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REPORT

Comprehensive genotype-phenotype analysis in POLR3-related disorders

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Summary

RNA polymerase III (RNA Pol III)-related disorders (POLR3-RDs) are a group of clinical entities characterized by causal variants in genes encoding RNA Pol III subunits, including *POLR3A*, *POLR3B*, *POLR1C*, *POLR1D*, *POLR3D*, *POLR3E*, *POLR3F*, *POLR3GL*, *POLR3H*, and *POLR3K*. These typically cause developmental phenotypes affecting the central nervous system; the eyes; connective tissues including bones, teeth, and endocrine axes; and the reproductive system. Similar phenotypes can be caused by variants in separate subunit genes (multigenic). In contrast, variants in the same gene can cause different phenotypes (pleiotropy), making genotype-phenotype correlation challenging. POLR3-RDs, though individually rare, have never been analyzed collectively. To bridge this gap, we developed an extensive database encompassing all published and unpublished cases of POLR3-RDs and conducted the first comprehensive genotype-phenotype correlation study across their entire spectrum. This work contributed new cases, representing 13% of all documented cases in the literature, along with 31 novel variants, accounting for 8% of all identified variants. This database was constructed by systematically reviewing the literature and integrating data from patients under the care of our international network of collaborators. The dataset includes genotype curation, bioinformatics, prior publications, and individual patient outcome information. By leveraging these comprehensive data, we were able to establish clear genotype-phenotype correlations for some pathogenic variants, which will help provide optimal clinical care and genetic counseling (including insights into disease phenotypes and progression) and offer valuable guidance for future clinical trial design and patient stratification.

RNA polymerase III (RNA Pol III)-related disorders (POLR3-RDs) are a group of clinical entities characterized by pathogenic variants in genes encoding Pol III subunits, including *POLR3A*, *POLR3B*, *POLR1C*, *POLR1D*, *POLR3D*, *POLR3E*,

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POLR3F, *POLR3G*, *POLR3GL*, *POLR3H*, and *POLR3K* (see Tables 1 and S1).[1–191] RNA Pol III is a constitutively expressed seventeen-subunit polymerase enzyme complex responsible for the transcription of essential non-coding RNAs involved in transcription, RNA processing, and translation (see Zhou and Van Bortle, 2023).[192–195] Canonical RNA Pol III transcripts include the entire pool of tRNAs and 5S ribosomal RNA, essential for translation; the small nuclear RNA U6, central to the spliceosome; and Alu-derived transcripts, including 7SL and BC200 RNAs functional in ubiquitous and neuron-specific signal recognition particles, respectively.[196,197] An expanding pool of research on RNA Pol III has produced conflicting results on its role in double-strand DNA repair[198–200] and novel implications in the control of the RNA Pol II transcriptome,[201] further expanding its role in biological processes.[201,202] RNA Pol III has unique subunits encoded by *POLR3** genes and shares some subunits with RNA Pol I and RNA Pol II, encoded by *POLR1** and *POLR2** genes, respectively. Complete loss of RNA Pol III-specific subunits is incompatible with life, underscoring that RNA Pol III is a foundational element of cellular function. Hypomorphic variants, which reduce but do not eliminate RNA Pol III function, are the underlying cause of the majority of this group of rare disease entities, often leading to significant morbidity and early mortality.

The most common POLR3-RD is POLR3-related hypomyelinating leukodystrophy (HLD; Phenotype MIM (PMIM): 607694, 614381, 616494, and 619310), also known as 4H leukodystrophy for its cardinal features of hypomyelination, hypodontia, and hypogonadotropic hypogonadism.[15,30,31,160] Other clinical presentations with predominant neurological involvement associated with pathogenic variants in genes encoding RNA Pol III subunits include hereditary spastic paraparesis and spastic ataxia (HSP/SA)[72]; mild and severe striatal variant forms (SVFs)[25,88]; isolated cerebellar atrophy (ICA)[142]; and ataxia, spasticity, demyelinating neuropathy with/without epilepsy (ASDN).[138,163] Other phenotypes such as intellectual disability or global developmental delay (GDD) with various overlapping features have also been reported.[135,154,157] Some subtypes of POLR3-RD have significant involvement of non-neurologic tissues or systems, with or without neurologic involvement. These include Wiedemann-Rautenstrauch syndrome (WRS; PMIM: 264090),[21,22,92] Treacher Collins syndrome (TCS; PMIM: 248390),[2] isolated hormonal disorder (IHD; e.g., idiopathic hypogonadotropic hypogonadism),[74,75,156] augmented susceptibility to *Varicella zoster* infection (VZV),[83,172] endosteal hyperostosis with oligodontia (EHO; PMIM: 619234),[170,185] and primary ovarian insufficiency (POI).[176] Collectively, reports of these clinical entities describe the spectrum of phenotypes caused by variants impacting RNA Pol III (refer to Table S2 for complete references).

Understanding POLR3-RD biology necessarily involves elucidating the relationships between genotypes and phenotypes. This has been challenging due to the large number of variants and combinations thereof and the fact that affected RNA Pol III subunit genes and their associated disease subtypes show complex and sometimes overlapping patterns of relationships (Table 1). Furthermore, phenotypes associated with subunits shared between RNA polymerases may be linked to effects on both RNA Pol III and the other polymerases. For example, craniofacial malformations seen in TCS are thought to be driven primarily by impaired RNA Pol I function, not by impaired RNA Pol III function. Furthermore, in some instances, different genes are associated with the same phenotype (i.e., multigenic disorders), and in other instances, unrelated phenotypes are associated with pathogenic variants in the same gene (i.e., pleiotropic disorders). When considering how to study and clarify these relationships, there are additional complicating factors to consider, including variable disease severity, variable expressivity of disease features, and overlapping phenotypes, i.e., the presence of features characteristic of more than one disorder (e.g., HLD and TCS).[4] Although genotype-phenotype correlations are key to understanding any genetic disease, only limited comparisons between specific phenotypes have been published,[15,33] leaving a description of genotype-phenotype relationships among all currently known disease presentations a key gap in POLR3-RD research.

To address this, we systematically searched the literature for information on POLR3-RD genotypes and phenotypes (see Figures 1 and S1 for search details). Literature was obtained from Medline & Embase, Embase Classic (Ovid), and Web of Science (Clarivate). Our search period included all database history until October 31, 2024. The Medline & Embase records were deduplicated automatically using Ovid and EndNote. A manual filtering process was first completed based on titles and abstracts, along with an additional record review for those with uncertain relevance. Records were automatically reviewed for the presence of genotype/phenotype information if they discussed human research and referred to a POLR3-RD diagnosis, RNA Pol III subunit, or *POLR* gene/genotype. Records were automatically excluded if they were associated exclusively with pathogenic variants in genes encoding non-RNA Pol III subunits (e.g., *POLR1A*). Literature was divided and reviewed by five authors (M.A.M.-R., S. G., Q.S., S.P., and A.D.) to ascertain the clinical information for patients reported in the literature, including supplementary data. The final database included patients from 195 published records that reported genetic associations between RNA Pol III subunits and rare developmental or neurological pathologies.

