



## A prospective trial comparing programmable targeted long-read sequencing and short-read genome sequencing for genetic diagnosis of cerebellar ataxia

Haloom Rafehi, Liam G. Fearnley, Justin Read, et al.

*Genome Res.* 2025 35: 769-785 originally published online February 27, 2025  
Access the most recent version at doi:[10.1101/gr.279634.124](https://doi.org/10.1101/gr.279634.124)

---

**References** This article cites 80 articles, 6 of which can be accessed free at:  
<http://genome.cshlp.org/content/35/4/769.full.html#ref-list-1>

**Creative Commons License** This article is distributed exclusively by Cold Spring Harbor Laboratory Press for the first six months after the full-issue publication date (see <https://genome.cshlp.org/site/misc/terms.xhtml>). After six months, it is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at <http://creativecommons.org/licenses/by-nc/4.0/>.

**Email Alerting Service** Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).



---

To subscribe to *Genome Research* go to:  
<https://genome.cshlp.org/subscriptions>

## Research

# A prospective trial comparing programmable targeted long-read sequencing and short-read genome sequencing for genetic diagnosis of cerebellar ataxia

Haloom Rafehi,<sup>1,2,43</sup> Liam G. Fearnley,<sup>1,2,43</sup> Justin Read,<sup>3,4,43</sup> Penny Snell,<sup>3</sup> Kayli C. Davies,<sup>3,5</sup> Liam Scott,<sup>1</sup> Greta Gillies,<sup>3</sup> Genevieve C. Thompson,<sup>3,5</sup> Tess A. Field,<sup>3</sup> Aleena Eldo,<sup>3</sup> Simon Bodek,<sup>6</sup> Ernest Butler,<sup>7</sup> Luke Chen,<sup>8</sup> John Drago,<sup>9,10</sup> Himanshu Goel,<sup>11</sup> Anna Hackett,<sup>11,12</sup> G. Michael Halmagyi,<sup>13,14</sup> Andrew Hannaford,<sup>15,16,17</sup> Katya Kotschet,<sup>18</sup> Kishore R. Kumar,<sup>19,20,21,22</sup> Smitha Kumble,<sup>23,24</sup> Matthew Lee-Archer,<sup>25</sup> Abhishek Malhotra,<sup>26</sup> Mark Paine,<sup>27</sup> Michael Poon,<sup>28</sup> Kate Pope,<sup>3</sup> Katrina Reardon,<sup>9,29</sup> Steven Ring,<sup>30</sup> Anne Ronan,<sup>12,31</sup> Matthew Silsby,<sup>15,16,17</sup> Renee Smyth,<sup>32</sup> Chloe Stutterd,<sup>23</sup> Mathew Wallis,<sup>33,34</sup> John Waterston,<sup>4</sup> Thomas Wellings,<sup>35</sup> Kirsty West,<sup>36</sup> Christine Wools,<sup>37,38</sup> Kathy H.C. Wu,<sup>32,39,40</sup> David J. Szmulewicz,<sup>41,42</sup> Martin B. Delatycki,<sup>3,5,23</sup> Melanie Bahlo,<sup>1,2,44</sup> and Paul J. Lockhart<sup>3,5,44</sup>

The cerebellar ataxias (CAs) are a heterogeneous group of disorders characterized by progressive incoordination. Seventeen repeat expansion (RE) loci have been identified as the primary genetic cause and account for >80% of genetic diagnoses. Despite this, diagnostic testing is limited and inefficient, often utilizing single gene assays. This study evaluates the effectiveness of long- and short-read sequencing as diagnostic tools for CA. We recruited 110 individuals (48 females, 62 males) with a clinical diagnosis of CA. Short-read genome sequencing (SR-GS) was performed to identify pathogenic RE and also non-RE variants in 356 genes associated with CA. Independently, long-read sequencing with adaptive sampling (LR-AS) was performed to identify pathogenic RE. SR-GS provided a genetic diagnosis for 38% of the cohort (40/110) including seven non-RE pathogenic variants. RE causes disease in 33 individuals, with the most common condition being SCA27B ( $n = 24$ ). In comparison, LR-AS identified pathogenic RE in 29 individuals. RE identification for the two methods was concordant apart from four SCA27B cases not detected by LR-AS due to low read depth. For both technologies manual review of the RE alignment enhances diagnostic outcomes. Orthogonal testing for SCA27B revealed a 15% and 0% false positive rate for SR-GS and LR-AS, respectively. In conclusion, both technologies are powerful screening tools for CA. SR-GS is a mature technology currently used by diagnostic providers, requiring only minor changes in bioinformatic workflows to enable CA diagnostics. LR-AS offers considerable advantages in the context of RE detection and characterization but requires optimization before clinical implementation.

[Supplemental material is available for this article.]

<sup>1</sup>Population Health and Immunity Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia;

<sup>2</sup>Department of Medical Biology, University of Melbourne, Parkville, Victoria 3052, Australia; <sup>3</sup>Bruce Lefroy Centre, Murdoch

Children's Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia; <sup>4</sup>Department of Neuroscience, Central

Clinical School, Monash University, The Alfred Centre, Melbourne, Victoria 3004, Australia; <sup>5</sup>Department of Paediatrics, University of

Melbourne, Royal Children's Hospital, Parkville, Victoria 3052, Australia; <sup>6</sup>Austin Health, Heidelberg, Victoria 3084, Australia;

<sup>7</sup>Monash Medical Centre, Clayton, Victoria 3168, Australia; <sup>8</sup>Department of Neurology, Alfred Hospital, Melbourne, Victoria 3004,

Australia; <sup>9</sup>Department of Medicine, St Vincent's Hospital, University of Melbourne, Fitzroy, Victoria 3065, Australia; <sup>10</sup>Florey Institute

of Neuroscience and Mental Health, Parkville, Victoria 3052, Australia; <sup>11</sup>Hunter Genetics, Hunter New England Health Service,

<sup>43</sup>These authors are joint first authors and contributed equally to this work.

<sup>44</sup>These authors are joint senior authors and contributed equally to this work.

Corresponding author: [paul.lockhart@mcri.edu.au](mailto:paul.lockhart@mcri.edu.au)

Article published online before print. Article, supplemental material, and publication date are at <https://www.genome.org/cgi/doi/10.1101/gr.279634.124>.

© 2025 Rafehi et al. This article is distributed exclusively by Cold Spring Harbor Laboratory Press for the first six months after the full-issue publication date (see <https://genome.cshlp.org/site/misc/terms.xhtml>). After six months, it is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at <http://creativecommons.org/licenses/by-nc/4.0/>.

Waratah, New South Wales 2298, Australia;<sup>12</sup>University of Newcastle, Callaghan, New South Wales 2308, Australia;<sup>13</sup>Neurology Department, Royal Prince Alfred Hospital, Camperdown, New South Wales 2050, Australia;<sup>14</sup>Central Clinical School, University of Sydney, Camperdown, New South Wales 2050, Australia;<sup>15</sup>Department of Neurology, Westmead Hospital, Hawkesbury Westmead, New South Wales 2145, Australia;<sup>16</sup>Brain and Nerve Research Centre, Concord Clinical School, University of Sydney, Camperdown, New South Wales 2050, Australia;<sup>17</sup>Department of Neurology, Concord Repatriation General Hospital, Concord, New South Wales 2139, Australia;<sup>18</sup>Department of Clinical Neurosciences, St Vincent's Hospital, University of Melbourne, Fitzroy, Victoria 3065, Australia;<sup>19</sup>Molecular Medicine Laboratory and Neurology Department, Concord Repatriation General Hospital, Concord, New South Wales 2139, Australia;<sup>20</sup>Faculty of Medicine and Health, The University of Sydney, Camperdown, New South Wales 2050, Australia;<sup>21</sup>Genomics and Inherited Disease Program, The Garvan Institute of Medical Research, Darlinghurst, New South Wales 2010, Australia;<sup>22</sup>School of Medicine, University of New South Wales, Sydney, New South Wales 2052, Australia;<sup>23</sup>Victorian Clinical Genetics Services, Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia;<sup>24</sup>Department of Clinical Genetics, Austin Health, Viewbank, Victoria 3084, Australia;<sup>25</sup>Department of Neurology, Launceston General Hospital, Launceston, Tasmania 7250, Australia;<sup>26</sup>Department of Neuroscience, University Hospital Geelong, Geelong, Victoria 3220, Australia;<sup>27</sup>Department of Neurology, Royal Brisbane and Women's Hospital, Herston, Queensland 4006, Australia;<sup>28</sup>Neurology Footscray, Footscray, Victoria 3011, Australia;<sup>29</sup>Department of Neurology, St Vincent's Hospital, University of Melbourne, Fitzroy, Victoria 3065, Australia;<sup>30</sup>Albury Wodonga Health, West Albury, New South Wales 2640, Australia;<sup>31</sup>Newcastle Medical Genetics, Lambton, New South Wales 2299, Australia;<sup>32</sup>St Vincent's Clinical Genomics, St Vincent's Hospital, Darlinghurst, New South Wales 2010, Australia;<sup>33</sup>Tasmanian Clinical Genetics Service, Tasmanian Health Service, Royal Hobart Hospital, Hobart, Tasmania 7001, Australia;<sup>34</sup>School of Medicine and Menzies Institute for Medical Research, University of Tasmania, Hobart, Tasmania 7000, Australia;<sup>35</sup>Department of Neurology, John Hunter Hospital, New Lambton Heights, New South Wales 2305, Australia;<sup>36</sup>Genomic Medicine, The Royal Melbourne Hospital, Parkville, Victoria 3052, Australia;<sup>37</sup>Department of Neurology, Calvary Health Care Bethlehem, Caulfield South Victoria 3162, Australia;<sup>38</sup>Department of Neurology, The Royal Melbourne Hospital, Parkville, Victoria 3052, Australia;<sup>39</sup>School of Medicine, University of Notre Dame, Darlinghurst, New South Wales 2010, Australia;<sup>40</sup>Discipline of Genomic Medicine, Faculty of Medicine and Health, University of Sydney, Camperdown, New South Wales 2050, Australia;<sup>41</sup>Royal Victorian Eye and Ear Hospital, East Melbourne, Victoria 3002, Australia;<sup>42</sup>Bionics Institute, East Melbourne, Victoria 3002, Australia

Genetic technologies are transforming healthcare by enabling genomic medicine, an emerging discipline that uses genetic data to improve clinical care and outcomes. High-throughput sequencing (HTS) is an important tool for genomic medicine, underpinning discovery, diagnostics, and understanding of disease mechanisms (Rehm 2017). Genomic medicine provides diagnostic certainty, key information for prognosis, genetic counseling, and reproductive planning and facilitates the development and delivery of improved treatments targeted to disease mechanisms (McCarthy et al. 2013; García-Foncillas et al. 2021). There is a significant treatment pipeline for personalized therapies, including gene and pharmacological therapies. For instance, treatment for spinocerebellar ataxia 27B (SCA27B) shows promising therapeutic outcomes with 4-aminopyridine (4-AP) (Wilke et al. 2023). However, genetic technologies have had limited success in extending genomic medicine's benefits to disorders caused by pathogenic nucleotide repeat expansions (REs).

RE disorders typically have significant neurological and/or neuromuscular outcomes. They occur when a segment of repetitive DNA, termed a short tandem repeat (STR), expands beyond a gene-specific threshold. STR is composed of tandem arrays of 1–12 bp sequence motifs and constitute ~6% of the human genome (Willems et al. 2014; Mousavi et al. 2019). To date, the genetic basis of 79 RE disorders has been described (for reviews, see Cortese et al. 2024; Rajan-Babu et al. 2024), including polyglutamine disorders such as Huntington's disease, fragile X syndrome, and hereditary cerebellar ataxias (CA). RE can occur within exonic, intronic, untranslated, or intergenic regions and are associated with various pathogenic mechanisms including loss-of-function, gain-of-function, transcriptional dysregulation, protein misfolding/aggregation, and repeat-associated non-AUG (RAN) translation. Some disorders result from combinations of these

mechanisms (for review, see Depienne and Mandel 2021). Collectively, RE disorders cause some of the most common genetic disorders seen by neurologists (Paulson 2018). Moreover, the RE-mediated disease burden is significantly underestimated. RE is difficult to amplify using standard molecular technologies such as polymerase chain reaction (PCR) (Schlötterer and Tautz 1992) and there is evidence suggesting that additional RE remains to be identified (Depienne and Mandel 2021). In addition, RE is often located in noncoding DNA, "hidden" from the gene, panel- and exome-based discovery approaches. Pathogenic RE embedded deep within nonpathogenic STR (Rosenbohm et al. 2022) or composed of novel motifs presents additional challenges (Dolzhenko et al. 2020). The timeline of RE identification demonstrates the issues. While the first pathogenic RE was described in 1991 (La Spada et al. 1991; Oberlé et al. 1991; Verkerk et al. 1991), ~50% of pathogenic RE identified to date have been described in the last 10 years. The acceleration in discovery has been driven largely by the development of PCR-free short-read and long-read genomic sequencing technologies and associated bioinformatic tools (Depienne and Mandel 2021; Gall-Duncan et al. 2022; Read et al. 2023).

