41
Hypomyelination score										
Hypomyelination, T1[a]	0	0	0	nt	0	0	0	0	0	0
Frontal WM; subc., T2	0	0	0	nt	0	0	0	0	0	0
Frontal WM; periv., T2	0	1	1	nt	0	0	1	1	0	0
Frontoparietal border area; subc., T2	0	0	0	nt	0	0	0	0	0	0
Frontoparietal border area; periv., T2	1	1	1	nt	1	1	1	1	0	0
Parietooccipital WM; subc., T2	0	1	0	nt	0	0	0	0	0	0
Parieto-occipital WM; periv., T2	0	1	1	nt	0	0	1	1	0	0
Int. capsule; ALIC, T2	0	0	0	nt	0	0	0	0	0	0
Int. capsule; pyramidal tracts in PLIC, T2	1	1	1	nt	1	1	1	2	1	1
Corpus callosum; genu, T2	0	0	0	nt	0	0	0	0	0	0
Corpus callosum; splenium, T2	0	0	0	nt	0	0	0	0	0	0
MCP, T2	1	1	2	nt	0	1	1	1	0	0
Total WM score (0-44)[b]	**3**	**6**	**6**	**nt**	**2**	**3**	**5**	**6**	**1**	**1**
Atrophy score										
Supratentorial (bicaudate ratio) score	0	1	0	2	1	0	0	2	0	2
Corpus callosum	0	0	0	0	0	0	0	0	0	0
Brainstem (pons diameter) score	0	0	0	0	0	0	0	0	2	0
Cerebellar vermis	1	1	1	1	1	1	0	1	1	1
Cerebellar hemispheres	1	1	0	0	0	1	0	0	0	0
Total atrophy score (0-10)[c]	**2**	**3**	**1**	**3**	**2**	**2**	**0**	**3**	**3**	**3**
Total score (WM and atrophy)										
Total score (0-54)	**5**	**9**	**7**	**nt**	**4**	**5**	**5**	**9**	**4**	**4**
Superior cerebellar peduncles score										
SCP, T1	0	1	1	0	0	0	0	0	0	0
SCP, T2	2	2	2	2	1	2	1	2	1	2
SCP, FLAIR	2	2	2	2	2	2	2	2	2	2
Spinal cord										
AP-dia C5-level[d]	6	6	6	5	6	6	5	5	6	6

ALIC = anterior limb of the internal capsule; AP-dia = anteriorposterior diameter; DD = disease duration (years); FLAIR = fluid attenuated inversion recovery; int. = internal; MCP = middle cerebellar peduncles; nt = not possible to assess due to movement artefacts; periv. = periventricular; PLIC = posterior limb of internal capsule; SCP = superior cerebellar peduncles; subc. = subcortical; T = tesla; T1 = T1-weighted; T2 = T2-weighted; WM = white matter.

[a] T1 hypomyelination; Same areas examined as in T2-weighted images, all scored 0.

[b] White matter scores: T1; 0 = hyperintense; 1 = isointense; 2 = hypointense in relation to caudate nucleus; T2/FLAIR; 0 = hypointense; 1 = isointense; 2 = hyperintense in relation to caudate nucleus. Not possible to evaluate in patient F4-2 due to movement artefacts.

[c] Atrophy scores: 0 = normal; 1 = slightly thinned/mild atrophy; 2 = severely thinned/severe atrophy (see reference Vrij-van den Bos *et. al.*, 2017).

[d] Anteroposterior diameter of the spinal cord at level C5 (mm) (see reference: Frostell *et al.*, 2016).

3 Molecular investigations

Prior molecular investigations of the patients included repeat expansions investigations of *FXN*, *FMR1*, and the most common spinocerebellar ataxias (*ATXN1*, *ATXN2*, *ATXN3*, *CACNA1A*, *ATXN7* and *ATN1*), and sequencing and MLPA of HSP genes; *SPAST*, *ATL1*, *REEP1*, *SPG7* and *SPG11*. In addition, analyses of gene panels for movement disorders based on high throughput whole exome sequencing data had been performed in a number of patients. In one of the ten identified *POLR3A*-families there was consanguinity (F10), the other families had no known common ancestors.