We next collated these published records with records from our international site-specific databases to create a list reflecting all currently known patients. Moreover, an international cross-sectional study was performed from 2019 to 2024, including a retrospective review of patients previously published by our lab and those of

Table 1. References to publications by POLR3-RD gene

Gene	OMIM #	Reference	Associated disorder categories
POLR1C	610060, 616494, 248390	Ching-López et al.[1]; Dauwerse et al.[2]; Di Bella et al.[3]; Gauquelin et al.[4]; Geschwind et al.[5]; Ghesh et al.[6]; Han et al.[7]; Kashiki et al.[8]; Kraoua et al.[9]; Liu et al.[10]; Mirchi et al.[11]; Naseer et al.[12]; Pelletier et al.[13]; Schlüter et al.[14]; Thiffault et al.[15]; Vanderver et al.[16]; Yadav et al.[17]; Yan et al.[18]	hypomyelinating leukodystrophy; Treacher Collins syndrome
POLR3A	614258, 607694, 264090	Ahmed et al.[19]; Al Yazidi et al.[20]; Arboleda et al.[21]; Arboleda et al.[22]; Arboleda et al.[23]; Arslan et al.[24]; Azmanov et al.[25]; Baviera-Muñoz et al.[26]; Bayram et al.[27]; Bekiesinska-Figatowska et al.[28]; Benkirane et al.[29]; Bernard et al.[30]; Bernard et al.,[31]; Buyukyilmaz et al.[32]; Báez-Becerra et al.[33]; Campopiano et al.[34]; Casamassa et al.[35]; Chen et al.[36]; Chorin et al.[37]; Cohen et al.[38]; Córdoba et al.[39]; D'Amore et al.[40]; Daoud et al.[41]; Di Bella et al.[3]; Di Donato et al.[42]; Dulski et al.[43]; Fang et al.[44]; Fellner et al.[45]; Fogel et al.[46]; French et al.[47]; Fu et al.[48]; Furukawa et al.[49]; Harting et al.[50]; Hengel et al.[51]; Hiraide et al.[52]; Hiraide et al.[53]; Infante et al.[54]; Jay et al.[55]; Ji et al.[56]; Khalifa et al.[57]; Khan et al.[58]; Kovalskaia et al.[59]; Kunii et al.[60]; Kyle et al.[61]; La Piana et al.[62]; Ledesma et al.[63]; Lessel et al.[64]; Lessel et al.[65]; Liu et al.[10]; Lynch et al.[66]; Macaron et al.[67]; Majethia et al.[68]; Maltese et al.[69]; McKenna et al.[70]; Midori et al.[71]; Minnerop et al.[72]; Mirchi et al.[11]; Monteiro et al.[73]; Montenegro et al.[74]; Montenegro et al.[75]; Moon et al.[76]; Morales et al.[77]; Morales-Rosado et al.[78]; Mrabet et al.[79]; Musumeci et al.[80]; Neocleous et al.[81]; Nikkhah et al.[82]; Ogunjimi et al.[83]; Paolacci et al.[84]; Paolacci et al.[85]; Patel et al.[86]; Pelletier et al.[13]; Peng et al.[87]; Perrier et al.[88]; Perrier et al.[89]; Potic et al.[90]; Potic et al.[91]; Rautenstrauch et al.[92]; Ruan et al.[93]; Ruggiero et al.[94]; Rydning et al.[95]; Saitsu et al.[96]; Schlüter et al.[14]; Schobers et al.[97]; Schüle et al.[98]; Shima et al.[99]; Shimojima et al.[100]; Sigatullina et al.[101]; Sun et al.[102]; Sytsma et al.[103]; Tailland et al.[104]; Tamura et al.[105]; Temel et al.[106]; Terao et al.[107]; Tewari et al.[108]; Timmons et al.[109]; Travaglini et al.[110]; Uygun et al.[111]; Venkatraman et al.[112]; Vázquez-López et al.[113]; Wambach et al.[114]; Wang et al.[115]; Wolf et al. Backspace[116]; Wolf et al.[117]; Wolf et al.[118]; Wu et al.[119]; Yan et al.[18]; Yang et al.[120]; Yang et al.[121]; Yau et al.[122]; Yoon Han et al.[123]; Yu et al.[124]; Zanette et al.[125]; Zea Vera et al.[126]; Zhang et al.[127]; de Assis Pereira Matos et al.[128]	hypomyelinating leukodystrophy; isolated hormonal disorder, mild and severe striatal forms; hereditary spastic paraparesis/hereditary spastic ataxia; Wiedemann-Rautenstrauch syndrome; VZV infection susceptibility
POLR3B	614366, 614381, 619742	Al Yazidi et al.[20]; Ando et al.[129]; Bai et al.[130]; Battini et al.[131]; Borella et al.[132]; Cayami et al.[133]; Colona et al.[134]; Daoud et al.[41]; Darvish et al.[135]; De Dominicis et al.[136]; DeGasperis et al.[137]; Djordjevic et al.[138]; Fogel et al.[46]; Fukuda et al.[139]; Gach et al.[140]; Geroldi et al.[141]; Ghoumid et al.[142]; Gutierrez et al.[143]; Hamdan et al.[144]; Haneda et al.[145]; Jurkiewicz et al.[146]; Krygier et al.[147]; Kulhánek et al.[148]; La Piana et al.[62]; Lin et al.[149]; Mahdieh et al.[150]; Mattijssen et al.[151]; Milovanova et al.[152]; Mirchi et al.[11]; Montenegro et al.[74]; Montenegro et al.[75]; Muirhead et al.[153]; Najmabadi et al.[154]; Ozgen et al.[155]; Pelletier et al.[13]; Perrier et al.[88]; Richards et al.[156]; Saghi et al.[157]; Saitsu et al.[96]; Schlüter et al.[14]; Symonds et al.[158]; Synofzik et al.[159]; Tétreault et al.[160]; Vanderver et al.[16]; Verberne et al.[161]; Wolf et al.[116]; Wolf et al.[117]; Wolf et al.[118]; Wu et al.[162]; Xue et al.[163]; Yang et al.[164]; Yang et al.[165]; Zhang et al.[166]	hypomyelinating leukodystrophy; isolated cerebellar atrophy; cerebellar hypoplasia + endosteal sclerosis/hyperostosis; isolated hormonal disorder; ataxia, spasticity, demyelinating neuropathy with/without epilepsy
POLR3D	187280	Macintosh et al., 2023[167]	hypomyelinating leukodystrophy
POLR3K	606007, 619310	Dorboz et al.[168]; Perrier et al.[169]	hypomyelinating leukodystrophy

References to literature sources for individual disorders are categorized by affected gene with appropriate Online Mendelian Inheritance in Man (OMIM) accessions.

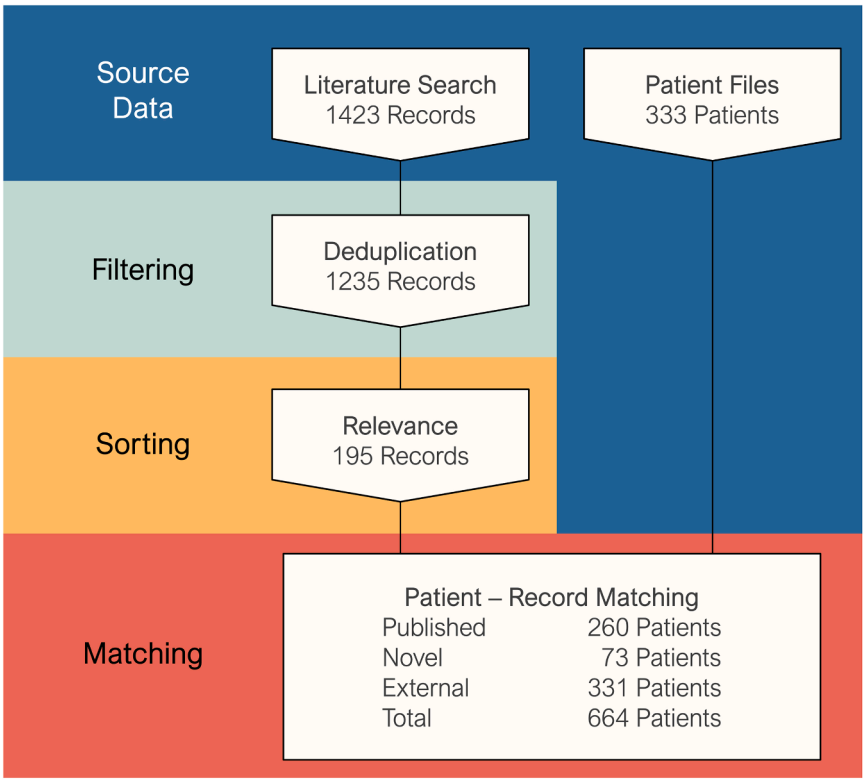

Figure 1. Assembly of patient data by collation of affected individuals from the literature with internal database entries