RE disorders also challenge diagnostic service providers, impacting the implementation of genomic medicine. One issue is the relatively low incidence of some RE disorders; in many settings, it is not economically viable to provide current, RE-specific diagnostic testing for loci/conditions with low prevalence (Stevanovski et al. 2022). In addition, technological challenges exist in the molecular characterization of expanded repeat DNA. Traditional diagnostic techniques such as Southern blot, PCR sizing, and repeat-primed PCR (RP-PCR) analysis are labor-intensive, imprecise and do not scale easily to large cohorts requiring testing; generally, each individual RE requires a separate assay with specific probes or primers (Bahlo et al. 2018). Exome analysis using short-

read HTS has become a mainstay diagnostic methodology for diagnosing pathogenic single nucleotide variants (SNVs), small insertions and deletions (indel), or copy number variants (CNVs) but is yet to be widely implemented in RE diagnostics (Lappalainen et al. 2019). While the technology can successfully identify RE (Tankard et al. 2018), there are limitations. Genomic DNA is fragmented and size-selection is performed to isolate inserts of ~250–1000 bp. Therefore, larger RE may not be represented in this process. In addition, the capture probes used may be disrupted by the RE and not efficiently hybridize to the expanded allele. Moreover, like other high-throughput diagnostic technologies such as multiplex amplicon sequencing and targeted gene panel analysis, exome sequencing uses PCR amplification of the captured target sequences, which also may not work efficiently, or at all, with large and complex RE. While bioinformatic tools now available in the research space allay concerns about the effects of PCR stutter and nonunique mapping of repetitive HTS reads (Bahlo et al. 2018; Tankard et al. 2018), they are yet to be widely implemented in diagnostic pipelines. In addition, another shortcoming of exome sequencing for diagnosing pathogenic RE is that most loci are outside coding regions and therefore not captured by current “off-the-shelf” library preparation kits.

Hereditary CAs exemplify the diagnostic and healthcare challenges of RE disorders. CA is a heterogeneous group of rare, incurable, and often life-limiting disorders characterized by progressive incoordination (Jayadev and Bird 2013). There are ~100 clinically recognized CAs, with similar numbers caused by autosomal dominant and autosomal recessive mechanisms. The prevalence of these disorders varies widely depending on genetic ancestry and geographical location, with estimates of a global average of ~6:100,000 (Ruano et al. 2014; Rudaks et al. 2024). These debilitating disorders predominantly impact locomotion, hand coordination, speech, swallowing, and vision. Apart from the recent approval of omaveloxolone for Friedreich ataxia (FRDA) (Lee 2023), there are no disease-modifying treatments for these conditions. Treatment consists of symptom management, such as using adaptive devices and ongoing physical and occupational therapy (de Silva et al. 2019). The predominant genetic cause of CA, accounting for over 80% of diagnoses, is a pathogenic RE in one of the 17 loci associated with CA identified to date. Non-RE pathogenic variants in ~100 other genes also contribute to disease prevalence (Beaudin et al. 2019; Rudaks et al. 2024), presenting a daunting panel of unusual variants and genes requiring examination. Diagnostic testing methodologies and outcomes for CA vary broadly. In Australia, standard clinical diagnostic testing uses single PCR/capillary array assays for between five and seven RE loci. Our center, servicing a population of ~6 million, only tests for six RE-causing CAs (spinocerebellar ataxia [SCA]1/2/3/6/7 and FRDA) with a diagnostic rate of ~5% (unpublished data 2015–2022). A higher diagnostic rate can be achieved by utilizing multiple testing methodologies to ensure the evaluation of multiple genetic variant types, including RE, SNV/indel, and CNV, with yields ranging between ~30% and 60% (Hadjivassiliou et al. 2017; Kang et al. 2019). However, these comprehensive, long-term studies were performed in a research setting. Clinical service providers prefer to deploy a single frontline test with high sensitivity and specificity to maximize yield and minimize cost. Notably, the yield for CA with short-read exome sequencing, arguably the most used and cost-effective frontline diagnostic test, is only ~25% (Rexach et al. 2019; Ngo et al. 2020).

Two technologies have recently been proposed as potential rapid and comprehensive diagnostic methods for RE disorders.

Single-molecule long-read sequencing (LRS) routinely generates reads >10 kb in length and supports de novo assembly and detection of all variant types including structural variants (Amarasinghe et al. 2020). The two leading LRS providers are Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT). PacBio performs sequencing by synthesis and requires the construction of a library of circular DNA molecules. In contrast, ONT is a nanopore-based technology. The DNA sequence is determined by measuring nucleotide-specific current changes as the molecule passes through a nanopore. While both technologies have strengths and weaknesses, one recent application of ONT appears to have considerable potential for RE. Single-molecule long-read sequencing using adaptive sampling (LR-AS, ONT) enables user-defined target selection in real time (Payne et al. 2021). This technology allows simultaneous screening of multiple target loci, capturing up to ~4% of the genome. Two recent proofs of concept pilot studies have demonstrated the method’s potential utility to diagnose RE disorders by simultaneous analysis of 37 (Stevanovski et al. 2022) or 59 (Miyatake et al. 2022a) RE loci in small retrospective cohorts of 37 and 22 patients, respectively. Alternatively, short-read genome sequencing (SR-GS) is emerging as a potential replacement for exome sequencing in rare disorders. This shift is driven by recognition of the gains achievable in diagnostic rates and the availability and decreasing cost of genome sequencing. Standard diagnostic analysis pipelines previously had limited capability to assess RE loci (Ashley 2016; Turro et al. 2020); however, recent advances in bioinformatic tools and technologies mean that it is now feasible to efficiently detect both non-RE and RE pathogenic variants in SR-GS (Leitão et al. 2024). Notably, a large study recently demonstrated high sensitivity and specificity (>97%) to detect 13 pathogenic RE in a retrospective cohort of 404 patients. The prospective analysis of these 13 RE in 11,631 patients identified 81 RE with a false discovery rate of 16% (Ibañez et al. 2022).

Here, we report a prospective trial comparing diagnostic outcomes of standard clinical testing in the Australian context (five pathogenic RE) with both short-read and long-read technologies in a cohort of 110 individuals with a clinical diagnosis of CA and clearly demonstrate the substantial diagnostic gains achievable for individuals and families affected by CA.

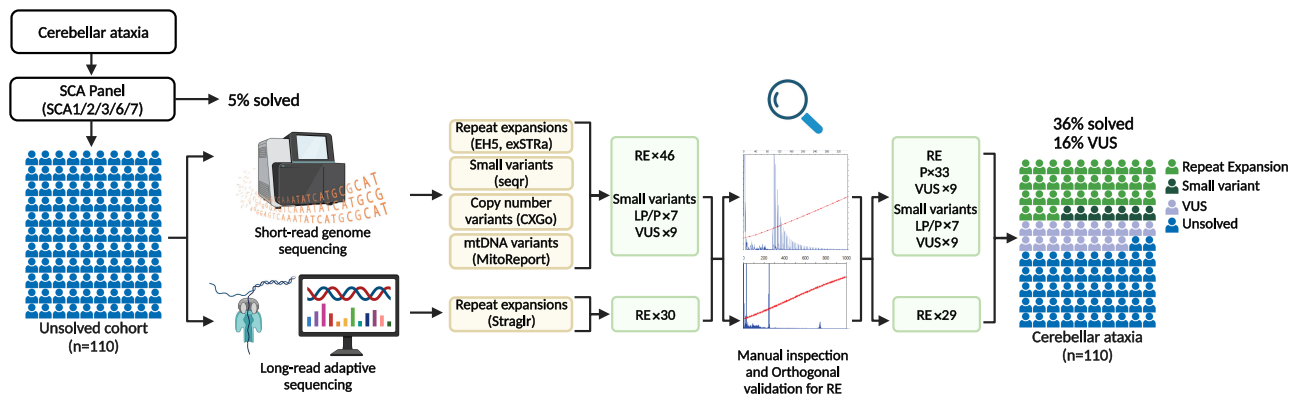
## Results

### Participant recruitment and details

The study design is summarized in Figure 1. A cohort of 110 individuals with a clinical diagnosis of CA, referred for diagnostic testing, were recruited to the research program over a 2-year period (2022–2023). Individuals were excluded if there was clinical suspicion of an acquired cause of ataxia, based on the history of acute injury or illness, toxic exposure, or rapid onset. All participants were singletons, 17% had a family history of ataxia, and included 48 female/62 male individuals with adult-onset ataxia. The mean age at onset was  $56 \pm 14$  years (range 15–77) and the mean age at testing  $68 \pm 13$  years (range 29–88). All individuals completed diagnostic testing for five pathogenic RE in SCA1, SCA2, SCA3, SCA6, and SCA7 before research-based genetic testing. Any individuals with a positive diagnostic test result were excluded from the trial.

### RE identification with short-read genome sequencing

The primary purpose of this trial was to compare the performance of SR-GS and LR-AS for the identification of pathogenic RE. At the



**Figure 1.** Overview of the study and investigations performed. (SCA) spinocerebellar ataxia, (EH5) ExpansionHunter5, (RE) repeat expansion, (LP) likely pathogenic, (P) pathogenic, (VUS) variant of uncertain significance.

time of trial initiation (2022), there were 67 known pathogenic REs, 17 directly associated with CA, and the remainder with a broader range of neurogenetic, neuromuscular, and other health conditions. Therefore, all 67 REs were interrogated as part of this program. However, given the cohort composition and clinical indication for study inclusion was CA, a specific focus of the analysis was the 22 REs listed in Table 1. This included the 17 REs associated with CA and five additional pathogenic REs that cause neurological movement disorders that could possibly be a differential

diagnosis for our cohort. In addition, these five REs are also commonly tested with single gene assays at our center (Victorian Clinical Genetics Services, VCGS) and more broadly. Therefore, they represent appropriate, cost-effective targets for inclusion in a diagnostic single test.

ExpansionHunter5 (EH5) (Dolzhenko et al. 2019) was used to genotype the 22 RE loci in the SR-GS data. A postanalysis custom *k*-mer filter was used to remove genotype calls where the predominant motif detected in the reads does not match the motif tested

**Table 1.** Repeat expansion target loci for CAs and clinically significant noncerebellar ataxia conditions (non-CAs), adapted from Tankard et al. (2018) and Depienne and Mandel (2021)

	Gene	Disease	Inheritance	Repeat motif	Pathogenic threshold
CA	<i>ATXN1</i>	SCA1	AD	(CAG) <i>n</i>	>38
	<i>ATXN2</i>	SCA2	AD	(CAG) <i>n</i>	>31
	<i>ATXN3</i>	MJD; SCA3	AD	(CAG) <i>n</i>	>54
	<i>CACNA1A</i>	SCA6	AD	(CAG) <i>n</i>	>19
	<i>ATXN7</i>	SCA7	AD	(CAG) <i>n</i>	>36
	<i>ATXN8/ATXN8OS</i>	SCA8	AD	(CAG) <i>n</i> /(CTG) <i>n</i>	>73
	<i>ATXN10</i>	SCA10	AD	(ATTCT) <i>n</i>	>280
	<i>PPP2R2B</i>	SCA12	AD	(CAG) <i>n</i>	>42
	<i>TBP</i>	SCA17	AD	(CAG) <i>n</i> /(CAA) <i>n</i>	>42
	<i>FGF14</i>	SCA27B	AD	(GAA) <i>n</i>	>249 <sup>b</sup>
	<i>BEAN1</i>	SCA31	AD	(TGGA) <i>n</i> <sup>a</sup>	>109
	<i>NOP56</i>	SCA36	AD	(GGCCTG) <i>n</i>	>649
	<i>DAB1</i>	SCA37	AD	(ATTTC) <i>n</i> <sup>a</sup>	>30
	<i>ATN1</i>	DRPLA	AD	(CAG) <i>n</i>	>47
	<i>RFC1</i>	CANVAS	AR	(AAGGG) <i>n</i> /others <sup>a</sup>	>249
	<i>FXN</i>	FRDA	AR	(GAA) <i>n</i>	>65
	<i>FMR1</i>	FXTAS	XLD	(CGG) <i>n</i>	55–200
Non-CA	<i>FMR1</i>	FXS	XLD	(CGG) <i>n</i>	>200
	<i>AR</i>	SMA1; SBMA	XLR	(CAG) <i>n</i>	>37
	<i>C9orf72</i>	FTDALS1	AD	(GGGGCC) <i>n</i>	>30
	<i>HTT</i>	HD	AD	(CAG) <i>n</i>	>35
	<i>DMPK</i>	DM1	AD	(CTG) <i>n</i>	>49
	<i>CNBP</i>	DM2	AD	(CCTG) <i>n</i>	>50

<sup>a</sup>Repeat motif not found in GRCh38 reference at this locus.

<sup>b</sup>Intermediate expansion range of uncertain significance 180–249.