3.1 Whole exome sequencing

Exome data of 95 probands without molecular diagnosis prior to the current project was included in the analysis. Seventeen of these originated from a research project in our department, while the remaining 78 were sequenced as part of routine diagnostics.

3.1.1 Sequencing method and variant calling

Sequencing method and variant calling were the same for all 95 probands. DNA was extracted from whole blood. The exome was enriched using the SureSelectXT V5 exome kit (Agilent, Böblingen, Germany) and sequenced on a HiSeq2500 sequencer (Illumina, San Diego, CA, USA). Reads were mapped against the human reference genome (hg 19, UCSC Genome Browser). Read mapping and variant calling were performed following Genome Analysis Tool Kit version 3.3.0 (https://software.broadinstitute.org/gatk/best-practices/workflow?id=11145) best practices recommendations. Variant annotation was performed with ANNOVAR (Wang *et al.*, 2010) along with an in-house script.

3.1.2 Original filtering procedures

The original filtering procedures differed slightly between the two groups. In the group of 78 exomes with diagnostic origin, 77 were analysed at the Department of Medical Genetics at Oslo University Hospital (OUH), Norway, with an *in silico* gene panel for movement disorders developed in-house containing 240 (version 01) or 412 (version 02) genes (gene list "Bevegelsesforstyrrelser v01/v02": https://oslo-universitetssykehus.no/avdelinger/klinikk-for-laboratoriemedisin/avdeling-for-medisinsk-genetikk/bevegelsesforstyrrelser). The last exome (Family F9) was analysed at the Department of Medical Genetics at Telemark Hospital, Norway. Downstream analysis followed routine pipelines for exome sequencing.

The remaining 17 exomes were originally analysed in a research project, using the software FILTUS (Vigeland *et al.*, 2016). Variant filters were applied in order to retain exonic and splice-site variants with minor frequency less than 1% in public databases (ExAC; 1000 Genomes) or our inhouse database. Variants not passing quality control (non-PASS variants) were excluded, as well as variants in repeat regions and segmental duplications.

With these filtering procedures, no disease-causing variants in other genes than *POLR3A* were identified.

3.1.3 *POLR3A*-specific analysis

For the specific *POLR3A* analysis, 94 exomes (F9-1 excluded) were analysed in FILTUS with the same filters as before, but without removing intronic variants. Identified variants were manually scrutinised, leaving five different variants which were possibly disease-causing.

Supplementary Table 5: Variants identified in POLR3A

Variant (NM_007055.3)	**c.1909+22G>A**	**c.3655G>T**	**c.1682G>A**	**c.1378_1380del**	**c.1771-6C>G**
Protein change	p.Tyr637Cysfs*14	p.Gly1219Ter	p.Arg561Gln	p.Val460del	p.Pro591Metfs*9
Present in families	F1-F9	F1-F6	F7	F8-F9	F10
Chromosomal location NC_000010.10 GRCh37 (hg19)	g.79769273C>T	g.79742016C>A	g.79769710C>T	g.79777384_ 79777386del	g.79769439G>C
Exon/intron number	intron 14	exon 28	exon 13	exon 10	intron 13
rs-number	rs191875469	na	na	na	rs115020338
Type	intron	stopgain	missense	non-frameshift deletion	intron/splice region
Frequency in-house database	1/400	1/400	0/400	3/400	0/400
Frequency in Norwegian variation database[a]	2/1590	0/1590	0/1590	1/1590	0/1590
gnomAD	386/282828	3/251280	2/ 251420	not found	17/ 282210
CADD score PHRED	na	49	35	na	na
GERP	3.81	5.99	5.42	na	3.61
Pathomechanism	Aberrant splicing, partial deficiency of full-length transcript, NMD	NMD	Structural change affecting the enzymatic activity of Pol III	Structural change in the hydrophobic core, affects protein folding	Aberrant splicing, partial deficiency of full-length transcript
Reference	Minnerop *et al.*, 2017, la Piana *et al.*, 2016, Travaglini *et al.*, 2018	Novel	Novel	Novel	Azmanov *et al.*, 2017