The literature was searched using Medline, Embase, and Web of Science for literature pertaining to *POLR3** genes and disease until October 31, 2024. The search details can be reviewed in Figure S1. Source data from the primary search yielded 1,422 records, from which 195 relevant records were filtered. Internally, we started with 333 patient files. These were collated together and reviewed for duplicates through a process of matching patients and previous publication records. The process was conservative, meaning that all patient entries are unique and mapped correctly to prior publications, but several patients that were potential duplicates have been excluded as a result of having the same variants and associated clinical data (e.g., birth year, sex, and/or case description).

collaborators. Patients were included if they had pathogenic variants, likely pathogenic variants, and/or variants of uncertain significance (VUS) in genes encoding RNA Pol III subunits and clinical disease consistent with a POLR3-RD. This research was approved by the Montreal Children's Hospital and McGill University Health Centre Research Ethics Boards (11-105-PED and 2019-4972). Informed consent was obtained from patients and/or legal guardians. Genetic variants from patient cohorts in our international group were identified by Sanger sequencing of specific genes, gene panels, or exome/genome sequencing using genomic DNA extracted from blood samples using standard protocols, with familial segregation analysis completed to validate variants when DNA was available, as previously described.[88]

In sum, we combined patient data from numerous international experts, including five major university hospitals (McGill University Health Centre [Canada], Children's Hospital of Philadelphia [United States], Amsterdam UMC [the Netherlands], University Hospital Düsseldorf [Germany], and Bambino Gesù Children's Hospital IRCCS [Italy]). Data collection included patient research identifiers, information on prior publication(s), genotype, and POLR3-RD diagnosis subtype. The data were collated with those of published cases, and duplicate entries were identified and removed using a conservative process (see Figure S1 for further detail on deduplication; Table S2). Deduplication of cases was performed across multiple variables, including variants, clinical information such as age of onset, familial history, clinical phenotype, year of birth, and referring physician or publication authorship.

In total, 664 affected individuals were included in the POLR3-RD database. All previously reported patients were included in the database, but not all were included in the described analysis. We excluded 113 published individuals with 64 unique variants from further analysis (analysis cohort: 551/664 cases, 83.0%) based on the following criteria: known non-RNA Pol III disorder (n = 51, e.g., patients with TCS and therefore a disorder mainly driven by RNA Pol I dysfunction and not RNA Pol III), uncertain RNA Pol III impact or atypical gene involvement (n = 23), incomplete genetic diagnosis (n = 16, e.g., ≥1 variant not defined), known alternate variant(s) that may be causal (n = 12), or limited clinical information (n = 11). For previously reported disease, *POLR3** variant allele frequency was not an exclusion criterion. For new cases, allele frequencies >0.01% were excluded as part of our variant-filtering process. Notably, the analysis cohort includes 73 novel affected individuals (73/551, 13.2%), including 36 novel *POLR3A* (36/325, 11.1% of *POLR3A*), 33 novel *POLR3B* (33/179, 18.4% of *POLR3B*), and 3 novel *POLR1C* (3/39, 7.7% of *POLR1C*) patients. Further breakdown of the prior publication statistics for affected individuals is given in Figures 2A and 2B and Table 2.

Genotypes and associated bioinformatics data included in the database were developed after all variants were curated using MobiDetails,[203] and nomenclature was normalized using VariantValidator[204] (Table S3). Identifiers for variants used in the database include HUGO Gene Nomenclature Committee (HGNC) and Human Genome Variation Society (HGVS) nomenclature. We used National Center for Biotechnology Information (NCBI) accessions (chromosome, transcript, and protein)

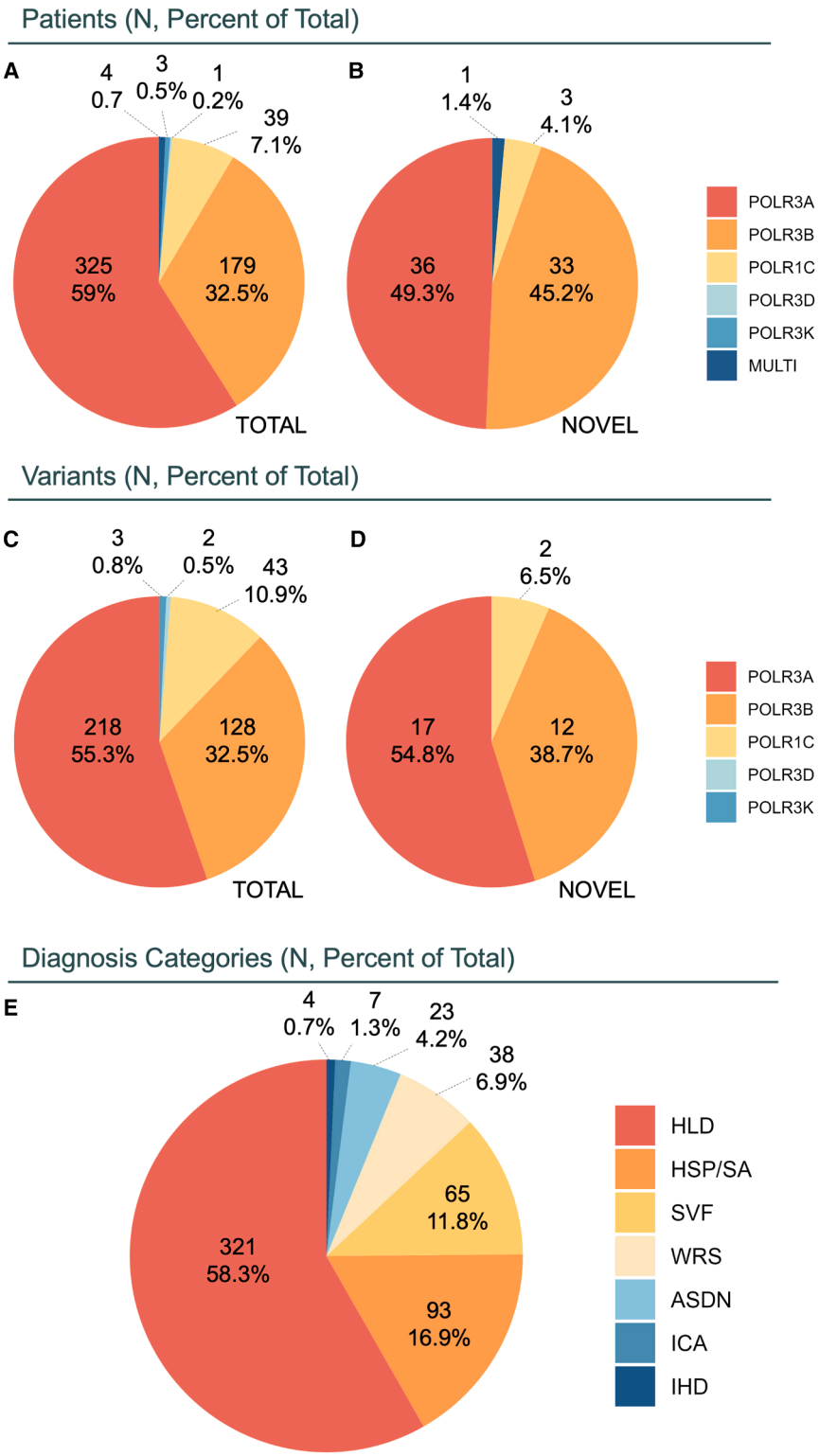

Figure 2. POLR3-RD database breakdown and summary of affected individuals

Patients with POLR3-RD in the analysis cohort are broken down by their causal genes and their status in the literature or as assessed by our team (for previously unpublished patients). Numeric values represent frequencies (counts) and percentages of the total.