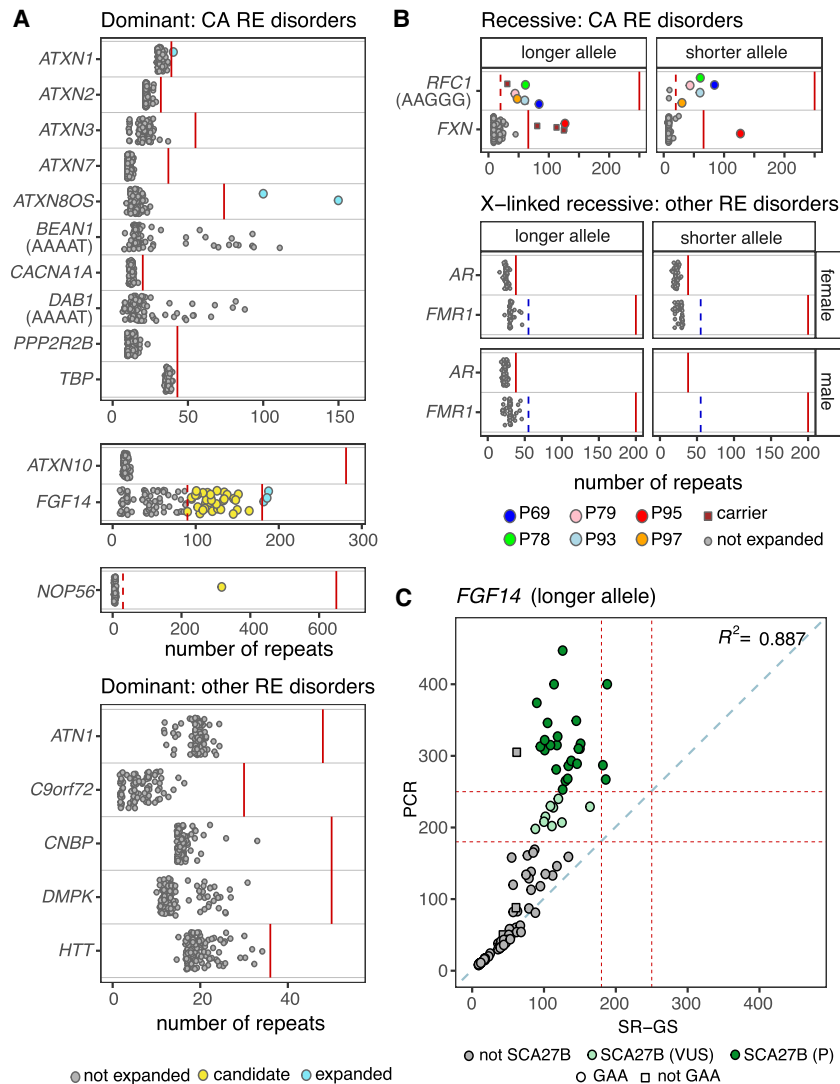
by EH5 (<https://doi.org/10.5281/zenodo.11514479>). All individuals with potential expansions were also manually reviewed in the Integrative Genomics Viewer (IGV) (Robinson et al. 2011) to confirm motif composition. We detected two individuals with dominant *ATXN8OS* expansions greater than the pathogenic threshold for SCA8 (Fig. 2A). One individual was found to have an expanded allele for *NOP56*; however, the estimated size (317 re-

peats) is lower than the pathogenic threshold (650 repeats) for SCA36 (Fig. 2A). We have previously shown that expansions in *NOP56* are typically underestimated by EH5 due to their large size vastly exceeding the read length (Rafehi et al. 2020). In addition, the *NOP56* STR is stable in the general population, with most alleles reported in gnomAD in the 4–10 repeats range, and only three alleles between 15 and 21 repeats. Therefore, any individual with an *NOP56* expansion  $\geq 30$  repeats is further tested to confirm or exclude an SCA36 diagnosis. Manual review confirmed a GGCCT expansion at the locus.

*FGF14* STR size is also known to be underestimated by RE calling in SR-GS data. Our previous work identified high concordance between PCR and SR-GS sizing for STR up to  $\sim(\text{GAA})_{100}$ ; however, larger RE is typically underestimated by RE genotyping tools such as EH5 (Rafehi et al. 2023). In contrast to *NOP56*, in which outliers are easy to detect, the *FGF14* GAA STR is highly unstable and there is significant variation in the length in the general population. As a result, it is not possible to distinguish pathogenic expansions ( $\geq 250$ ) from alleles of  $\sim 100$ –249 using EH5. Based on previous experience with this locus, we prioritize any individual with an EH5 genotype call of  $\geq 90$  repeats as candidates for SCA27B. Using this approach, we identified 37 individuals suspected to have SCA27B (Fig. 2A). Manual review confirmed that all 37 REs were composed of pure (GAA)<sub>n</sub>.

Expanded sequences were also detected for the benign reference motif (AAAAT) in *BEAN1* and *DAB1* (Fig. 2A), but neither of the pathogenic motifs (TGGA) nor (ATTTC) were identified in the short-read data. Although expansions of the reference motif alone are not pathogenic for these two loci, we cannot exclude the possibility that these individuals have a pathogenic motif embedded deep within the reference motif as these cannot be detected with SR-GS (Rosenbohm et al. 2022).

Analysis of recessive loci also identified potential pathogenic RE in *FXN* and *RFC1*. One individual has two expanded alleles in *FXN*, consistent with a diagnosis of FRDA (Fig. 2B). We also identified four individuals with a single expanded *FXN* allele (Supplemental Table S1). We tested for the possibility of a second pathogenic small variant or CNV on the other *FXN* allele utilizing research tools seq and CXGo; however, no candidate variants were identified, suggesting these individuals are heterozygous carriers. Testing for the nonreference pathogenic AAGGG motif in *RFC1* did not identify



**Figure 2.** Targeted RE screening in SR-GS identifies potential RE diagnoses. STR genotypes were determined for the 22 loci associated with CA (Table 1) with EH5. (A) Genotypes are shown for the longer allele in dominant RE disorders that cause ataxia (top 3 plots, loci separated based on maximum allele size) and those that cause other disorders (bottom). Blue circles indicate individuals with an expansion in the respective allele that is larger than the pathogenic threshold (solid red line), while yellow circles are individuals who exceed an EH5-specific threshold (dashed red line) and are candidates for further investigation. Benign AAAAT motifs do not have a threshold. (B) Genotypes for the shorter and longer alleles are shown for autosomal recessive RE ataxia disorders (top) and X-linked recessive disorders other than ataxia, split by sex (bottom). Individuals who are heterozygous carriers for an allele expanded beyond the pathogenic threshold (solid red line) or an allele larger than the EH5-specific threshold (dashed red line) are shown as brown squares. For *FMR1*, the blue dashed line indicates the threshold for FXTAS while the solid red line is the threshold for FXS. Individuals who carry two alleles expanded beyond either the pathogenic or EH5-specific threshold are shown as colored circles. (C) Concordance plot showing a comparison of the *FGF14* STR EH5 genotypes from SR-GS compared to PCR sizing (longer allele only). The pathogenic ( $\geq 250$  repeats) and VUS ( $\geq 180$  repeats) thresholds are shown as dashed red lines. A 1:1 correlation is shown as a dashed blue line. The  $R^2$  is a Pearson's correlation. Dark green circles are individuals with a confirmed SCA27B diagnosis and those in light green are SCA27B VUS. Circles indicate GAA motifs and squares are non-GAA motifs, determined by observation in IGV.

any individuals with an RE greater than the pathogenic threshold (>250 repeats) (Dominik et al. 2023). However, we have previously shown that AAGGG RE sizing of *RFC1* is underestimated in SR-GS (Rafehi et al. 2019). Therefore, any individual with an AAGGG allele  $\geq 30$  repeats is considered likely to carry a pathogenic RE in *RFC1*. Using this threshold, we identified five individuals with pathogenic biallelic RE in *RFC1*, consistent with the clinical presentation of CA, neuropathy, and vestibular areflexia syndrome (CANVAS) in these individuals (Table 2). In addition, we identified three individuals with a heterozygous pathogenic AAGGG *RFC1* allele (Supplemental Table S1) but failed to identify a second pathogenic variant on the other allele, suggesting they are carriers but that this is not the cause of their presentation.

Overall, this analysis identified 46 individuals with potential pathogenic RE causing their clinical presentation, including heterozygous RE in *ATXN8/ATXN8OS* (2 $\times$ ), *NOP56* (1 $\times$ ), and *FGF14* (37 $\times$ ), and biallelic RE in *FXN* (1 $\times$ ) and *RFC1* (5 $\times$ ).

### Molecular validation of RE identified by SR-GS

We and others have shown that SR-GS has high sensitivity and specificity for the identification and sizing of pathogenic RE when the DNA repeat length is similar or smaller than the standard SR-GS read length of 150 bp (Dolzhenko et al. 2017; Tankard et al. 2018). In contrast, while SR-GS demonstrates high specificity/sensitivity for larger pathogenic RE, such as those causing SCA36 and myotonic dystrophy 2 (DM2), it significantly underestimates the size of the pathogenic allele (Day et al. 2003; Rafehi et al. 2020). We previously demonstrated that size estimates of the RE in *FGF14* that cause SCA27B are unreliable when the expansion is greater than  $\sim(\text{GAA})_{100}$  (Rafehi et al. 2023), suggesting SR-GS may have poor specificity and/or sensitivity for this RE. Therefore, we performed orthogonal molecular testing of all individuals with potential pathogenic RE identified by SR-GS (*FXN* via clinical diagnostic test; *NOP56* and *RFC1* via RP-PCR; *ATXN8/ATXN8OS* and *FGF14* via RP-PCR and flanking PCR/capillary array sizing). This analysis confirmed the SR-GS results for the nine individuals with pathogenic RE in *ATXN8/ATXN8OS*, *FXN*, *NOP56*, and *RFC1* (Table 2). Of the 37 individuals with a potential pathogenic RE in *FGF14* only 24 were confirmed to be expanded above the current pathogenic threshold of  $(\text{GAA}) \geq 250$  (Pellerin et al. 2023; Rafehi et al. 2023). We subsequently used long-range PCR to also size the *FGF14* locus in the remaining 73 individuals in the cohort, which SR-GS suggested were nonpathogenic. This analysis demonstrated significant divergence in the size of the larger allele estimated by EH5 compared to PCR (Fig. 2C). These results demonstrate that SR-GS has a sensitivity of 100% and specificity of 85% to identify pathogenic RE in *FGF14* when utilizing an EH5 estimate of 90 repeats as the pathogenic threshold (Supplemental Table S2). In addition, the PCR analysis identified nine individuals with an *FGF14* GAA allele >179 but <250 repeats; these were classified as variants of uncertain significance (VUSs) (Supplemental Table S3; Mohren et al. 2024). Overall, while SR-GS identified 46 individuals with a potentially pathogenic RE (42% of the cohort), the actual diagnostic rate achieved was 33/110 (30%), the discrepancy being the result of 13 false positive *FGF14* diagnoses (i.e., a GAA repeat <250 repeats).

### Identification of non-RE pathogenic variants with SR-GS

In addition to RE, a variety of other pathogenic variants can cause CA (Beaudin et al. 2019; Rudaks et al. 2024). Therefore, we analyzed the SR-GS data for SNV/indel, CNV, and mitochondrial

DNA (mtDNA) variants. No CNV or mtDNA variants were identified but an additional seven patients were solved by identification of likely pathogenic/pathogenic (LP/P) variants in *ANO10* (autosomal recessive spinocerebellar ataxia 10; SCAR10), *CACNA1G* (SCA42), *HEXA* (Tay Sachs disease), *PNPT1* (SCA25), *SPG7* (spastic paraplegia 7; SPG7), *STUB1* (SCA48), and *TTBK2* (SCA11) (Table 3). This analysis also identified an additional nine individuals with suspicious variants that do not meet ACMG guidelines for classification as LP/P (Richards et al. 2015) and were, therefore, classified as VUS (Supplemental Table S4). The overall diagnostic yield achieved by SR-GS was 38% (33 $\times$  RE, 7 $\times$  non-RE), with a false positive and negative rate of 12% and 0%, respectively, for *FGF14* RE (SCA27B).

### RE identification with long-read adaptive sequencing

Targeted sequencing is an effective tool that provides many advantages for genomic medicine, prioritizing relevant candidate genes for phenotype and significantly reducing per test cost and analysis burden. However, multiplex PCR-based enrichment and sequencing technologies have several limitations in the context of characterizing pathogenic RE, including PCR stutter affecting both RE size and composition and an inability to amplify large pathogenic RE. Alternative physical enrichment technologies that do not require PCR amplification have been developed, including CRISPR–Cas9 enrichment. However, these enrichment approaches are bespoke, difficult to multiplex, and are limited to  $\sim 50$  targets per library preparation. Recently, the development of LR-AS has provided a mechanism to couple multiplex targeting with LRS at a reasonable cost. Therefore, LR-AS was performed independently to SR-GS, targeting 334 genomic regions (111 Mb/ $\sim 3.59\%$  of the GRCh38 [hg38] reference genome), which included 67 genes with a pathogenic RE and other non-RE genes associated with CA (Supplemental Table S5). Each sample was analyzed using a single MinION flow cell. Summary statistics are presented in Figure 3 and detailed individual sample/locus results are described in Supplemental Table S5. The on-target mean read length was 4727 bp (95% CI 2728, 6014) compared to 480 bp (95% CI 444, 493) for off-target sequence, confirming rapid and efficient rejection of nontargeted regions of the genome (Fig. 3A). The on-target mean sequencing depth was 22.1 (95% CI 12.7, 31.0) compared to 3.0 (95% CI 1.7, 4.1) for off-target regions (Fig. 3B), resulting in a mean 8.2-fold (95% CI 5.3-fold, 10.4-fold) target enrichment (Fig. 3C). Consistent with the SR-GS analysis, we focused analysis on the 22 REs listed in Table 1. The distribution of read counts across all samples at these 22 loci (Fig. 3D,E) ranged from a mean of 16.8 reads (*AR*) to 28.2 reads (*C9orf72*) across the 110 individuals in the cohort.