CADD = Combined Annotation Dependent Depletion; na = not available; NMD = nonsense-mediated decay.
[a] http://invitro.hpc.uio.no:8082/vcf-miner/

After filtering, 10 probands remained with two variants in *POLR3A* (nine compound heterozygous; one homozygous). Five different *POLR3A* variants were identified, of which two were previously described (see Supplementary Table 5).

None of the five variants were present in a heterozygous state in the remaining 85 exomes.

The frequency of *POLR3A* variants in our patient material are subject to selection bias and different selection criteria, and cannot be directly compared to the results presented by Minnerop *et al*. and Gauquelin *et al*.. Also, our data is not suited to test for association of the c.1909+22G>A variant in the general ataxia population.

3.2 Sanger sequencing of DNA

In families F7-F10 the *in trans* status was confirmed by Sanger sequencing of the variants in available family members (see Fig. 1A).

EDTA blood was drawn from selected healthy family members, and DNA extracted according to in-house procedures. DNA was amplified using *POLR3A* specific primers (2.5 pmol of each, Supplementary Table 6) in a 20 µL reaction containing 0.5 U AmpliTaq Gold 360 DNA Polymerase (ThermoFisher Scientific) supplemented with GC-enhancer, buffer, dNTPs, and $MgCl_2$ according to manufacturer's instructions. Cycling conditions were as follows: 95°C for 10 min, followed by 30 cycles of 95°C for 30 sec / 53°C for 30 sec / 72°C for 60 sec, and a final step of 72°C for 7 min. Reactions were purified by Agencourt AMPure XP beads (Beckman Coulter), followed by direct sequencing of amplicons using a 1:40 dilution of BigDye Terminator v3.1 chemistry, clean up with CleanSEQ beads (Beckman Coulter), and separation on a 3730 capillary sequencer (Applied Biosystems). Further details regarding Sanger sequencing are available upon request.

Patients shown from exome sequencing to carry c.1909+22G>A in combination with c.1682G>A or c.1378_1380del were confirmed compound heterozygous by sequencing of healthy family members. Biallelic status of the c.1771-6C>G variant in patient F10-1 was verified by sequencing of the parents, who were heterozygous carriers.

Supplementary Table 6: Primers for PCR and Sanger sequencing of DNA

Mutation of interest	Forward primer	Reverse primer
c.1378_1380del (exon 10)	agagggagaaacttggtgag	aaaagcagtgctactcacca
c.1682G>A (exon 13)		
c.1771-6C>G (intron 13)	gaggtagatcctagttggagga	tcgcgaaggtaccagcaaag
c.1909+22G>A (intron 14)		

3.3 RNA analyses

To investigate whether variants were *in trans*, and the occurrence of nonsense-mediated decay (NMD), analyses of RNA were performed.