(A) Total patients with POLR3-RD, including published and previously unpublished patients broken down by their causal genes.

(B) Previouly published affected individuals broken down by their causal genes.

(C) Total variants in the literature regardless of publication status, broken down by their causal genes.

(D) Novel variants that have not yet been published, broken down by genes affected.

(E) POLR3-RD affected individuals grouped by diagnosis category.

and included the corresponding Ensembl MANE (matched annotation from NCBI and EBI) transcript. Where discrepancies arose between validated nomenclature and published variants, the corrected variant and original variants were both included, but the corrected variant was used for analysis. Chromosome, coding sequence (CDS), and protein positions were obtained using validated variants. The Ensembl Variant Effect Predictor[205] was used to generate and/or fetch bioinformatic data including codon, exon/intron numbers, location annotation, nonsense-mediated decay (NMD) escape predictions, and UTR annotation. Pathogenicity of variants was established through manual variant curation in accordance with the American College of Medical Genetics (ACMG), Association for Molecular Pathology (AMP), and the College of American Pathologists (CAP) variant interpretation guidelines. GeneBe was used for manual curation,[206] followed by validation with Franklin (Genoox, Palo Alto, California). Automated curation by both tools was supplemented by manual curation for terms specific to patient phenotype (PP4), familial segregation (PP1 and BS4), *de novo* (PS2 and PM6), *in trans* variants (PM3 and BP2), and functional data (PS2 and PM6). Specific aspects of variant pathogenicity were assessed by *in silico* tools (SIFT,[207] PolyPhen2,[208] AlphaMissense,[209] EVE,[210] dbscSNV,[211] and SpliceAI[212]) for amino acid conservation, mutation tolerance, structural consequence, functional impact, and splicing effects. Codon sequences before and after variants were ascertained from Ensembl using the primary transcript as reference, and matching codon frequencies were matched to frequency values from the Kasuza Codon Usage Database.[213]

Table 2. Observations of published and novel/unpublished patients by affected gene

Gene	Patients (*n*)	Published	Unpublished	Novel (%)
POLR3A	325	289	36	11.1
POLR3B	179	146	33	18.4
POLR1C	39	36	3	7.7
POLR3D	1	1	0	–
POLR3K	3	3	0	–
Multiple*	4	3	1	25.0
Sum	**551**	**478**	**73**	**13.2**

Asterisk (*) denotes patients with >1 RNA Pol III subunit involvement. This group includes 1 novel patient harboring *POLR3A* and *POLR3B* variants (patient ID #598; see Table S2) and 3 patients with *POLR3A* and *POLR3B* variants previously published and known to our group (patient IDs #380, #381, and #403; see Table S2).[13]

Next, we sought to describe the cohort's variants at the population level. The number of novel/unpublished pathogenic variants in the database is broken down by RNA Pol III subunit gene in Table 3 and Figures 2C and 2D. In the analysis cohort, 551 patients harbored 394 unique variants, of which 31 are previously unpublished (31/394, 7.9%; Table S4). These variants were found as part of 388 unique genotypes (i.e., combinations of variants) in the analysis cohort. Among these unique genotypes, 95 (95/388, 24.5%) were reported in more than one patient, and 27 (27/388, 7.0%) were reported in more than two patients (frequency: 2–9 patients/genotype; Table S4). We observed no discrepancies in diagnoses among patients with the same genotype. 18 patients harbored more than one variant on any individual RNA Pol III subunit gene copy, i.e., had multiple variants in *cis* ("complex/multiallelic variants," 18/551, 3.3%; see Table S2). Among the three more commonly mutated genes (*POLR3A*, *POLR3B*, and *POLR1C*), variants associated with individual disease outcomes did not show a high degree of spatial bias/clustering at the transcript or protein level (Figures 3A–3J). Taken together, most POLR3-RDs are recessive, and affected patients have bi-allelic variants in a single RNA Pol III subunit gene, excepting rare cases (e.g., *de novo POLR3B* variants causing ASDN). While 25% of individual genotypes have been observed more than once, they result in the same diagnosis (e.g., *POLR3A*: c.1909+18G>A (p.Y637Cfs*14); *POLR3A*: c.1909+18G>A in 9 patients with HLD).

The database contains affected individuals with diagnoses categorized into 12 groups, 7 of which are included in the analysis cohort (HLD, HSP/SA, SVF, WRS, ASDN, ICA, and IHD; Figure 2E). The most frequent diagnosis was HLD, accounting for 321 cases (321/551, 58.3%). Patients are often classified in the literature as being either "typical" or "mild/adult-onset" when features are less severe or when patients are diagnosed incidentally/in adulthood. This suggests a potential underdiagnosed cohort with less severe clinical presentations that may be seen in adult clinics with minimal symptoms or that may not have been seeking medical attention. Genetic screening would increase diagnostic success in this cohort. The typical form is more common, accounting for 312 cases (312/321, 97.2%). However, there was a substantial number of cases where the age of onset was not explicitly discussed in the literature, and the number of mild affected individuals may be underestimated. For example, 3 of 5 known patients with homozygous c.1568T>A (p.V523E) *POLR3B* variants were diagnosed incidentally, suggesting a milder clinical course. Among the HLD cases, there were more patients with *POLR3B* (n = 143) than *POLR3A* (n = 131) bi-allelic pathogenic variants, even though *POLR3A* has a higher number of unique HLD-associated variants (*POLR3A* 121 vs. *POLR3B* 97). The difference in the number of known variants is not entirely explained by a relative difference in gene size (*POLR3A* chr10:77975149–78029515, 54.4 kb, vs. *POLR3B* chr12:106357748–106510198, 152.5 kb, [GRCh38.p14])

Table 3. Observations of published and novel/unpublished variants by affected gene

Gene	Variants (*n*)	Published	Unpublished	Novel (%)
POLR3A	218	201	17	7.8
POLR3B	128	116	12	9.4
POLR1C	43	41	2	4.7
POLR3D	2	2	0	–
POLR3K	3	3	0	–
All	**394**	**363**	**31**	**7.9**

Variants in the database are organized by gene and publication status.