Given that clinical testing excluded individuals with SCA1/2/3/6 and 7 from the cohort, we first tested if LR-AS was effective in identifying the corresponding pathogenic RE by analyzing individuals with known pathogenic RE status. Affected individuals, not part of the cohort, with known pathogenic RE in *ATXN1*, *ATXN2*, *ATXN3*, *CACNA1A*, and *ATXN7* were subject to LR-AS and genotypes were compared to allele sizes determined by diagnostic testing (flanking PCR and capillary array analysis). LR-AS identified two alleles, one pathogenic and one nonpathogenic, for *ATXN1*, *ATXN2*, *ATXN3*, and *ATXN7*. The calculated allele sizes were concordant with those obtained by diagnostic testing (Supplemental Table S6). However, Straglr only identified a single allele (14 repeats) for *CACNA1A*, compared to the diagnostic result of two alleles of 23 and 12 repeats. Visual inspection in IGV

## Diagnosis of cerebellar ataxia by sequencing

**Table 2.** Repeat expansion variant findings

Individual ID	Sex	Age at testing	Age at onset	FHx of CA	Gene	Disease	Inheritance	Variant motif	EHS RE size	Straglr RE size	PCR RE size	Zygoty	Clinical presentation
P066	F	49	47	–	<i>ATXN8/ATXN8OS</i>	SCA8	AD	(CAG)n/(CTG)n	100	124	134	Het	CA
P110	F	81	76	–	<i>ATXN8/ATXN8OS</i>	SCA8	AD	(CAG)n/(CTG)n	150	768	Expanded <sup>e</sup>	Het	CABV
P003 <sup>a</sup>	M	74	62	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	134	288	286	Het	CABV, ANS dysfunction, and chronic cough
P004 <sup>a</sup>	F	71	55	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	105	327 <sup>b</sup>	346	Het	CA and chronic cough
P005 <sup>a</sup>	M	76	58	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	101	328 <sup>b</sup>	308	Het	CABV, ANS dysfunction and hyperreflexia
P013 <sup>a</sup>	M	60	47	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	126	445 <sup>b</sup>	447	Het	CA
P014 <sup>a</sup>	M	77	70	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	150	260	310	Het	CABV, hyperreflexia and parkinsonism
P022 <sup>a</sup>	M	69	ND	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	114	108 <sup>b,c</sup>	>400	Het	CA and spasticity
P025	F	74	44	+	<i>FGF14</i>	SCA27B	AD	(GAA)n	118	311	315	Het	CABV and chronic cough
P026 <sup>a</sup>	F	71	69	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	138	16 <sup>b,c</sup>	293	Het	CA
P038 <sup>a</sup>	M	57	46	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	95	324 <sup>b</sup>	313	Het	CA and spasticity
P040 <sup>a</sup>	F	69	58	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	188	126 <sup>b</sup>	>400	Het	CAUV, ANS dysfunction, and hyperreflexia
P042 <sup>a</sup>	F	80	59	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	126	252	253	Het	CABV
P045	M	72	ND	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	145	329	349	Het	CABV
P050 <sup>a</sup>	M	73	67	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	130	270 <sup>b</sup>	265	Het	CABV
P052 <sup>a</sup>	F	87	77	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	117	277 <sup>b</sup>	281	Het	CA
P063	F	76	ND	+	<i>FGF14</i>	SCA27B	AD	(GAA)n	119	325 <sup>b</sup>	327	Het	CA and Hashimoto's thyroiditis
P064	F	76	71	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	182	255 <sup>b</sup>	287	Het	CA
P067	M	82	69	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	186	265	267	Het	CABV
P068	M	76	71	–	<i>FGF14</i>	SCA27B	AD	(GAA)n	133	249 <sup>b</sup>	268	Het	CA
P072	M	54	51	+	<i>FGF14</i>	SCA27B	AD	(GAA)n	146	288	289	Het	Episodic CA
P090	M	77	70	+	<i>FGF14</i>	SCA27B	AD	(GAA)n	109	310	315	Het	CA
P100	F	76	65	+	<i>FGF14</i>	SCA27B	AD	(GAA)n	90	378	374	Het	CABV
P101	M	70	50	+	<i>FGF14</i>	SCA27B	AD	(GAA)n	151	316	317	Het	Episodic CA
P106	F	64	60	+	<i>FGF14</i>	SCA27B	AD	(GAA)n	148	316	310	Het	CA
P107	M	77	ND	+	<i>FGF14</i>	SCA27B	AD	(GAA)n	101	325	322	Het	CA
P095	M	32	15	–	<i>FXN</i>	FRDA	AR	(GAA)n	127 127	947 295	Expanded <sup>d</sup> Expanded <sup>d</sup>	Hom	CA and upgoing plantar reflexes
P023	M	61	56	+	<i>NOP56</i>	SCA36	AD	(GGCCTG)n	317	5 <sup>b</sup>	Expanded <sup>e</sup>	Het	CA
P069	M	70	ND	–	<i>RFC1</i>	CANVAS	AR	(AAGGG)n	84 84	<i>No call</i>	Expanded <sup>e</sup>	Hom	CANVAS
P078	M	74	ND	–	<i>RFC1</i>	CANVAS	AR	(AAGGG)n	61 61	872 <sup>c</sup>	Expanded <sup>e</sup>	Hom	CANVAS

(continued)

**Table 2.** *Continued*

Individual ID	Sex	Age at testing	Age at onset	FHx of CA	Gene	Disease	Inheritance	Variant motif	EH5 RE size	Straglr RE size	PCR RE size	Zygoty	Clinical presentation
P079	F	79	ND	–	<i>RFC1</i>	CANVAS	AR	(AAGGG) <i>n</i>	44 44	794 <sup>c</sup>	Expanded <sup>e</sup>	Hom	CANVAS
P093	M	67	63	–	<i>RFC1</i>	CANVAS	AR	(AAGGG) <i>n</i>	60 60	944 <sup>c</sup>	Expanded <sup>e</sup>	Hom	CANVAS
P097	M	81	55	–	<i>RFC1</i>	CANVAS	AR	(AAGGG) <i>n</i>	30 48	<i>No call</i>	Expanded <sup>e</sup>	Hom	CANVAS

M, male; F, female; ND, no data available; FHx, family history; CA, cerebellar ataxia; –, absent; +, present; SCA, spinocerebellar ataxia; FRDA, Friedreich ataxia; CANVAS, cerebellar ataxia neuropathy and vestibular areflexia syndrome; AD, autosomal dominant; AR, autosomal recessive; EH5, ExpansionHunter5; RE, repeat expansion; Het, heterozygous; Hom, homozygous; CABV, cerebellar ataxia and bilateral vestibulopathy; ANS, autonomic nervous system; CAUV, cerebellar ataxia and unilateral vestibulopathy.

<sup>a</sup>Previously reported in Rafehi et al. (2023).

<sup>b</sup>Less than seven supporting reads.

<sup>c</sup>Only one allele size is called at this locus.

<sup>d</sup>On clinical diagnostic testing.

<sup>e</sup>RP-PCR showing an expanded allele.

Italicized sizing numbers are those not called as expanded (only applicable to Straglr).

showed four spanning reads with 22–23 repeats, and 10 spanning reads with 12 repeats. It is not clear why Straglr was unable to call two alleles; it may be that the relatively low read depth and allelic bias (4 reads vs. 10 reads) confounded the analysis. However, *ATXN7* was called successfully despite lower read depth and similar allelic bias. Alternatively, the small relative difference in size between pathogenic and nonpathogenic alleles may have contributed to only a single allele being identified. Analysis of the LR-AS data using the alternate algorithm *vamos*, which can interrogate both motif size and composition (Ren et al. 2023) similarly failed to call the pathogenic RE in the SCA6-positive sample (Supplemental Table S6). While previous studies have suggested that LR-AS can effectively identify pathogenic RE in these five genes, they also highlighted the utility of manual inspection/visual confirmation of bioinformatic allele size estimations (Miyatake et al. 2022a; Stevanovski et al. 2022). This is a limitation that also impacts SR-GS as described above.

We subsequently extended the LR-AS analysis to the trial cohort, identifying individuals with potentially pathogenic RE by determining if their allele size estimates exceeded thresholds in a process similar to that used for SR-GS (Methods; Table 1). Overall, before any manual review, Straglr identified RE that supported 30 potential diagnoses. For the dominant CA disorders, Straglr identified heterozygous pathogenic RE in *ATXN8OS* in two individuals (Fig. 4). One RE was estimated to be 124 repeats, similar to the size estimated by orthogonal PCR analysis (134 repeats), while the second RE was estimated to be 768 repeats (Table 2), which is larger than can be determined by PCR. Heterozygous pathogenic REs in *FGF14* ( $\geq 250$  GAA) were identified in 20 individuals and showed very high concordance with the PCR sizing results ( $R^2 = 0.997$ ) (Fig. 5A; Table 2). Expanded sequences were also detected for the benign reference motif (AAAAT) in *BEAN1* and *DAB1* (Fig. 4A) but not the pathogenic motifs (TGGAA) or (ATTTC). Manual review of the aligned reads did not provide any evidence of pathogenic motifs embedded deep within the expanded sequence, which would have been missed with SR-GS (Supplemental Fig. S1).

Analysis of recessive loci also identified expanded RE in *FXN* and *RFC1*. Pathogenic *FXN* RE of 947 and 296 repeats were identified in one individual, confirming a molecular diagnosis of FRDA. Three other individuals were identified with a pathogenic GAA RE

in *FXN*, with estimated sizes of 629, 570, and 160 repeats, respectively (Fig. 4B). However, the second allele for each individual was in the normal range (32, 21, and 8 repeats, respectively), identifying these individuals as heterozygous carriers (Supplemental Table S1). Pathogenic RE in *RFC1* was identified by Straglr in six individuals. Five of these were called “homozygous” REs for the pathogenic AAGGG, i.e., only a single allele size was reported. The estimated allele sizes of 872, 794, 945, 395, and 1109 repeats exceed the current pathogenic threshold of  $>250$  repeats (Dominik et al. 2023). Three of these were confirmed as biallelic expansions by visual review of aligned reads (Table 2), while the other two (395 and 1109 repeats) were shown to be heterozygous for a single pathogenic AAGGG RE. In both cases, Straglr did not identify (call) the second nonpathogenic allele. On visual inspection in IGV, the samples appeared to be heterozygous in *motif* as well as size, with both samples having one pathogenic AAGGG allele, and a second AAAAG allele. Subsequent flanking PCR and gel electrophoresis confirmed the presence of a nonpathogenic AAAAG allele in both P010 and P005 (Supplemental Fig. S2A,B). In addition, one individual was called “homozygous” RE for the pathogenic AAAGG, with an estimated allele size of 640 repeats, exceeding the current pathogenic threshold of  $>500$  repeats for this motif (Dominik et al. 2023). However, manual review determined that the motif is actually AAGGG. Additionally, Straglr did not call the second nonpathogenic 130 repeat AAAGG allele (Supplemental Fig. S2C); therefore, this individual is also a heterozygous carrier.