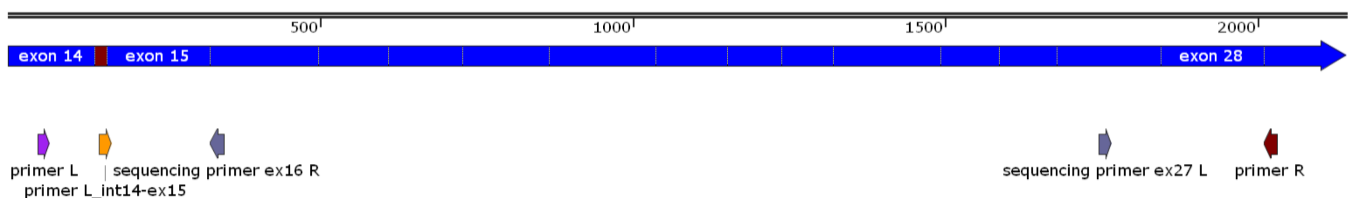
3.3.1 Patient material

From patients in families F1-F6, RNA was extracted from PAXgene RNA blood tubes (PreAnalytiX) using the PAXgene Blood RNA Kit (Qiagen) according to manufacturer's procedure. For analysis of NMD, RNA was extracted from peripheral blood lymphocytes from patient F1-2 and F2-1. In short, lymphocytes were cultured from Lithium Heparin blood for four days in Medium 199 (BioWhittaker, supplementations available upon request), after which half of the cells were treated with NMD-inhibitor cycloheximide (Sigma-Aldrich, dissolved in DMSO) to a final concentration of 100 µg/mL medium, while the other half was left untreated (DMSO only). After 5 hours cells were washed, and RNA extracted by RNeasy Mini kit (Qiagen). Further details are available upon request. RNA concentration and purity measures were obtained from NanoDrop 8000 spectrophotometer (ThermoFischer Scientific).

3.3.2 Reverse transcription and gene specific cDNA amplification

The OneStep RT-PCR Kit (Qiagen) was used for reverse transcription (RT) and gene specific cDNA amplification, using one fourth of the volumes given in the protocol (no Q-solution) and 15 ng input RNA. Location of primers are shown in Supplementary Fig. 2, and primer sequences are given in Supplementary Table 7.

Supplementary Figure 2: Location of primers for RT-PCR and sequencing of RT-PCR products



Primer pair L_int14-ex15 + R amplifies aberrantly spliced transcript only (containing 19 nt of intron 14). Pair L + R amplifies both WT transcript and aberrant transcript. Sequencing primer ex16_R reads through the 19 nt insertion, while sequencing with primer ex27_L covers c.3655G>T in exon 28.

Supplementary Table 7: Primers for RT-PCR and sequencing of RT-PCR products

Primer name	Sequence
L	GCCTAGCGATGACAATCCAG
L_int14-ex15	gacggaactcggATGTTACAA
R	GATGCCCAGAGTTTTCTCCA
ex27_L	CTGTTCATGGTGAGGCTGTG
ex16_R	CCAATTGAGAAACCACGGTTAG

All RNA samples were reverse transcribed and amplified by primer pair L_int14-ex15 + R. In addition, RNA from cultured lymphocytes was transcribed and amplified with primer pair L + R. Cycling conditions were as follows: 50°C for 30 min, 95°C for 15 min, followed by 38 cycles of 94°C for 45 sec / 55°C for 30 sec / 72°C for 2 min, and a final step of 72°C for 10 min. L_int14-ex15 + R amplicons were subject to automated electrophoresis on TapeStation 4200, using the D5000 High Sensitivity Kit (Agilent). All RT-PCR products were purified and sequenced as described above (Sanger sequencing of DNA), using sequencing primers ex16_R and ex 27_L.

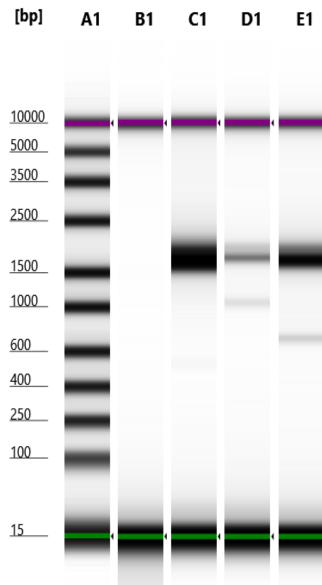
3.3.3 Results of RNA analyses

In families F1-F6, sequencing of the aberrantly spliced transcripts produced by the c.1909+22A allele revealed compound heterozygosity in all patients presenting with the c.1909+22G>A and c.3655G>T variants.