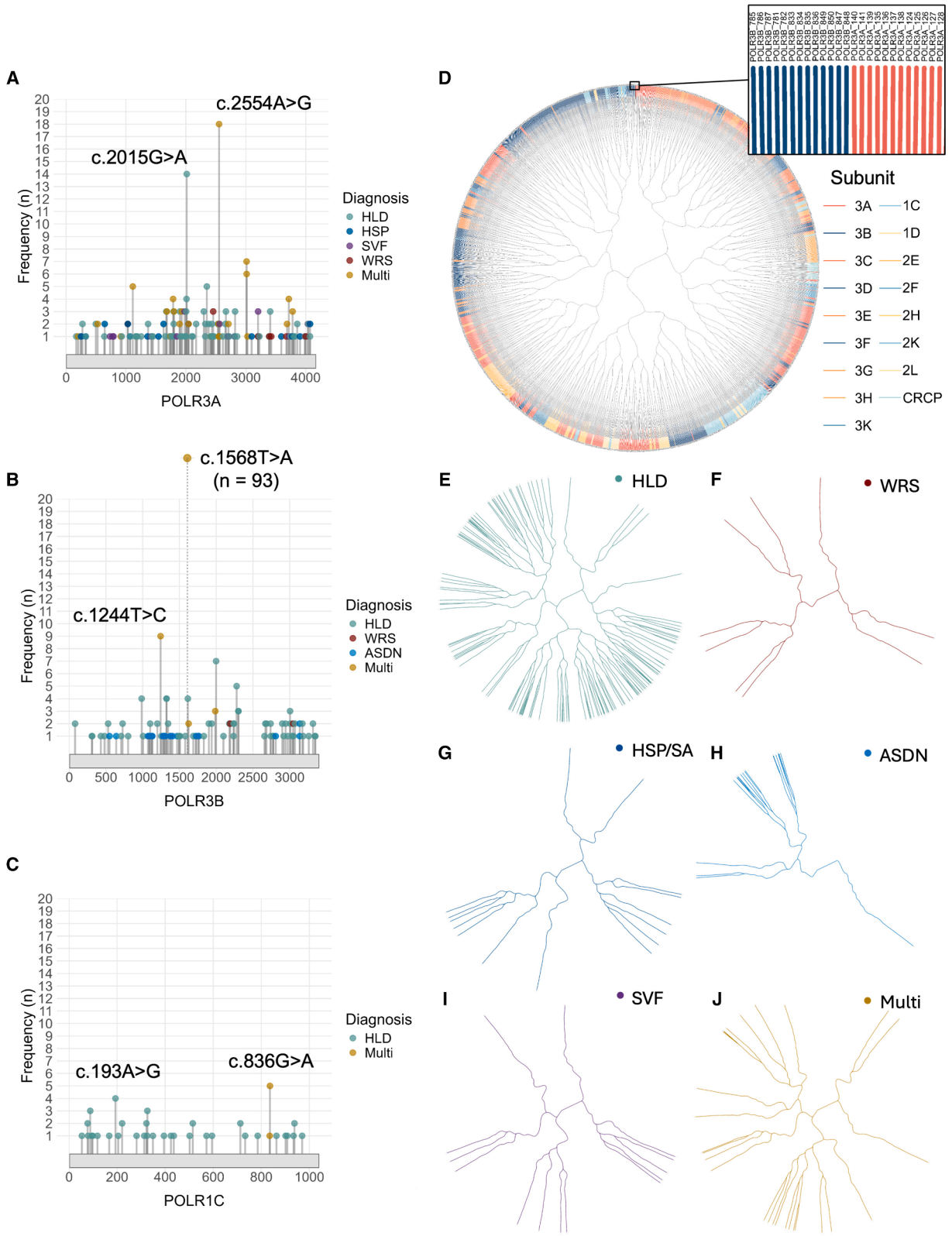

Figure 3. Structural relationships of missense variants associated with specific diagnostic categories at the coding sequence and protein levels

(A–C) Lollipop charts of the most frequently mutated genes, *POLR3A*, *POLR3B*, and *POLR1C*, demonstrating that missense variants typically occur throughout these genes and do not show spatial bias at the level of messenger RNA.

(D–J) Two-dimensional dendrogram representation of the apo protein structure (PDB: 7A6H) reveals that residue positions affected by missense variants causing non-HLD outcomes are sometimes associated with spatial bias/clusters (e.g., ASDN) but often occur in clusters with promiscuous variants.

(legend continued on next page)

but may be explained by the difference in transcript size given that most patients are diagnosed with exome sequencing (*POLR3A* 6,610 bp vs. *POLR3B* 4,183 bp). Among HLD variants, *POLR3A* exhibits greater diversity in variant types, while *POLR3B* is linked to a higher proportion of non-missense variants (Figures 4A and 4B). This is due to the high frequency of *POLR3B* c.1568T>A mutations among patients with *POLR3B* variants (96/193, 49.7%). Additionally, this release includes three novel cases of *POLR1C*-HLD, as well as the only known patient with disease-causing variants in *POLR3D* (Table S5),[167] and a recently published case involving a large deletion in *POLR3K*.[169] Together, HLD variants remain the largest population and most diverse group of variant types among POLR3-RDs (Figures 4A–4E).

HSP/SA is the second most frequent disorder category among patients with RNA Pol III subunit gene variants (Figure 5A). HSP/SA is associated with 57 defined *POLR3A* variants but is primarily defined by a splice-modifying variant that is found in all 93 cases (c.1909+22G>A, p.Y637Cfs*14). The *POLR3A* c.1909+22G>A variant is not exclusive to HSP/SA but shows a strong association. It is also found in 8 patients with Wiedemann Rautenstrauch syndrome (WRS) that have multiallelic genotypes where the c.1909+22G>A variant is always found in *cis* with an additional *POLR3A* variant and another pathogenic variant in *trans*. Notably, none of the patients with HSP/SA with this splicing variant have a second variant in *cis*, further outlining the strong genotype-phenotype correlation. The only known patient with two *POLR3A* c.1909+22G>A variants is categorized as having a mild SVF phenotype due to a concomitant *POLR3A* c.1771−7C>G (p.G548_Y637del/p.P591Mfs*9) (patient ID #66; see Table S2).[72] Indeed, the homozygous *POLR3A* c.1909+22G>A genotype is expected to cause no/extremely mild disease; 10 cases of homozygosity have been reported in gnomAD, suggesting that individuals harboring this genotype may be asymptomatic or perhaps incidentally diagnosed with mild findings late in life.[214] The similar and nearby *POLR3A* c.1909+18G>A variant is not associated with HSP/SA; however, it has been reported in 9 cases of HLD in homozygous genotypes and 2 cases of WRS in compound-heterozygous genotypes. This evidence thus suggests that the *POLR3A* c.1909+22G>A variant itself is mild, producing an HSP/SA outcome in combination with another variant in *trans*, while having an additional variant in *cis* favors WRS, a more severe outcome.

The SVFs of POLR3-RDs account for 65 cases and are associated with 41 unique variants. Like HSP/SA, SVF cases are also defined by the involvement of a canonical locus: two adjacent variants located in a polypyrimidine tract in intron 13 of *POLR3A* (Figure 5B). Each affected individual has at least one copy of *POLR3A* c.1771−6C>G (p.G548_Y637del/p.P591Mfs*9) (25/65, 38.5%) or *POLR3A* c.1771−7C>G/A (39/65, 60.0%) or both *in trans* (1/65, 1.5%). Both of these variants cause similar leaky splicing defects and are associated with specific disease manifestations and MRI features characterized by a neurodegenerative process affecting the striatum.[25,88] We hypothesized that disease severity is related to the combination of alleles, including the variant inherited *in trans* with the canonical splice variant (here, c.1771−6C>G or c.1771−7C>G). The hypothesis postulates that individuals with two canonical variants have the mildest course and cases with a canonical variant and an amorphic variant have the most severe course, while the severity of cases having other types of variants *in trans* (e.g.,