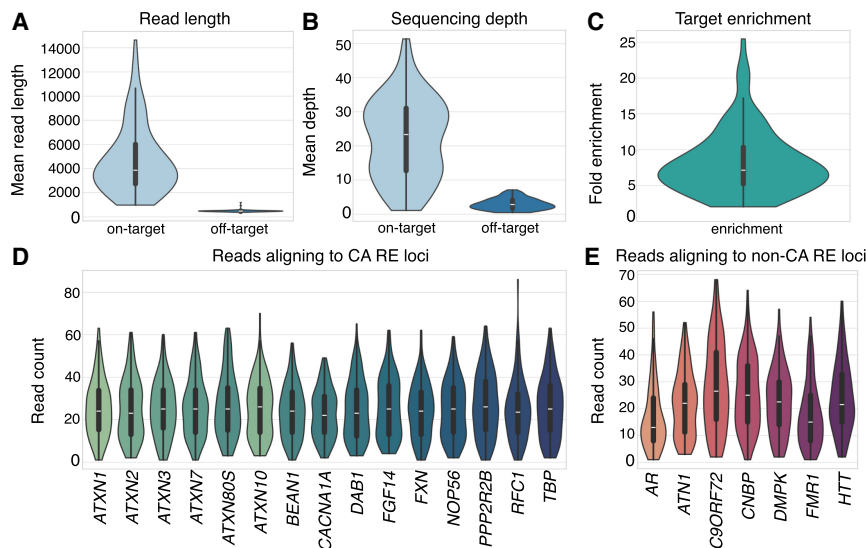
### Comparison of testing methods

Independent analysis of the cohort by LR-AS, SR-GS, and for some loci orthogonal PCR methodologies enabled us to compare and contrast the results across the different platforms. Allele sizing was broadly concordant between LR-AS and SR-GS data up to an RE size of  $\sim 450$  bp (Fig. 4; Supplemental Fig. S3). This observation is consistent with previously published work performing PCR validation of long-read RE sizing methods (Ibañez et al. 2022). Expansions of more than 450 bp were consistently sized by LR-AS as being larger than SR-GS due to the longer read length afforded by the ONT platform. There was a very good correlation between LR-AS and orthogonal sizing methodologies (Fig. 4; Table

**Table 3.** Single nucleotide and small insertion or deletion variant findings

Individual ID	Sex	Age at testing	Age at onset	FHx of CA	Gene	Disease	Inheritance	Genomic variant (hg38)	HGVSc	HGVSp	Zygoty	Consequence	Classification	Clinical presentation
P074	F	57	39	-	ANO10	SCAR10	AR	Chr3:43574873T>C	c.1163-9A>G		Hom	Splice acceptor	P	CA
P109	M	61	ND	+	CACNA1G	SCA42	AD	Chr17:50617560G>A	c.5144G>A	p.(Arg1715His)	Het	Missense	P	CA
P018	M	30	26	-	HEXA	Tay Sachs disease	AR	Chr15:72350518C>T Chr15:72375740C>T	c.805G>A c.233G>A	p.(Gly269Ser) p.(Trp78*)	Het Het	Missense Stop gained	P P	CA and reduced muscle power
P049	F	57	17	+	PNPT1	SCA25	AD	Chr2:55643216G>C	c.2014-3C>G		Het	Splice acceptor	LP	CA
P096	M	75	68	-	SPG7	SPG7	AR	Chr16:89546737C>T	c.1529C>T	p.(Ala510Val)	Hom	Missense	P	CA, spasticity and osteoarthritis
P020	M	68	ND	-	STUB1	SCA48	AD	Chr16:681504CGAA>C	c.433_435del	p.(Lys145del)	Het	Inframe deletion	LP	CA, akathisia and hyperreflexia
P059	M	76	72	+	TTBK2	SCA11	AD	Chr15:42777132ATC>A	c.1306_1307del	p.(Asp436Tyrfs*14)	Het	Frameshift	P	CA, laryngeal tremor, sensorimotor neuropathy and dementia

M, male; F, female; ND, no data available; FHx, family history; CA, cerebellar ataxia; -, absent; +, present; SCAR10, autosomal recessive spinocerebellar ataxia type 10; SPG7, spastic paraplegia 7; AD, autosomal dominant; AR, autosomal recessive; Het, heterozygous; Hom, homozygous; P, pathogenic; LP, likely pathogenic.  
Transcripts: ANO10, NM\_018075.5; CACNA1G, NM\_018896.5; HEXA, NM\_000520.6; PNPT1, NM\_033109.5; SPG7, NM\_003119.4; STUB1, NM\_005861.4; TTBK2, NM\_173500.4.



**Figure 3.** Performance metrics for adaptive sequencing in a targeted panel of RE loci. (A) Mean read length in target regions significantly exceeds that of off-target regions. (B) Sequencing depth in on-versus off-target regions. (C) Genome-wide enrichment of on- versus off-target regions in individual experiments. (D) Number of reads aligning to each of the targeted CA repeat loci. (E) Number of reads aligning to each targeted clinically significant non-CA locus.

2), indeed LR-AS with read lengths >5 kb exceeds the sizing capabilities of PCR/capillary array technology (limited to <2 kb).

Straglr did not size the single sample with a pathogenic *NOP56* expansion (P026) due to a lack of any reads completely spanning the expanded allele. However, manual review clearly identified reads demonstrating the presence of a pathogenic heterozygous expansion at this locus (Supplemental Fig. S4A). Similarly, two samples with pathogenic biallelic *RFC1* expansions (P069, P097, independently identified by SR-GS and PCR testing) were not identified by Straglr due to low read depth; in each case, only a single read spanned the entire RE. However, manual review confirmed the majority of reads at this locus encoded the pathogenic AAGGG motif, suggesting the individuals had biallelic pathogenic *RFC1* RE (Supplemental Fig. S4B,C).

Overall, manual review of LR-AS was an important step in the correct interpretation and diagnoses of very large RE. Our results also demonstrate the need for orthogonal methodologies for accurate interpretation of *RFC1* alleles, which can demonstrate significant size and motif heterogeneity. LR-AS identified 29 pathogenic REs and achieved a genetic diagnosis for 26% of the cohort. This included heterozygous RE in *ATXN8/ATXN8OS* (2×), *NOP56* (1×), and *FGF14* (20×), and biallelic RE in *FXN* (1×) and *RFC1* (5×). This is four less than the 33 pathogenic REs identified using SR-GS, all of which were individuals with *SCA27B*. In one of these four cases, LR-AS determined an allele size of 249 repeats, compared to 268 repeats by PCR. This falls just under the current pathogenic threshold and therefore was classified as a VUS. For the other three, the read depth was low (<7), and only a single allele (<250 repeats) was called by Straglr (Table 2).

## Discussion

There are over 100 clinically recognized disorders encompassed by CA making genetic diagnosis challenging. CA can present with nonspecific and overlapping clinical features, providing minimal guidance for prioritizing genetic candidates and optimal diagnos-

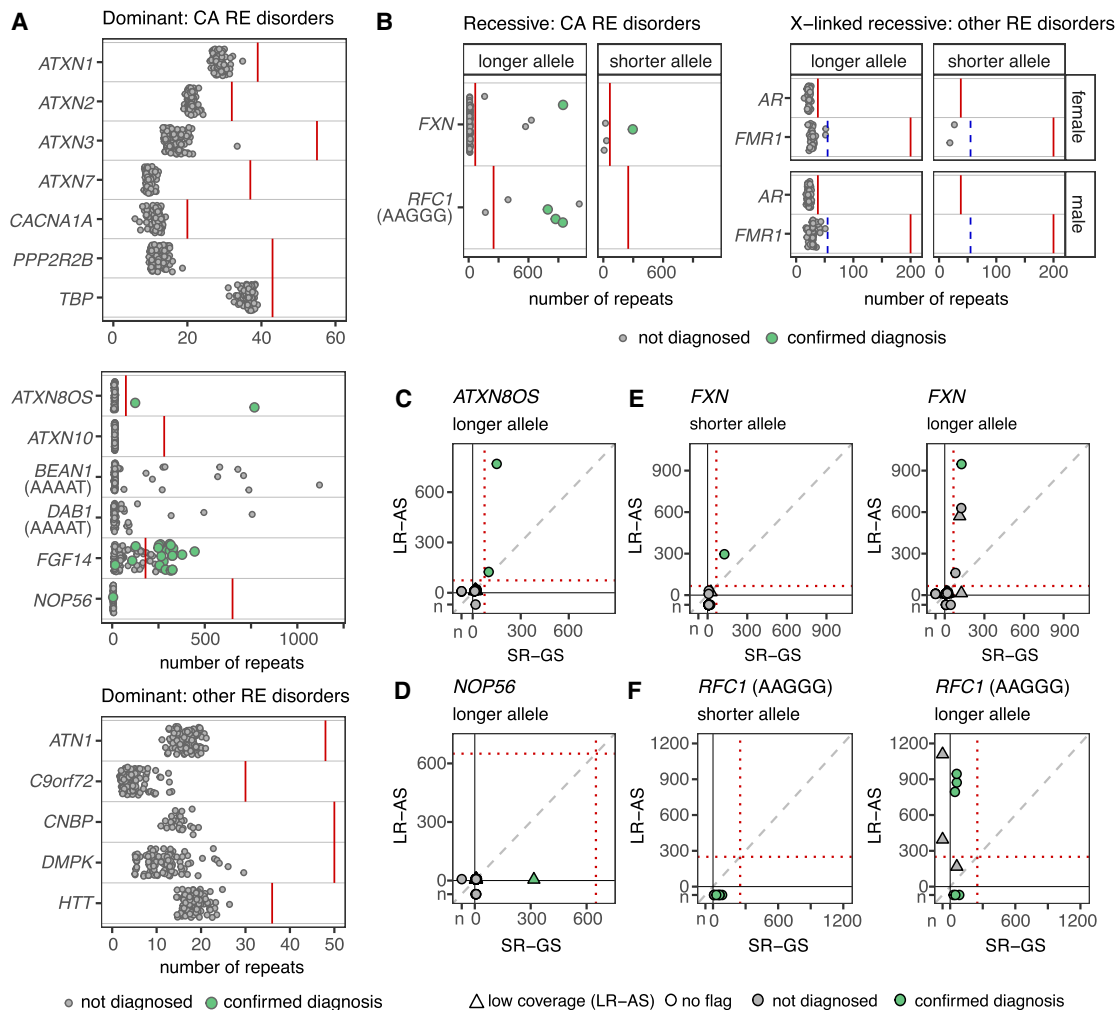
tic methodologies. RE is the predominant genetic cause of CA but until recently they were difficult to diagnose with existing molecular tools. Therefore, CA testing has been fragmented, inefficient, and often restricted to a single or a small number of RE loci. Patients often endure a long diagnostic odyssey with sequential testing, frequently to no avail (Németh et al. 2013; Daker-White et al. 2015; Rexach et al. 2019; Ngo et al. 2020). This study compares two promising technologies for comprehensive genetic diagnosis of CA, focusing on identifying pathogenic RE.

The cohort characteristics are broadly consistent with expectations for adult-onset cerebellar disorders, with similar numbers of males and females. Symptom onset ( $56 \pm 14$  years) considerably preceded mean age at testing ( $68 \pm 13$  years), reflecting a slowly progressive disease course. Diagnoses included *SCA8* (2×), *SCA27B* (24×), *SCA36* (1×), *CANVAS* (5×), and *FRDA* (1×). Our data confirm RE as the most common cause of CA in

Australia, even in a cohort depleted of five commonly tested REs (*SCA1*, 2, 3, 6, and 7). Notable was the high frequency of *SCA27B* (22%). This is consistent with observations in European cohorts with unsolved ataxia, where the reported frequency ranges from 15% to 30% (Iruzubieta et al. 2023; Pellerin et al. 2023; Rafehi et al. 2023). However, there does seem to be variability associated with genetic ancestry, with a frequency of ~1% reported in Japanese cohorts (Ando et al. 2024; Mizushima et al. 2024). It is possible that biases in cohort collection may have influenced disorder frequency compared to true Australian population prevalence. Individuals were predominantly recruited from a single center and therefore may not be broadly representative. In addition, cohort recruitment was mediated by the clinician referring patients for diagnostic testing, introducing potential bias depending on clinician expertise and enthusiasm for research.

SR-GS achieved 40 diagnoses (40/110, 36% yield) including 33 pathogenic RE and seven other non-RE causes. One advantage of SR-GS is its maturity and well-established pipelines for different types of genetic variation. It is most efficient in identifying small variants (SNV and indel) with LP/P variants accounting for 6% of the cohort. In addition, nine clinically suspicious variants, scoring 4 or 5 ACMG classification points and with a potential phenotype match were identified as VUS, requiring future clinical follow-up and functional studies to confirm pathogenicity. Overall, clinically relevant small variants were found in 15% of cases, consistent with the literature on their contribution to CA (Rudaks et al. 2024). Notably, all of these variants affected protein coding or nearby flanking sequences and therefore are detectable by standard exome sequencing.

Bioinformatic tools capable of identifying pathogenic RE in SR-GS data are a relatively recent development and have yet to be widely embraced in diagnostic pipelines (Bahlo et al. 2018; Leitão et al. 2024). This study demonstrates SR-GS's utility in identifying pathogenic RE-causing CA. Analysis of 17 RE loci associated with CA yielded 33 genetic diagnoses, including both dominant and recessive disorders. Tools like EH5 estimate STR sizes and

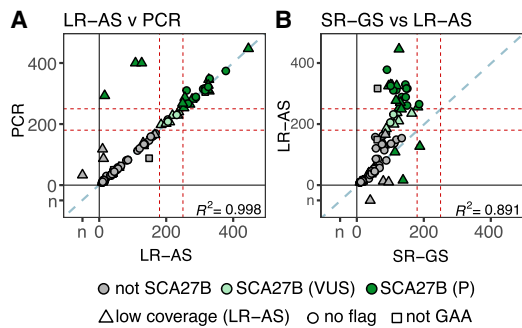


**Figure 4.** Targeted RE screening panel in LR-AS and comparison to SR-GS. STR genotypes were determined for short-listed loci with Straglr. Green points indicate individuals with a confirmed clinical diagnosis for the respective locus. (A) Genotypes are shown for the longer allele in dominant RE disorders that cause ataxia (top 2 plots, loci separated based on maximum allele size) and those that cause other disorders (*bottom*). (B) Genotypes for the shorter and longer alleles are shown for autosomal recessive RE ataxia disorders (*left*) and X-linked recessive disorders other than ataxia, split by sex (*right*). Comparison of allele genotyping with SR-GS and LR-GS is shown for loci with a confirmed diagnosis for the longer allele in dominant disorders. (C) *ATXN8OS* (SCA8), (D) *NOP56* (SCA36), and for both alleles in recessive disorders for (E). *FXN* (FRDA) and (F). *RFC1* (CANVAS, AAGGG motif only). LR-AS with low coverage ( $\leq 7$  reads) are shown as triangles, those with no coverage issues flagged ( $> 7$  reads) are shown as circles. Green circles indicate individuals with an expansion in the respective allele that is larger than the pathogenic threshold (solid or dashed red line). Benign AAAAT motifs do not have a threshold. For *FMR1*, the blue dashed line indicates the threshold for FXTAS while the solid red line is the threshold for FXS.

can use reported pathogenic thresholds for RE, such as *ATXN8OS*, which are smaller than the read length. However, larger pathogenic RE requires the empirical establishment of thresholds that maximize the specificity and sensitivity of RE identification. This is straightforward for disorders such as CANVAS, where a nonreference motif causes disease. In our experience detection of 10 or more nonreference pathogenic motifs provides strong suspicion of a pathogenic RE. Nonreference motif conditions like SCA31 and SCA37 present challenges as pathogenic motifs may be embedded within expanded nonpathogenic motifs (Rosenbohm et al. 2022). Thousands of alleles in gnomAD have nonpathogenic reference motif (AAAAT) expansions  $> 30$  repeats for SCA31 and SCA37 (out of 18,511 individuals). SR-GS methods are mostly unable to detect cases where a pathogenic RE is embedded deep within nonpathogenic motifs due to short DNA fragment libraries and

reliance on sequencing reads aligning to nonrepetitive regions adjacent to a putative RE.