With regards to the occurrence of NMD, both variants (c.1909+22G>A and c.3655G>T) were shown to be subject to NMD. Sequencing of RT-PCR-products showed that patient lymphocytes cultured in the presence of NMD inhibitor produced both an aberrantly spliced transcript and a wild type (WT) transcript from the 1909+22A allele. The aberrant splice product contained an inclusion of the first 19 nucleotides of intron 14, introducing a premature stop-codon, as previously shown by Minnerop *et al.* (Minnerop *et al*., 2017). There was considerably more of the aberrant RT-PCR product when lymphocytes had been cultured with NMD inhibitor versus no inhibitor, confirming that the aberrantly spliced mRNA is subject to NMD (Supplementary Fig. 3).

Sequencing of RT-PCR products from patient lymphocytes verified that also the c.3655G>T nonsense variant leads to NMD-mediated degradation. There were minor amounts of RT-PCR product from the c.3655T allele when cells had been cultured without NMD inhibitor, while addition of inhibitor led to a marked increase in product from this allele (see Fig. 1C).

Supplementary Figure 3: NMD-analysis of aberrantly spliced mRNA produced by the c.1909+22A allele



TapeStation electrophoresis image of reverse transcribed and amplified mRNA from lymphocytes cultured with and without inhibitor of NMD. Primer pair L_int14-ex15 + R was used in order to amplify aberrant splice product only. (A1) Ladder. (B1) Patient F1-2 without inhibitor. (C1) Patient F1-2 with inhibitor. (D1) Patient F2-1 without inhibitor. (E1) Patient F2-1 with inhibitor.

3.4 Haplotype analysis of the c.1909+22G>A and c.3655G>T variants

A haplotype analysis was done to investigate whether founder effects could account for the relatively high frequencies of variants c.1909+22G>A and c.3655G>T in our cohort. In the absence of phasing information, we did an approximate analysis using incompatible homozygosity in the exome data to mark the outer limits of possible haplotype sharing. More precisely, after removing low quality variants (Filters: PASS; DP>5; GQ>29; RepeatMaster; SegDup) we identified the closest variants on either side of *POLR3A* where homozygosity for different alleles was observed among the probands carrying the c.1909+22G>A variant. A similar analysis was conducted for the subset of probands carrying the c.3655G>T variant. Finally, the same technique was applied to each pair of probands (excluding F10-1 who did not share causal *POLR3A* variants with any of the others), see Supplementary Table 9. The variants used, and the inferred limits of haplotype sharing are summarised in, see Supplementary Tables 8 and 9.