(A) *POLR3A* missense variants occur throughout the coding sequence and are associated with multiple diagnoses. *POLR3A* missense variants causing specific disease outcomes have low spatial bias. The most frequently observed *POLR3A* missense variants are c.2554A>G (p.M852V), associated with multiple outcomes, and c.2015G>A (p.G672E), associated uniquely with HLD outcomes.
(B) *POLR3B* missense variants occur throughout the coding sequence and are associated with multiple diagnoses. *POLR3B* missense variants causing specific disease outcomes have low spatial bias. The most frequently observed *POLR3B* missense variants are c.1568T>A and c.1244T>C (p.M415T), both associated with multiple outcomes.
(C) *POLR1C* missense variants occur throughout the coding sequence and are associated primarily with HLD outcomes. The most frequently observed *POLR1C* missense variants are c.836G>A (p.R279Q) and c.193A>G (p.M65V). The former is associated with a position affected in multiple disease outcomes.
(D) A two-dimensional circular dendrogram representation of the apo RNA Pol III structure developed by Girbig et al. (PDB: 7A6H) was assembled using alpha carbon positions of individual residues, and clustering was done using Ward's minimum variance method. As pictured, variants closer to each other along the perimeter of the dendrogram are more closely associated in the three-dimensional RNA Pol III structure. Cluster height is represented as (r = log2(cluster height)), and increasingly peripheral nodes merge clusters with lower spatial variance (i.e., residues that are closer together merge at more peripheral nodes). Residues of specific RNA Pol III subunits are colored according to the legend pictured on the right of the diagram.
(E) RNA Pol III residues affected by missense variants implicated in HLD occur broadly throughout the polymerase and display low spatial bias.
(F) RNA Pol III residues affected by missense variants implicated in WRS are rare. Many occur in clusters populated by residues that are also implicated in association with multiple outcomes.
(G) RNA Pol III residues affected by missense variants implicated in HSP/SA often occur in clusters populated by residues that are also implicated in association with multiple outcomes.
(H) Missense variants causing ASDN occur in *POLR3B*. These variants typically fall into one of three unique spatial clusters of POLR3B residues that are thought to be involved in nucleic acid interactions.
(I) Missense variants associated with SVF outcomes are typically found in clusters populated by residues that are also implicated in association with multiple outcomes.
(J) Residue positions affected by missense variants causing multiple outcomes (i.e., promiscuous variants) are pictured.
Underlying data for D-J can be found in Table S6.

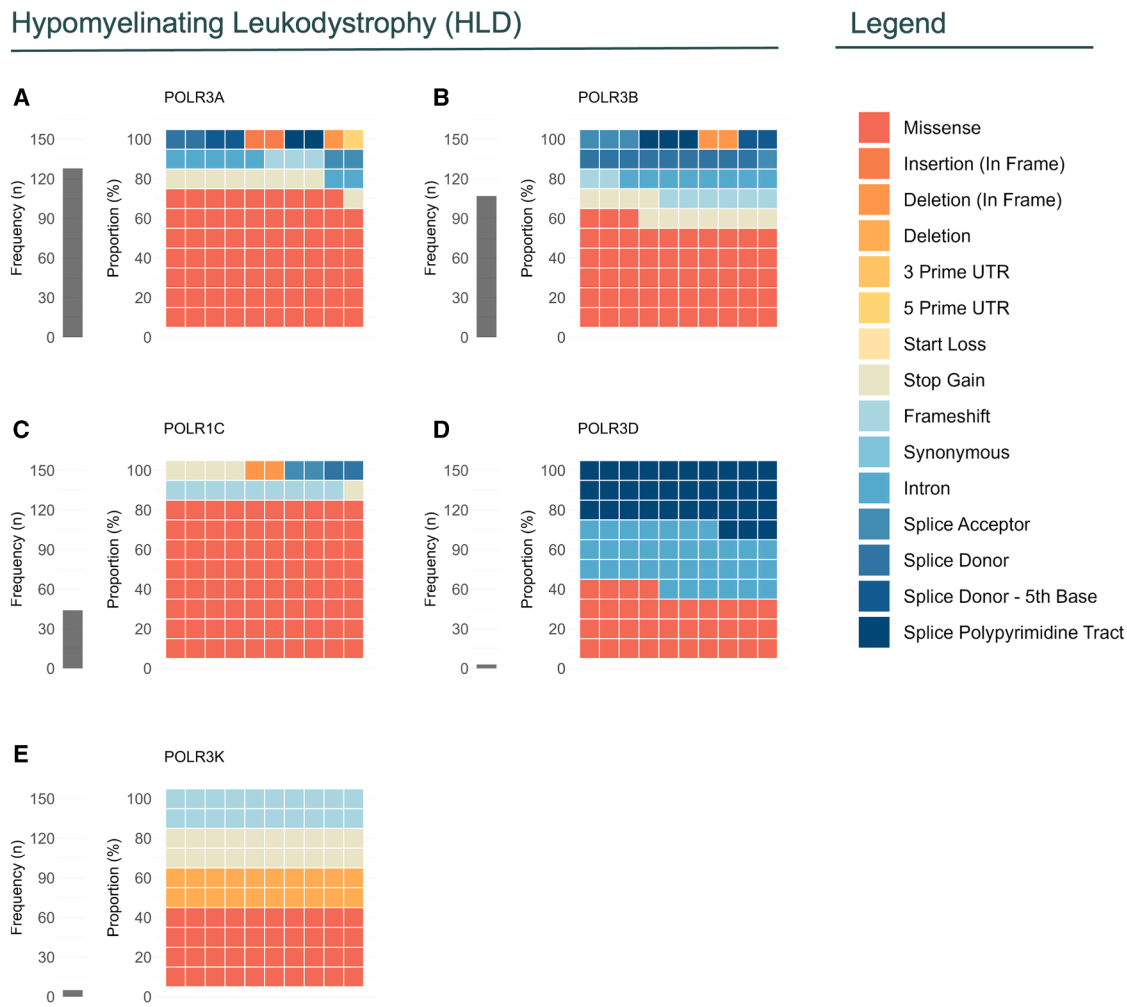

Figure 4. Hypomyelinating leukodystrophy variant frequency by gene (*n*) and type (proportion)
Frequency (no. of unique variants) and relative proportion (percentage) of types of variants among patients with POLR3-related hypomyelinating leukodystrophy. Variants can have multiple type classifications; the relative proportion represents the frequency of variants of a particular gene and type divided by the total number of instances of classification for that gene.
(A) HLD is associated with 121 unique *POLR3A* variants in the database, of which the majority are missense variants ($n = 88$). The next most frequent variant types are splice ($n = 16$) and stop-gain ($n = 12$) variants.
(B) *POLR3B* is associated with 97 unique variants. The most frequent are missense ($n = 57$) followed by splice ($n = 19$) and stop-gain ($n = 12$) variants.
(C) *POLR1C* variants ($n = 43$) are mainly missense ($n = 35$). The next most frequent are splicing ($n = 4$) and frameshift ($n = 4$) variants.
(D) *POLR3D* variants ($n = 2$) include an intronic variant in a polypyrimidine tract and another that is a missense variant.
(E) *POLR3K* is known for two missense variants and a large deletion involving the entirety of exon 3 (the final exon), predicted to cause frameshift/stop gain. The underlying basis of the low diversity among *POLR3D* and *POLR3K* variants is the low frequency of total observations in the population and not a relationship between these subunits and a particular type of variant.

missense or splice) is related to their severity. We tested this hypothesis using a total of 39 of 42 genotypes—excluding 3 with conflicting reports of severity.[10,44,50,115]

SVF cases ($n = 16$) with homozygous or compound heterozygous canonical variants (i.e., homozygous c.1771−6C>G or c.1771−7C>G; compound heterozygous c.1771−6C>G; c.1771−7C>G) all have mild disease. Of 21 SVF cases with missense variants found in *trans* with the canonical variant, 20 have mild disease (95.2%). Additionally, a single known SVF individual with a canonical variant and an in-frame deletion *in trans* exhibits mild disease.[123] In SVF cases where variants found in *trans* cause premature termination of translation (i.e., predicted loss of function), all 13 affected individuals are classified as having severly affected individuals. Of all severe cases ($n = 18$), 5 are not obviously associated with premature termination of translation. In 2 individuals, there are non-canonical splice variants in *trans* (patient IDs #251 and #252; see Table S2),[50] 2 are previously unpublished patients where the canonical allele is found in the context of a multiallelic genotype (>2 alleles, patient IDs #421 and #422; see Table S2), and the final affected individual is known to have a missense variant in *trans* that does not cause premature termination of translation (patient ID #253; see Table S2).[50] This *POLR3A* c.2713G>A (p.D905N) variant produces a deleterious substitution in a region of the RNA Pol III foot domain. A homologous domain in