Our findings show that SCA27B, caused by pathogenic expansion of the reference GAA motif, is potentially the most common genetic cause of adult-onset ataxia in Australia. Although no diagnostic test is currently available in Australia for this condition, our results demonstrate SR-GS's effectiveness as a screening tool. Despite considerable population variability in nonpathogenic GAA allele size, applying an empirical threshold  $\geq 90$  repeats using EH5 provides optimal sensitivity (100%) but a false positive rate (15%). These outcomes are consistent with an independent retrospective cohort study, which showed good predictive value (70%) and excellent sensitivity (100%) using STRling for allele size estimates (Mohren et al. 2024). Collectively, the two studies suggest that while SR-GS has a high sensitivity for *FGF14* RE, the



**Figure 5.** Comparison of *FGF14* STR sizing between LR-AS, SR-GS, and PCR identifies the strengths of LR-AS. Comparison of *FGF14* STR sizing is shown as concordance plots for (A). LR-AS compared to PCR and (B). LR-AS compared to SR-GS. The  $R^2$  is a Pearson's correlation. Dark green circles are individuals with a confirmed SCA27B diagnosis (P) and those in light green are SCA27B VUS. Triangles indicate low coverage ( $\leq 7$  reads) on LR-AS, squares indicate non-GAA repeat motifs, and circles samples with coverage  $> 7$  reads and GAA motifs. The pathogenic ( $\geq 250$  repeats) and VUS ( $\geq 180$  repeats) thresholds are shown as dashed red lines. A 1:1 correlation is shown as a dashed blue line. The  $R^2$  is a Pearson's correlation. Individuals with no genotype call from either LR-AS, SR-GS, or PCR are indicated separately to the numbered axis and are labeled nc (i.e., no genotype call).

false positive rate is likely to limit the utility of the method as a diagnostic test specifically for SCA27B. In the research context, there is a need to follow-up a potential positive result with targeted orthogonal analysis to accurately determine RE size in cases identified by SR-GS, especially given SCA27B's recent identification and uncertainty regarding pathogenic thresholds. While the original studies suggest a pathogenic threshold  $\geq 250$  repeats (Pellerin et al. 2023; Rafehi et al. 2023), subsequent studies suggest higher ( $\geq 300$ ) (Méreux et al. 2024) or lower ( $\geq 180$ ) (Mohren et al. 2024) thresholds. We identified nine individuals (8% of the cohort) with *FGF14* GAA alleles in the 180–249 range and reported them as VUS. No alternative pathogenic variants were found in other loci associated with CA in these cases, providing additional support that this range may be pathogenic.

The second most common cause of CA identified in our cohort was biallelic RE in *RFC1* (5/110, 5% yield). Our results demonstrate SR-GS's ability to prioritize cases with potential pathogenic RE at this locus, but extensive orthogonal testing, expert variant interpretation, and clinical input were essential for accurate *RFC1*-related disorder diagnosis. Awareness of diverse pathogenic motifs and thresholds for this disorder is growing, presenting challenges for effective molecular diagnosis utilizing SR-GS (Miyatake et al. 2022b; Scriba et al. 2023). While additional bioinformatic tools can address some issues (Sullivan et al. 2024), SR-GS will likely function best as a prescreening method for *RFC1* diagnostics. A long-read diagnostic tool capable of haplotype-resolved read alignments and full motif composition interrogation will be needed for effective genetic analysis and interpretation of this locus.

LR-AS is a nanopore-based LRS application that enables in silico target selection in real time and has considerable diagnostic potential. Advantages include prioritizing of likely disease-causing loci/variants, reducing curation time, and improving resource utilization by enabling higher sequence multiplexing. In RE disorders, including CA, sequencing depth is critical as knowledge of RE size and composition affects disease prognosis and management (Hannan 2018; GeM-HD 2019). This study achieved a

mean approximately eightfold target enrichment and on-target mean sequencing depth of 22 (95% CI 12.7, 31.0) (Fig. 3). This was insufficient for local assembly of diploid alleles and motif composition interrogation for a majority (75%) of the 2420 RE ( $22 \times 110$ ) targeted. Our results contrast with reports of successful haplotype-resolved allele interrogation with similar metrics ( $\sim 5\times$  enrichment,  $\sim 8\text{--}40\times$  target coverage) (Miller et al. 2021; Stevanovski et al. 2022). Reliable and reproducible data are essential for the accreditation of a diagnostic test, and it is possible these divergent results are due to the choice of bioinformatic tools implemented by the different studies. A current limitation of LR-AS and indeed the ONT platform more broadly, is the lack of widely accepted best-practice pipelines for bioinformatic analysis of data. The Epi2Me Labs wf-human-variation pipeline produced by ONT's Customer Workflows group is the closest analog to the GATK best practices broadly used in processing short-read sequencing data. We designed our analysis after the Epi2Me pipeline, using the most recent, highest-accuracy basecalling model available, and selecting minimap2 (Li 2018) as recommended by recent benchmarks of alignment tools (LoTempio et al. 2023; Helal et al. 2024). Similarly, we selected Straglr (Chiu et al. 2021) as our primary analysis algorithm for its compatibility with minimap2-aligned data, as it is the currently recommended STR caller in Epi2Me's human-variation pipeline.

Despite read depth limitations associated with some targets, LR-AS performed exceptionally well in this trial, achieving 29 diagnoses (29/110, 26% yield). Greater manual review and interpretation of LR-AS data were required to support a diagnosis at specific loci compared to SR-GS. For example, Straglr analysis did not identify potential RE in *NOP56*, despite 4/8 reads indicating a pathogenic RE was likely present (Supplemental Fig. S4). Similarly, while Straglr identified six individuals with potential biallelic pathogenic RE in *RFC1*, extensive manual curation and orthogonal testing showed that only three had true biallelic RE. Moreover, Straglr was unable to generate an allele size estimate for two individuals that SR-GS and orthogonal analysis identified as biallelic. Pathogenic RE was identifiable in aligned reads, but low read depth and few spanning reads compromised allele identification and sizing. However, LR-AS data provided superior diagnostic outcomes at other loci, including *FGF14*.

This study was not designed to efficiently benchmark STR callers and indeed different tools have strengths and weaknesses dependent on the program design. However, we did reanalyze the LR-AS data using an alternate algorithm (vamos), which can interrogate both motif size and composition (Ren et al. 2023). Vamos and Straglr results were broadly concordant (Supplemental Fig. S5) and as a general comment, neither tool could be described as superior to the other. Missing calls were observed with both and these were predominantly due to low sequencing depth; manual review and interpretation were similarly required for vamos. Comparison of Straglr and vamos calls for the 22 loci where there is a biallelic or heterozygous pathogenic RE revealed some discordant results (Supplemental Table S7), which likely reflect the underlying differences in how the algorithms function. Straglr uses a statistical model of length alone that fits Gaussian mixtures to repeat length estimates to produce allele sizes. Straglr may miscall a heterozygote as a homozygote if depth is low. Vamos performs assembly on phased reads and then assesses repeat composition and length from a catalog derived from reference assemblies. Therefore, vamos may perform better where two alleles have repeat sizes that are close to one another, as these are difficult to distinguish from homozygous alleles in the absence of phased reads. We believe vamos

is more sophisticated than Straglr when used on aligned reads that have been phased, but is much more sensitive to the specification of the catalog parameters. For example, *vamos*' calls on the non-expanded *HTT* locus using the "efficient motif catalog" provided by the authors were 3× longer than expected; the use of a CAG-only catalog resulted in *HTT* calls in the normal range. Similarly, *vamos* did not produce calls for *FGF14* until we used a simplified motif catalog testing GAA only at this locus (Supplemental Table S8). In addition, standard implementation of *vamos* requires phased reads (e.g., from WhatsHap), and the variation in sequencing depth at RE loci can result in *vamos* performing assembly with as few as two reads. Our experience suggests that discordancy between algorithms is minimized by greater read depth and the best practice may be to use a pipeline with multiple algorithms. For example, Straglr can be used to identify expanded repeats, while *vamos* can provide redundancy for expansion detection and potentially provide information about motif composition.

RE identification by LR-AS and Straglr analysis was concordant with SR-GS, except for four individuals with SCA27B (Table 2). These diagnoses were missed due to low read depth at the *FGF14* locus, and do not constitute a systemic issue with interrogation of this locus. Indeed, LR-AS appears to have significant advantages over SR-GS for SCA27B diagnosis. No false positive results were observed and LRS can easily span the largest *FGF14* alleles reported to date (>900 repeats) (Mohren et al. 2024) using current diagnostic workflows for gDNA extraction. High correlation in repeat length estimates with LR-PCR (Fig. 5) ( $R^2=0.998$ ) suggests orthogonal validation will not be required when suitable read depth is achieved consistently.

Read depth is a major technical impediment to the diagnostic implementation of LR-AS. While there is a lack of consensus in the field, a read depth of ~33× supports phased de novo genome assembly (Porubsky et al. 2021). For diagnostic purposes 33× is likely the lower bound; ideally, all would be spanning reads to support accreditation benchmarks for consistent and accurate characterization of RE (and small variants). Reliably achieving these targets may be challenging given the competing need to minimize the target panel for maximum enrichment versus increasing panel size to reduce nanopore destruction due to polarity reversal associated with off-target rejection. Additional enrichment strategies, including CRISPR-Cas9, have been proposed with LR-AS, or indeed as an alternative enrichment protocol (Mizuguchi et al. 2021; Lopatriello et al. 2023; Leitão et al. 2024). This developing technology has now been deployed to characterize RE in both the research and diagnostic settings, for example, the PureTarget RE panel developed by PacBio. However, in the context of diagnostic application, CRISPR-Cas9 approaches are bespoke, require considerable optimization, and are limited in the number of targets that can be successfully multiplexed. A second limitation of LR-AS and indeed PCR-free ONT sequencing more broadly for diagnostic implementation is the preparation of the genomic DNA and sequencing library. Up to 10 times the amount of gDNA is needed compared to SR-GS and depending on the platform and experimental design shearing of the DNA may be required for optimal results. This is most reproducible using physical techniques such as Adaptive Focused Acoustics (Covaris) or hydropore shearing (Megaruptor), however, these techniques require additional experimental protocols, leading to potential increases in both the cost and time to return of results.

One major point of difference between SR-GS and LR-AS is the diversity of mutation types that can be interrogated. We used existing diagnostic grade pipelines for the identification of small vari-

ants, CNV, and alterations in mtDNA in the SR-GS data, showing pathogenic small variants as the second most common CA mutation class. We did not formally interrogate the LR-AS data for small variants given the read depth achieved and ONT sequence data error rates (Ni et al. 2023). However, visual inspection of aligned reads spanning pathogenic small variants identified by SR-GS revealed seven of eight variants were identifiable in the ONT reads, suggesting that LR-AS data analysis for RE and small variants could improve diagnostic yield (Supplemental Fig. S6). Notably, the *HEXA* variants in P018 were observed to be in trans. These variants are separated by 22 kb of genomic sequence, making phasing with singleton SR-GS challenging. Indeed, phasing of variants in late-onset conditions such as CA can often be difficult as parental samples cannot be obtained for orthogonal testing. While the accuracy of ONT base calling has been improving over time (>99%) it remains more error-prone than the alternative long-read technology PacBio (>99.9%) (Espinosa et al. 2024) and this may be a consideration for RE testing. Very recently a Tandem Repeat Genotyping Tool (TRGT) and an accompanying Tandem Repeat database have been developed specifically for RE characterization using PacBio HiFi data and this is likely to have utility for diagnostic testing of RE (Dolzhenko et al. 2024). A second major point of difference between SR-GS and long-read technologies more broadly is the ability of the latter to detect base modifications in single molecules of genomic DNA. Methylation of cytosine molecules, most commonly 5-mCpG or 5-hydroxymethylcytosine (5hmC), plays a key role in complex regulatory mechanisms controlling gene expression, ultimately impacting development and disease (Greenberg and Bourc'his 2019). Clinical epigenomics is also playing an increasingly important role in disease diagnosis and prognostication, driving the development of an array of tools to accurately assess genome-wide methylation in long-read sequence data (Sigurpalsdottir et al. 2024).