Supplementary Table 8: Variants and genotypes used in the haplotype analysis

POS	ID	REF	ALT	gnomAD	F1-2	F2-1	F3-1	F4-2	F5-1	F6-1	F7-1	F8-1
71921591	rs2271690	G	A	0.25	0/0	0/0	1/1	0/0	0/1	0/0	0/1	0/0
73472693	rs11599901	G	A	0.18	0/1	0/1	0/0	1/1	0/0	0/0	0/0	0/0
73537388	rs7917781	T	C	0.43	1/1	0/0		0/1	0/1	0/0	0/0	0/1
73822957	rs1530802	G	A	0.34	0/0	1/1	0/1	0/1	1/1	0/0	0/1	0/0
73827292	rs1530804	A	G	0.73	0/1	1/1	0/1	0/1	1/1	1/1	1/1	0/0
73856984	rs3312	A	G	0.5	1/1	0/1	0/1	0/1	1/1	1/1	0/0	1/1
74033899	rs55700783	C	T	0.024	0/0	1/1	0/0	0/0	0/0	0/1	0/0	0/0
74035041	rs1053639	T	A	0.39	0/1	0/0	0/0	0/0	0/0	0/1	1/1	0/1
74100642	rs9416018	G	A	0.42	0/1	0/1	0/1	1/1	1/1	0/1	0/1	0/0
75674740	rs2227571	C	T	0.41	0/0	1/1	1/1	1/1	1/1	0/1	0/1	1/1
76158177	rs2126763	C	T	0.65	0/1	1/1		0/1	0/1	0/1	0/0	1/1
76729632	rs3213967	C	T	0.32	0/0	0/0	0/1	0/1	1/1	0/0	0/0	0/1
76861680	rs6480771	T	C	0.56	0/1	0/0	1/1	0/0	0/0	0/0	0/0	0/1
77542558	rs2133705	C	T	0.42	0/1	0/0	1/1	0/0	1/1	0/0	0/1	0/1
77818594	rs7924288	C	T	0.4	1/1	1/1	0/1	0/1	0/0	1/1	0/1	1/1
78778734	rs16934182	G	A	0.54	1/1	1/1	0/0	0/1	0/0	0/1	0/0	0/1
79163867	rs596832	G	C	0.31	1/1	0/1	0/1	0/1	0/0	0/1	0/1	1/1
79742016	.	C	A	NA	0/1	0/1	0/1	0/1	0/1	0/1	0/0	0/0
79769273	rs191875469	C	T	0.0013	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
79769710	.	C	T	NA	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0
79777383	.	GCAC	G	NA	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1
81053475	rs1250560	G	A	0.43	0/1	0/0	0/1	0/0	0/0	1/1	0/1	0/1
81060474	rs1250549	T	C	0.55	0/1	0/0	0/1	0/0	1/1	1/1	0/1	0/1
81318565	rs17882088	C	T	0.14	0/0	0/0	0/1	0/0	1/1	0/0	0/0	0/0
81318621	rs17884396	C	T	0.34	0/0	0/0	0/0	0/0	1/1	0/0	0/0	0/0
81318820	rs17880349	G	A	0.55	1/1	1/1	1/1	0/0	0/0	0/1	0/1	1/1
81838656	rs3824727	A	C	0.1	0/0	0/1	0/0	0/0	0/0	0/0	1/1	0/0
81917708	rs1079242	A	G	0.44	0/1	1/1	0/1	0/0	0/0	0/0	0/0	0/1
82182328	rs1870143	G	A	0.35	0/1	0/1	0/1	0/0	0/0	0/0	0/1	1/1
82331347	rs59617064	A	G	0.25	0/0	0/1	1/1	0/0	0/0		1/1	0/0
82363340	rs7069048	C	A	0.34	0/0	0/1	1/1	0/1	0/1	0/0	0/1	0/0
84625107	rs478010	C	T	0.74	1/1	1/1	1/1	0/1	0/0	0/0	1/1	0/1
85957681	rs11200915	A	C	0.27	1/1	0/1	0/0	0/1	0/0	0/0	0/1	0/0
88445385	rs3740345	G	C	0.67	0/1	0/0	1/1	1/1	1/1	1/1	0/1	0/1
88451869	rs3740346	G	C	0.16	0/1	1/1	0/0	0/0	0/0	0/0	0/1	0/0
93000211	rs10881907	G	A	0.49	1/1	0/0	0/1	0/0	0/1	0/0	0/0	1/1

ALT = alternative allele; gnomAD = frequency of the ALT allele in the Genome Aggregation Database (http://gnomad.broadinstitute.org/); ID = reference SNP ID number; POS = genomic position on chromosome 10 (hg19); REF = reference allele.
Allelic codes in genotype columns: 0 = REF; 1 = ALT.
Colours: Blue = homozygous REF; orange = homozygous ALT; red = variants in *POLR3A*.