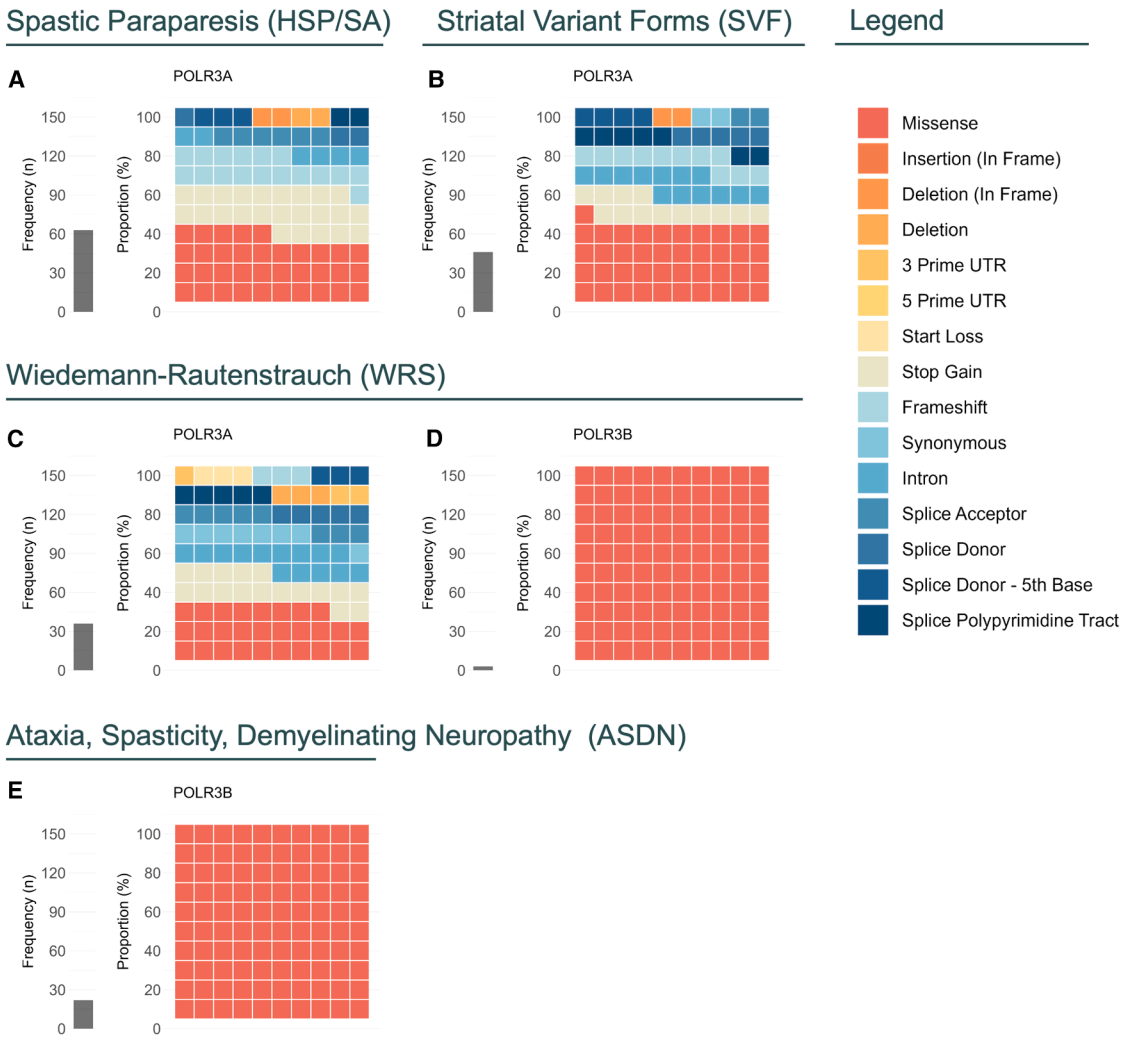

Figure 5. Variant frequency by gene (*n*) and type (proportion) among other presentations of POLR3-related disorders
Frequency (no. of unique variants) and relative proportion (percentage) of types of variants among patients with POLR3-related disorders. Variants can have multiple type classifications; the relative proportion represents the frequency of variants of a particular gene and type divided by the total number of instances of classification for that gene.
(A) HSP/SA is associated with 57 unique *POLR3A* variants. Observations of *POLR3A* missense variants ($n = 22$) are proportionally lower among patients with HSP/SA than among patients with HLD, while stop-gain variants ($n = 15$) are more frequent overall. The next most frequent variants among *POLR3A* are frameshift ($n = 11$) or splicing ($n = 11$) variants.
(B) SVF is associated exclusively with *POLR3A* variants ($n = 40$), and all cases have at least one allele that is either *POLR3A* c.1771−6C>G or *POLR3A* c.1771−7C>G. Examination of the total variant population reveals that most non-canonical variants are missense ($n = 19$), splicing ($n = 7$), stop gain ($n = 6$), or frameshift ($n = 5$). These exemplify typical variants found *in trans* in patients with SVF.
(C) Unique *POLR3A* variants ($n = 32$) associated with WRS are typically missense ($n = 10$), splice ($n = 10$), or stop gains ($n = 6$).
(D) There are 3 unique *POLR3B* missense variants associated with WRS.
(E) There are 22 unique *POLR3B* missense variants associated with cases of ASDN. These are all inherited *de novo*, making them unique as compared to all other variants causing POLR3-RD.

RNA Pol II has a role in regulating proximal promoter pausing and chromatin modification.[50,215,216] While these activities have not been shown for the homologous RNA Pol III domain, it has co-evolved with its RNA Pol II equivalent,[215] suggesting the possibility of an important functional role that produces effects similar to an amorphic variant when lost.

Of the 8 SVF affected individuals with a non-canonical splice variant in *trans* (non-c.1771−6C>G, non-c.1771−7C>G), 4 have mild disease (4/8, 50.0%). Bioinformatics predictions for splicing variants included here do not predict the severity of the outcome, while the predicted sequence ontology impact was not well correlated with clinical outcomes. Based on our understanding of the other cases, we hypothesize that severely affected individuals with non-canonical splice variants in *trans* should be those causing more substantial reductions in functional protein levels. To thoroughly address and validate this hypothesis, functional studies will be essential to draw definitive conclusions and provide a deeper understanding of the underlying mechanisms.

In the initial dataset, the TCS category included 45 cases implicating *POLR1D*, 5 cases implicating *POLR1C*, and 1 case with an 889 kb deletion involving *POLR3C*.[173] Previous work has shown that pathogenic variants causing POLR3-RDs, such as HLD, impact RNA Pol III, whereas those associated with TCS are known to impact RNA Pol I. Indeed, in TCS, pathogenic variants affect the dimerization interface between *POLR1C* and *POLR1D*, leading to disrupted RNA Pol I nucleolar localization and abnormal ribosomal biogenesis.[2,15,177,183,217–219] We observed that all patients harboring *POLR1C* variants with TCS have an allele with a small in-frame deletion or missense variant in the dimerization domain and a stop-gain variant on the other allele, except one patient who has a splicing variant predicted to cause loss of exon 8 (and loss of the dimerization domain) in *trans* with a missense variant (patient ID #40). By contrast, patients with HLD have missense-only genotypes or small deletions in *trans* with missense mutations but do not carry stop-gain variants in *trans*. Four patients with HLD and variants causing frameshift and premature termination of translation harbor missense variants outside of the dimerization domain in *trans* (patient IDs #319, #320, #322, and #324). Interestingly, it is possible to have phenotypic overlap between TCS and POLR3-RD. For example, one of the patients harboring a small in-frame deletion and a nonsense *POLR1C* variant is known to have both HLD and TCS features concurrently (patient ID #329; see Table S2).[4,11]

WRS is a diagnostic category comprising patients with a connective tissue disorder known to be caused by variants in *POLR3A* and *POLR3B* (Figures 5C and 5D). There are 35 patients with WRS caused by variants in *POLR3A*, and 3 with WRS caused by *POLR3B*. Of the 3 WRS-associated *POLR3B* variants, two are unique to WRS and are found in a compound heterozygous state (*POLR3B* c.2191G>C (p.E731Q); c.3046G>A (p.V1016M)),[162] while one that is homozygous in WRS (*POLR3B* c.1625A>G (p.N542S))[151] has also been observed in the heterozygous state in association with ICA (*POLR3B* c.1625A>G; c.707T>C (p.I236T)).