In conclusion, diagnosing CA remains challenging due to its complexity and variability. This study compared SR-GS and LR-AS for their efficacy in identifying pathogenic REs, the predominant genetic cause of CA. SR-GS demonstrated a higher diagnostic yield (36%) and benefits from its mature, established clinical use, capable of identifying various genetic variants. It also supports future reanalysis as new RE and CA-associated genes are identified. However, its reliance on orthogonal validation for certain RE and limitations in mapping large, complex RE limit its use primarily as a screening tool. Conversely, LR-AS showed promise with a 26% diagnostic yield and potential for more precise haplotype resolution and targeted analysis of clinically relevant regions. Despite requiring extensive manual data interpretation and facing challenges with read depth and error rates, LR-AS has the potential to develop into a standalone frontline diagnostic test for RE. Both technologies represent significant advancements in CA diagnostics that require continued investment in technological development and computational resources. Cost will also significantly impact clinical implementation. SR-GS capacity is established in many diagnostic facilities but reagent costs per test are unlikely to decrease significantly. LR-AS will require investment to establish equipment and computational infrastructure but reagent costs per test will likely decrease due to sample multiplexing and targeted array analysis. Ultimately, a substantial proportion of the cost for both technologies lies in variant curation and interpretation. The future integration of enhanced variant interpretation algorithms, including artificial general intelligence capability, into diagnostic pipelines will benefit both platforms.

## Methods

### Cohort recruitment and clinical phenotype

The Royal Children's Hospital Human Research Ethics Committee (HREC #28097) and the Walter and Eliza Hall Institute of Medical Research (HREC 18/06) approved the study. Multiple health practitioners in Australia referred individuals with a clinical diagnosis of CA for standard diagnostic testing of five RE loci (SCA1/2/3/6/7), mostly to a single center (VCGS). Informed consent for research genetic testing was obtained from 110 participants undergoing clinical testing and phenotype details were derived from the pathology test request form or the referring clinician. Blood-derived genomic DNA was isolated and quality control was performed using standard techniques as described in [Supplemental Methods](#).

### Short-read genome sequencing and analysis

Genome sequencing was performed by the VCGS in Melbourne, Australia with the TruSeq PCR-free DNA HT Library Preparation Kit and sequenced on the Illumina NovaSeq 6000 platform at targeted mean coverage of 30×. For the detection of pathogenic non-RE variants, data were bioinformatically processed by VCGS using commercially available pipelines. Alignment to the reference genome (GRCh38) and calling of nuclear/germline DNA variants was performed using the Dragen v.3.3.7 (Illumina) workflow. Alignment to the revised Cambridge Reference Sequence (rCRS) mitochondrial genome (NC\_012920.1) and calling of mtDNA variants was performed using an in-house analysis pipeline based on the Broad Institute best practice workflow (Mitoreport V1.2.1, see [Data access](#)). We used a research instance of *seqr* (Pais et al. 2022) implemented by Murdoch Children's Research Institute, Melbourne, Australia, to annotate and filter SNV and indel variants. Variants were prioritized for curation using the PanelApp Australia Ataxia\_Superpanel gene list (Version 3.13). Candidate variants were reviewed at a multidisciplinary team (MDT) meeting comprised of clinicians, genomic laboratory staff, and bioinformaticians and classified based on ACMG guidelines (Richards et al. 2015). CNV was screened for and interpreted using the CNV detection tool CXGo (Sadedin et al. 2018). mtDNA variant interpretation was performed by manual filtering, with a variant regarded as homoplasmic or apparently homoplasmic when it was present in at least 97%, respectively, of sequence reads aligned to the genomic position. For RE detection, alignment and variant calling were performed according to the GATK best practice pipeline, and analysis of 67 RE loci was performed utilizing *exSTRa* (v.1.1.0) (Tankard et al. 2018) and *ExpansionHunter* (v.5.0.0) (Dolzhenko et al. 2019) as previously described (Rafehi et al. 2023).

### Targeted long-read sequencing and analysis

LRS was performed on ONT MinION Mk1B sequencer using standard methods as described in [Supplemental Methods](#). Libraries were loaded onto FLO-MIN106D flow cells (ONT) and run for 16–20 h before performing a wash and reload using the Flow Cell Wash Kit (EXP-WSH004, ONT) and run for an additional 24–36 h. Targeted sequencing was performed using the *Readfish* software package (Payne et al. 2021). A panel of 334 regions of genome sequence was targeted, including 67 genes with a pathogenic RE associated with neurological/neuromuscular conditions, genes identified in the Ataxia\_Superpanel (PanelApp Australia Version 3.13), and other candidate genes potentially associated with CA. A full list of target genes and co-ordinates (including 100 kb buffer) is provided in [Supplemental Table S5](#). Libraries were processed using the *wf-human-variation* pipeline (ONT,

v1.8.3), with super-accuracy basecalling model *dna\_r9.4.1\_e8\_sup* from Dorado v0.3.1 on NVIDIA A100 and A30 GPUs. Minimum depth for the pipeline was set at 2× coverage. Reads were aligned to GRCh38 (*GRCh38\_full\_analysis\_set\_plus\_decoy\_hla*) using *minimap2* (v2.24-r1122). STRs were typed against a custom locus catalog containing 154 locus-motif pairs at 67 unique loci using *Straglr* (v1.4.1) or against a subset of loci from *vamos* efficient motif set (v2.1) using *vamos* (v2.1.3) on the haplotagged CRAM files produced by the *wf-human-variation* pipeline. Loci were manually reviewed with *IGV* (v.2.16.2).

### Molecular genetic studies

*FGF14* LR-PCR and RP-PCR were performed as previously described (Rafehi et al. 2023), but using LongAmp HotStart Taq (New England Biolabs). *RFC1* flanking PCR was performed as previously described (Rafehi et al. 2019). *RFC1* RP-PCR and LR-PCR were performed using Q5 High-Fidelity PCR Master Mix (New England Biolabs) and previously published primers (Cortese et al. 2019). *ATXN8/ATXN8OS* sizing PCR and RP-PCR were performed as previously described (Zhou et al. 2019). *NOP56* RP-PCR was performed as previously described (García-Murias et al. 2012). For all PCRs, fragment analysis of FAM-labeled PCR products was performed on the capillary array (ABI3730xl DNA Analyzer, Applied Biosystems) with a LIZ1200 size standard, and visualized using *PeakScanner 2* (Applied Biosystems).

### Statistical analyses

Pearson's correlation performed in R (version 4.0.5) (R Core Team 2024) was used to determine concordance for *FGF14* size estimates between SR-GS, LR-AS, and PCR. RE consisting of non-GAA motifs and low-coverage LR-AS calls were excluded from the calculation. Sensitivity and specificity for *FGF14* for SR-GS and LR-AS were calculated only for individuals with pure GAA motifs. For LR-GS, samples with read depth  $\leq 7$  were excluded from the calculations.

### Data access

All raw sequence data generated in this study have been submitted to the European Genome-phenome Archive (EGA) (<https://ega-archive.org/>) under accession numbers EGAS50000000573 (study ID) and EGAD50000000815 (data set ID). Source code for *mitoreport* is publicly available at GitHub (<https://github.com/bioinfomethods/mitoreport>) and as [Supplemental Code](#).

### Competing interest statement

The authors declare no competing interests.

### Acknowledgments

This work was supported by the Australian Government National Health and Medical Research Council grants (GNT2001513 and MRFF2007677) to P.J.L., M.B.D., H.R., M.B., and D.S. H.R. was supported by a NHMRC Emerging Leadership 1 grant (1194364) and M.B. was supported by an NHMRC Investigator grant (1195236). Additional funding was provided by the Independent Research Institute Infrastructure Support Scheme, the Victorian State Government Operational Infrastructure Program, the Serp Hills Foundation, and the JTM Foundation. K.R.K. reports financial support was provided by the Ainsworth 4 Foundation unrelated to the current study.

*Author contributions:* H.R., L.G.F., M.B., and P.J.L.: conceptualization and funding acquisition; H.R., L.G.F., J.R., P.S., K.C.D., L.S.,

G.G., G.C.T., T.A.F., A.E., S.B., E.B., L.C., J.D., H.G., A.H., G.M.H., A.H., K.K., K.R.K., S.K., M.L.A., A.M., M.Pa., M.Po., K.P., K.R., S.R., A.N., M.S., R.S., C.S., M.W., J.W., T.W., K.W., C.W., K.H.C.W., D.J.S., M.B.D., M.B., and P.J.L.: investigation; M.B. and P.J.L.: supervision; P.J.L.: writing—original draft; H.R., L.G.F., J.R., P.S., K.C.D., L.S., G.G., G.C.T., T.A.F., A.E., S.B., E.B., L.C., J.D., H.G., A.H., G.M.H., A.H., K.K., K.R.K., S.K., M.L.A., A.M., M.Pa., M.Po., K.P., K.R., S.R., A.N., M.S., R.S., C.S., M.W., J.W., T.W., K.W., C.W., K.H.C.W., D.J.S., M.B.D., M.B., and P.J.L.: writing—review and editing.