The maximal possible length of a shared haplotype shared by the probands carrying c.1909+22G>A, was 1.9 Mb. This was also the result when only including carriers of c.3655G>T. For the pairwise analysis, the upper limits for shared haplotypes ranged from 2.2 Mb to 17.3 Mb, with a median of 5.7 Mb. Since most of these maximal lengths were short, we concluded that a recent founder effect for either of the two variants was unlikely.

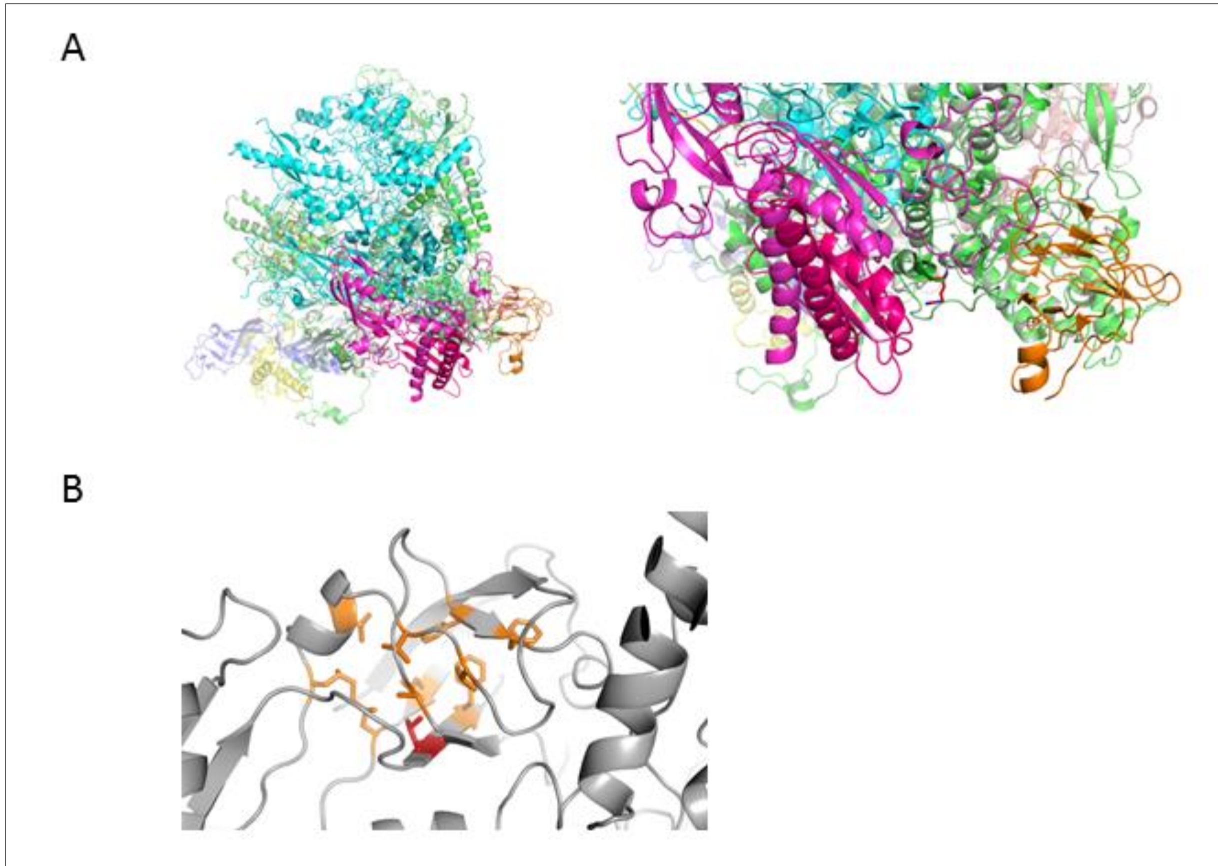
Supplementary Table 9: Maximum length of shared haplotypes