Overall, there are 29 genotypes associated with the WRS diagnosis category, including a variety of variant types. Start or stop sites are affected in 10 genotypes, causing initiator loss or premature termination of translation (10/29, 34.5%, $n = 13$ patients). There are 5 genotypes that have missense variants alone (5/29, 17.2%, $n = 9$ patients), including the only 2 *POLR3B* genotypes, 7 genotypes that have only splice variants (7/29, 24.1%, $n = 8$ patients), and 1 genotype that is homozygous for a synonymous *POLR3A* variant (1/29, 3.4%, $n = 1$ patient). The remaining genotypes, including 3 of the genotypes affected by premature termination of translation, have alleles with mixed downstream consequences. Additionally, 7 total genotypes are multiallelic and include the *POLR3A* c.1909+22G>A, c.3337−11T>C (p.I1113_E1143del) alleles found in *cis* (7/29, 24.1%, $n = 8$ patients with $n = 7$ genotypes; genotype IDs #89, #262, #271, #272, #279, #293, and #354; see Table S4).

We found that, invariably, genotypes causing WRS included at least one allele that could be considered more severe, for example, null-equivalent variants or missense variants with higher predicted functional impact according to bioinformatics tools (e.g., SIFT, PolyPhen2, AlphaMissense, and EVE). Although in many cases, the severity of variants was self-evident (e.g., variants causing premature termination), cases involving only splicing or missense variants are less so. Because splicing variants require experimental evaluation, we analyzed SIFT, PolyPhen2, and AlphaMissense severity of missense variants associated with each of the WRS cases having genotypes with missense alleles only. A non-parametric two-sided Wilcoxon rank-sum test with continuity correction (data are not normally distributed) was performed on the most severe missense allele for each WRS case. There was a statistical difference in SIFT and PolyPhen2 severity scores of these WRS variants vs. all other non-WRS variants in the database (SIFT $W = 6355.5$, $p = 1.23 \times 10^{-9}$; PolyPhen2 $W = 5507.5$, $p = 1.62 \times 10^{-4}$). Evaluation of the same groups' AlphaMissense severity using parametric two-tailed Student's t test with Welch's correction (data are normally distributed) revealed a statistically significant difference ($t = 3.022$, degrees of freedom(df) = 9.361, $p = 0.0138$). This result supports the validity of a potential association between exceptionally severe alleles and WRS outcomes in missense-only cases, but bioinformatics tools alone are not sufficient to conclude whether WRS cases are always associated with exceptionally severe variants. Further functional validation is required to support the hypothesis suggested by these *in silico* results. Moreover, it would be necessary to extend the analysis to confirm a similar association among cases with splicing variants only.

Finally, 22 known *de novo* missense variants in *POLR3B* are associated with ASDN ($n = 23$ cases; Figure 5E).[129,134,136,138,139,141,149,158,163] These variants are unique to this diagnosis and never associated with other POLR3-RDs, highlighting their unique mechanism, which is proposed to involve altered function at DNA-interacting elements in RNA Pol III.[138,158] There are a handful of even rarer presentations of POLR3-RDs, including IHD cases such as idiopathic hypogonadotropic hypogonadism and severe VZV cases caused by *POLR3A* variants that are dominant and specific to viral susceptibility, ICA, POI, and EHO. Together, these account for 32 cases (32/664, 4.8% of the total database). Among them, cases of IHD, VZV, and ICA account for 25 cases, or 78.1% of this category of rare presentations.

In conclusion, we developed a comprehensive database of all known patients with POLR3-RDs and their associated diagnoses and disease-causing variants. This allowed us to elucidate genotype-phenotype relationships, including notable associations between specific variants and clinical outcomes, such as the link between *POLR3A* c.1771−6C>G and c.1771−7C>G with SVF or *POLR3A* c.1909+22G>A and HSP/SA. Additionally, we explored

the molecular pathogenesis underlying distinct phenotypic presentations, such as WRS. Notably, this study introduces 73 new cases and 31 novel variants to the literature, significantly expanding the current knowledge of genotype-phenotype correlations across the POLR3-RD spectrum. These findings provide immediate value for patient and family counseling while also contributing to a deeper understanding of disease presentation and progression, which is critical for designing future clinical trials and ensuring effective patient stratification.

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Author contributions

M.A.M.-R. wrote the manuscript, developed the database, analyzed data, and contributed to the literature review. S.P. developed the database and contributed to literature review. S.G. developed the database, variant validation, and curation methods and data and contributed to literature review. A.D. developed the database and contributed to literature review. Q. S. developed variant validation and curation methods and data. M.G. developed RNA Pol III models and contributed to understanding variant severity. A.D.M. developed RNA Pol III models and contributed to understanding variant severity. A.M.P., D.L. R., D.D.A.P., P.O., L.M.K., S.K., K.C., L.G., B.B., M.M., B.L., T.G., M.E.S., R.S., E.B., F.N., D.T., A.V., and N.I.W. contributed patients and expertise and participated in the editing of the manuscript. C.W.M. contributed expertise, supervised student authors, and participated in developing the projects and editing the manuscript. G.B. contributed patients and expertise, supervised student authors, and participated in developing projects and editing the manuscript.

Declaration of interests

M.M. serves on the medical advisory board of the German Heredo Ataxia Society (DHAG) and received honoraria from Biogen, unrelated to this research. G.B. is/was a consultant for Calico (2023–present), Orchard Therapeutics (2023), Passage Bio, Inc. (2020–2022), and Ionis (2019). She is/was a site investigator/sub-I for trials and natural histories of Calico/Abbvie (2024–present), Ionis (2021–present), Shire/Takeda (2020–2021), Passage Bio (2021–2024), Bluebird Bio (2019), Regenxbio (2021–present), and Denali (2022–present). She has received an unrestricted educational grant from Takeda (2021–2022). She serves on the Scientific Advisory board of the Pelizaeus-Merzbacher Foundation, the Yaya Foundation Scientific and Clinical Advisory Council, and the Medical and Scientific Advisory Board of the United Leukodystrophy Foundation.

Web resources

AlphaMissense, https://alphamissense.hegelab.org
dbscSNV, http://www.liulab.science/dbscsnv.html
Ensemble VEP, https://ensembl.org/info/docs/tools/vep/index.html
EVE, https://evemodel.org
Franklin (Genoox), https://franklin.genoox.com/clinical-db/home
GeneBe, https://genebe.net
OMIM, https://www.omim.org
PDB, https://www.rcsb.org
PolyPhen2, http://genetics.bwh.harvard.edu/pph2/
SIFT, https://sift.bii.a-star.edu.sg
SpliceAI, https://spliceailookup.broadinstitute.org

Supplemental information

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