Samples compared	Max haplotype length (MB)	Closest upstream incompatible	Closest downstream incompatible
All c.1909+22G>A	1.89	79163867	81053475
All c.3655G>T	1.89	79163867	81053475
F1-2 vs. F2-1	17.33	75674740	93000211
F1-2 vs. F3-1	3.55	78778734	82331347
F1-2 vs. F4-2	5.64	75674740	81318820
F1-2 vs. F5-1	2.15	79163867	81318565
F1-2 vs. F6-1	11.09	73537388	84625107
F1-2 vs. F7-1	3.06	78778734	81838656
F1-2 vs. F8-1	10.28	75674740	85957681
F2-1 vs. F3-1	9.67	78778734	88445385
F2-1 vs. F4-2	7.28	74033899	81318820
F2-1 vs. F5-1	2.28	78778734	81060474
F2-1 vs. F6-1	7.23	73822957	81053475
F2-1 vs. F7-1	3.14	78778734	81917708
F2-1 vs. F8-1	14.42	74033899	88451869
F3-1 vs. F4-2	3.78	77542558	81318820
F3-1 vs. F5-1	4.46	76861680	81318621
F3-1 vs. F6-1	4.82	77542558	82363340
F3-1 vs. F7-1	4.98	76861680	81838656
F3-1 vs. F8-1	10.41	71921591	82331347
F4-2 vs. F5-1	3.52	77542558	81060474
F4-2 vs. F6-1	7.58	73472693	81053475
F4-2 vs. F7-1	7.80	74035041	81838656
F4-2 vs. F8-1	7.22	74100642	81318820
F5-1 vs. F6-1	3.23	77818594	81053475
F5-1 vs. F7-1	4.59	76729632	81318565
F5-1 vs. F8-1	2.15	79163867	81318565
F6-1 vs. F7-1	7.98	73856984	81838656
F6-1 vs. F8-1	8.36	73827292	82182328
F7-1 vs. F8-1	5.68	76158177	81838656

3.5 Protein structural analyses

The three-dimensional structure of the POLR3A protein was modelled using the Phyre2 protein fold recognition server (Kelley *et al.*, 2015). On the basis of the RBP1 subunit in the crystal structure of the Schizosacharomyces pombe RNA polymerase II at 3.6 Å resolution (PDB ID: 3H0G) (Spahr *et al.*, 2009), identified as the top rank template, POLR3A was modelled with 100% confidence. The homology model was then superimposed onto the equivalent RPB1 subunit in the S pombe RNA polymerase II structure.

Supplementary Figure 4: Structural modelling of the POLR3A variants c.1682G>A and c.1378_1380del



(A) **c.1682G>A may affect Pol III activity**. Structure of the large Pol II complex from S pombe (PDB ID: 3H0G), with the homology model of POLR3A in grey superimposed onto the equivalent RPB1 subunit in green. Arg561 is located between the subunits RPB8 (POLR2H) in orange and the heterodimer RPB3/RPB11 (POLR2C/POLR2J) in magenta and pink, respectively. (B) **c.1378_1380del disturbs the hydrophobic core**. The homology model of POLR3A shows that Val460, depicted in red, forms together with several other hydrophobic amino acids (depicted in orange) a hydrophobic core. Deletion of Val460 may potentially disturb this hydrophobic core and thereby affect the stability of the protein. Images have been generated with PyMol software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.).

4 Abbreviations

Arg	arginine
BAEP	brainstem auditory evoked potentials
CVLT	California Verbal Learning Test
CVMT	Continuous Visual Memory Test
EEG	electroencephalography
FLAIR	fluid-attenuated inversion recovery
Gln	glutamine
MLPA	multiplex ligation-dependent probe amplification
NCS	nerve conduction studies
nt	nucleotide
SEP	somatosensory evoked potentials
SER	spherical equivalent refraction
SNAP	sensory nerve action potentials
Val	Valine
VEP	visual evoked potentials
WES	whole exome sequencing

5 Supplementary references

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