## References

- Amarasinghe SL, Su S, Dong X, Zappia L, Ritchie ME, Gouil Q. 2020. Opportunities and challenges in long-read sequencing data analysis. *Genome Biol* **21**: 30. doi:10.1186/s13059-020-1935-5
- Ando M, Higuchi Y, Yuan J, Yoshimura A, Kojima F, Yamanishi Y, Aso Y, Izumi K, Imada M, Maki Y, et al. 2024. Clinical variability associated with intronic *FGF14* GAA repeat expansion in Japan. *Ann Clin Transl Neurol* **11**: 96–104. doi:10.1002/acn3.51936
- Ashley EA. 2016. Towards precision medicine. *Nat Rev Genet* **17**: 507–522. doi:10.1038/nrg.2016.86
- Bahlo M, Bennett MF, Degorski P, Tankard RM, Delatycki MB, Lockhart PJ. 2018. Recent advances in the detection of repeat expansions with short-read next-generation sequencing. *F1000Res* **7**: 736. doi:10.12688/f1000research.13980.1
- Beaudin M, Matilla-Duenas A, Soong BW, Pedroso JL, Barsottini OG, Mitoma H, Tsuji S, Schmahmann JD, Manto M, Rouleau GA, et al. 2019. The classification of autosomal recessive cerebellar ataxias: a consensus statement from the society for research on the cerebellum and ataxias task force. *Cerebellum* **18**: 1098–1125. doi:10.1007/s12311-019-01052-2
- Chiu R, Rajan-Babu IS, Friedman JM, Birol I. 2021. Straglr: discovering and genotyping tandem repeat expansions using whole genome long-read sequences. *Genome Biol* **22**: 224. doi:10.1186/s13059-021-02447-3
- Cortese A, Simone R, Sullivan R, Vandrovicova J, Tariq H, Yau WY, Humphrey J, Jaunmuktane Z, Sivakumar P, Polke J, et al. 2019. Biallelic expansion of an intronic repeat in *RFC1* is a common cause of late-onset ataxia. *Nat Genet* **51**: 649–658. doi:10.1038/s41588-019-0372-4
- Cortese A, Beecroft SJ, Facchini S, Curro R, Cabrera-Serrano M, Stevanovski I, Chintalaphani SR, Gamaarachchi H, Weisburd B, Folland C, et al. 2024. A CCG expansion in *ABCD3* causes oculopharyngodistal myopathy in individuals of European ancestry. *Nat Commun* **15**: 6327. doi:10.1038/s41467-024-49950-2
- Daker-White G, Kingston H, Payne K, Greenfield J, Ealing J, Sanders C. 2015. 'You don't get told anything, they don't do anything and nothing changes'. Medicine as a resource and constraint in progressive ataxia. *Health Expect* **18**: 177–187. doi:10.1111/hex.12016
- Day JW, Ricker K, Jacobsen JF, Rasmussen LJ, Dick KA, Kress W, Schneider C, Koch MC, Beilman GJ, Harrison AR, et al. 2003. Myotonic dystrophy type 2: molecular, diagnostic and clinical spectrum. *Neurology* **60**: 657–664. doi:10.1212/01.WNL.0000054481.84978.F9
- Depienne C, Mandel JL. 2021. 30 years of repeat expansion disorders: what have we learned and what are the remaining challenges? *Am J Hum Genet* **108**: 764–785. doi:10.1016/j.ajhg.2021.03.011
- de Silva R, Greenfield J, Cook A, Bonney H, Vallortigara J, Hunt B, Giunti P. 2019. Guidelines on the diagnosis and management of the progressive ataxias. *Orphanet J Rare Dis* **14**: 51. doi:10.1186/s13023-019-1013-9
- Dolzhenko E, van Vugt J, Shaw RJ, Bekritsky MA, van Blitterswijk M, Narzisi G, Ajay SS, Rajan V, Lajoie BR, Johnson NH, et al. 2017. Detection of long repeat expansions from PCR-free whole-genome sequence data. *Genome Res* **27**: 1895–1903. doi:10.1101/gr.225672.117
- Dolzhenko E, Deshpande V, Schlesinger F, Krusche P, Petrovski R, Chen S, Emig-Agius D, Gross A, Narzisi G, Bowman B, et al. 2019. ExpansionHunter: a sequence-graph-based tool to analyze variation in short tandem repeat regions. *Bioinformatics* **35**: 4754–4756. doi:10.1093/bioinformatics/btz431
- Dolzhenko E, Bennett MF, Richmond PA, Trost B, Chen S, van Vugt J, Nguyen C, Narzisi G, Gainullin VG, Gross AM, et al. 2020. ExpansionHunter denovo: a computational method for locating known and novel repeat expansions in short-read sequencing data. *Genome Biol* **21**: 102. doi:10.1186/s13059-020-02017-z
- Dolzhenko E, English A, Dashnow H, De Sena Brandine G, Mokveld T, Rowell WJ, Karniski C, Kronenberg Z, Danzi MC, Cheung WA, et al. 2024. Characterization and visualization of tandem repeats at genome scale. *Nat Biotechnol* **42**: 1606–1614. doi:10.1038/s41587-023-02057-3
- Dominik N, Magri S, Currò R, Abati E, Facchini S, Corbetta M, Macpherson H, Di Bella D, Sarto E, Stevanovski I, et al. 2023. Normal and pathogenic variation of *RFC1* repeat expansions: implications for clinical diagnosis. *Brain* **146**: 5060–5069. doi:10.1093/brain/awad240
- Espinosa E, Bautista R, Larrosa R, Plata O. 2024. Advancements in long-read genome sequencing technologies and algorithms. *Genomics* **116**: 110842. doi:10.1016/j.ygeno.2024.110842
- Gall-Duncan T, Sato N, Yuen RKC, Pearson CE. 2022. Advancing genomic technologies and clinical awareness accelerates discovery of disease-associated tandem repeat sequences. *Genome Res* **32**: 1–27. doi:10.1101/gr.269530.120
- García-Foncillas J, Argente J, Bujanda L, Cardona V, Casanova B, Fernández-Montes A, Horcajadas JA, Iñiguez A, Ortiz A, Pablos JL, et al. 2021. Milestones of precision medicine: an innovative, multidisciplinary overview. *Mol Diagn Ther* **25**: 563–576. doi:10.1007/s40291-021-00544-4
- García-Murias B, Quintáns B, Arias M, Seixas AI, Cacheiro P, Tarrío R, Pardo J, Millán MJ, Arias-Rivas S, Blanco-Arias P, et al. 2012. 'Costa da Morte' ataxia is spinocerebellar ataxia 36: clinical and genetic characterization. *Brain* **135**(Pt 5): 1423–1435. doi:10.1093/brain/aws069
- GeM-HD. 2019. CAG repeat not polyglutamine length determines timing of Huntington's disease onset. *Cell* **178**: 887–900.e14. doi:10.1016/j.cell.2019.06.036
- Greenberg MVC, Bourc'his D. 2019. The diverse roles of DNA methylation in mammalian development and disease. *Nat Rev Mol Cell Biol* **20**: 590–607. doi:10.1038/s41580-019-0159-6
- Hadjivassiliou M, Martindale J, Shanmugarajah P, Grünwald RA, Sarrigiannis PG, Beauchamp N, Garrard K, Warburton R, Sanders DS, Friend D, et al. 2017. Causes of progressive cerebellar ataxia: prospective evaluation of 1500 patients. *J Neurol Neurosurg Psychiatry* **88**: 301–309. doi:10.1136/jnnp-2016-314863
- Hannan AJ. 2018. Tandem repeats mediating genetic plasticity in health and disease. *Nat Rev Genet* **19**: 286–298. doi:10.1038/nrg.2017.115
- Helal AA, Saad BT, Saad MT, Mosaad GS, Aboshanab KM. 2024. Benchmarking long-read aligners and SV callers for structural variation detection in Oxford nanopore sequencing data. *Sci Rep* **14**: 6160. doi:10.1038/s41598-024-56604-2
- Ibañez K, Polke J, Hagelstrom RT, Dolzhenko E, Pasko D, Thomas ERA, Daugherty LC, Kasperaviciute D, Smith KR, WGS for Neurological Diseases Group, et al. 2022. Whole genome sequencing for the diagnosis of neurological repeat expansion disorders in the UK: a retrospective diagnostic accuracy and prospective clinical validation study. *Lancet Neurol* **21**: 234–245. doi:10.1016/S1474-4422(21)00462-2
- Iruzbieteta P, Pellerin D, Bergareche A, Albajar I, Mondragón E, Vinagre A, Fernández-Torrón R, Moreno F, Equiza J, Campo-Caballero D, et al. 2023. Frequency and phenotypic spectrum of spinocerebellar ataxia 27B and other genetic ataxias in a Spanish cohort of late-onset cerebellar ataxia. *Eur J Neurol* **30**: 3828–3833. doi:10.1111/ene.16039
- Jayadev S, Bird TD. 2013. Hereditary ataxias: overview. *Genet Med* **15**: 673–683. doi:10.1038/gim.2013.28
- Kang C, Liang C, Ahmad KE, Gu Y, Siow SF, Colebatch JG, Whyte S, Ng K, Cremer PD, Corbett AJ, et al. 2019. High degree of genetic heterogeneity for hereditary cerebellar ataxias in Australia. *Cerebellum* **18**: 137–146. doi:10.1007/s12311-018-0969-7
- Lappalainen T, Scott AJ, Brandt M, Hall IM. 2019. Genomic analysis in the age of human genome sequencing. *Cell* **177**: 70–84. doi:10.1016/j.cell.2019.02.032
- La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH. 1991. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature* **352**: 77–79. doi:10.1038/352077a0
- Lee A. 2023. Omaveloxolone: first approval. *Drugs* **83**: 725–729. doi:10.1007/s40265-023-01874-9
- Leitão E, Schröder C, Depienne C. 2024. Identification and characterization of repeat expansions in neurological disorders: methodologies, tools, and strategies. *Rev Neurol (Paris)* **180**: 383–392. doi:10.1016/j.neurol.2024.03.005
- Li H. 2018. Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics* **34**: 3094–3100. doi:10.1093/bioinformatics/bty191
- Lopatriello G, Maestri S, Alfano M, Papa R, Di Vittorio V, De Antoni L, Bellucci E, Pieri A, Bitocchi E, Delledonne M, et al. 2023. CRISPR/Cas9-mediated enrichment coupled to nanopore sequencing provides a valuable tool for the precise reconstruction of large genomic target regions. *Int J Mol Sci* **24**: 1076. doi:10.3390/ijms24021076
- LoTempio J, Delot E, Vilain E. 2023. Benchmarking long-read genome sequence alignment tools for human genomics applications. *PeerJ* **11**: e16515. doi:10.7717/peerj.16515
- McCarthy JJ, McLeod HL, Ginsburg GS. 2013. Genomic medicine: a decade of successes, challenges, and opportunities. *Sci Transl Med* **5**: 189sr184. doi:10.1126/scitranslmed.3005785

- Méreaux JL, Davoine CS, Pellerin D, Coarelli G, Coutelier M, Ewencyk C, Monin ML, Anheim M, Le Ber I, Thobois S, et al. 2024. Clinical and genetic keys to cerebellar ataxia due to *FGF14* GAA expansions. *EBioMedicine* **99**: 104931. doi:10.1016/j.ebiom.2023.104931
- Miller DE, Sulovari A, Wang T, Loucks H, Hoekzema K, Munson KM, Lewis AP, Fuerte EPA, Paschal CR, Walsh T, et al. 2021. Targeted long-read sequencing identifies missing disease-causing variation. *Am J Hum Genet* **108**: 1436–1449. doi:10.1016/j.ajhg.2021.06.006
- Miyatake S, Koshimizu E, Fujita A, Doi H, Okubo M, Wada T, Hamanaka K, Ueda N, Kishida H, Minase G, et al. 2022a. Rapid and comprehensive diagnostic method for repeat expansion diseases using nanopore sequencing. *NPJ Genom Med* **7**: 62. doi:10.1038/s41525-022-00331-y
- Miyatake S, Yoshida K, Koshimizu E, Doi H, Yamada M, Miyaji Y, Ueda N, Tsuyuzaki J, Kodaira M, Onoue H, et al. 2022b. Repeat conformation heterogeneity in cerebellar ataxia, neuropathy, vestibular areflexia syndrome. *Brain* **145**: 1139–1150. doi:10.1093/brain/awab363
- Mizuguchi T, Toyota T, Miyatake S, Mitsuhashi S, Doi H, Kudo Y, Kishida H, Hayashi N, Tsuburaya RS, Kinoshita M, et al. 2021. Complete sequencing of expanded *SAMD12* repeats by long-read sequencing and Cas9-mediated enrichment. *Brain* **144**: 1103–1117. doi:10.1093/brain/awab021
- Mizushima K, Shibata Y, Shirai S, Matsushima M, Miyatake S, Iwata I, Yaguchi H, Matsumoto N, Yabe I. 2024. Prevalence of repeat expansions causing autosomal dominant spinocerebellar ataxias in Hokkaido, the northernmost island of Japan. *J Hum Genet* **69**: 27–31. doi:10.1038/s10038-023-01200-x
- Mohren L, Erdlenbruch F, Leitão E, Kilpert F, Hönes GS, Kaya S, Schröder C, Thieme A, Sturm M, Park J, et al. 2024. Identification and characterisation of pathogenic and non-pathogenic *FGF14* repeat expansions. *Nat Commun* **15**: 7665. doi:10.1038/s41467-024-52148-1
- Mousavi N, Shleizer-Burko S, Yanicky R, Gymrek M. 2019. Profiling the genome-wide landscape of tandem repeat expansions. *Nucleic Acids Res* **47**: e90. doi:10.1093/nar/gkz501
- Németh AH, Kwasniewska AC, Lise S, Parolin Schneckenberg R, Becker EB, Bera KD, Shanks ME, Gregory L, Buck D, Zameel Cader M, et al. 2013. Next generation sequencing for molecular diagnosis of neurological disorders using ataxias as a model. *Brain* **136**: 3106–3118. doi:10.1093/brain/awt236
- Ngo KJ, Rexach JE, Lee H, Petty LE, Perlman S, Valera JM, Deignan JL, Mao Y, Aker M, Posey JE, et al. 2020. A diagnostic ceiling for exome sequencing in cerebellar ataxia and related neurological disorders. *Hum Mutat* **41**: 487–501. doi:10.1002/humu.23946
- Ni Y, Liu X, Simeneh ZM, Yang M, Li R. 2023. Benchmarking of Nanopore R10.4 and R9.4.1 flow cells in single-cell whole-genome amplification and whole-genome shotgun sequencing. *Comput Struct Biotechnol J* **21**: 2352–2364. doi:10.1016/j.csbj.2023.03.038
- Oberlé I, Rousseau F, Heitz D, Kretz C, Devys D, Hanauer A, Boué J, Bertheas MF, Mandel JL. 1991. Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science* **252**: 1097–1102. doi:10.1126/science.252.5009.1097
- Pais LS, Snow H, Weisburd B, Zhang S, Baxter SM, DiTroia S, O’Heir E, England E, Chao KR, Lemire G, et al. 2022. *seqr*: a web-based analysis and collaboration tool for rare disease genomics. *Hum Mutat* **43**: 698–707. doi:10.1002/humu.24366
- Paulson H. 2018. Repeat expansion diseases. *Handb Clin Neurol* **147**: 105–123. doi:10.1016/B978-0-444-63233-3.00009-9
- Payne A, Holmes N, Clarke T, Munro R, Debebe BJ, Loose M. 2021. Readfish enables targeted nanopore sequencing of gigabase-sized genomes. *Nat Biotechnol* **39**: 442–450. doi:10.1038/s41587-020-00746-x
- Pellerin D, Danzi MC, Wilke C, Renaud M, Fazal S, Dicaire MJ, Scriba CK, Ashton C, Yanick C, Beijer D, et al. 2023. Deep intronic *FGF14* GAA repeat expansion in late-onset cerebellar ataxia. *N Engl J Med* **388**: 128–141. doi:10.1056/NEJMoa2207406
- Porubsky D, Ebert P, Audano PA, Vollger MR, Harvey WT, Marijng P, Ebler J, Munson KM, Sorensen M, Sulovari A, et al. 2021. Fully phased human genome assembly without parental data using single-cell strand sequencing and long reads. *Nat Biotechnol* **39**: 302–308. doi:10.1038/s41587-020-0719-5
- Rafehi H, Szmulewicz DJ, Bennett MF, Sobreira NLM, Pope K, Smith KR, Gillies G, Diakumis P, Dolzhenko E, Eberle MA, et al. 2019. Bioinformatics-based identification of expanded repeats: a non-reference intronic pentamer expansion in *RFC1* causes CANVAS. *Am J Hum Genet* **105**: 151–165. doi:10.1016/j.ajhg.2019.05.016
- Rafehi H, Szmulewicz DJ, Pope K, Wallis M, Christodoulou J, White SM, Delatycki MB, Lockhart PJ, Bahlo M. 2020. Rapid diagnosis of spinocerebellar ataxia 36 in a three-generation family using short-read whole-genome sequencing data. *Mov Disord* **35**: 1675–1679. doi:10.1002/mds.28105
- Rafehi H, Read J, Szmulewicz DJ, Davies KC, Snell P, Fearnley LG, Scott L, Thomsen M, Gillies G, Pope K, et al. 2023. An intronic GAA repeat expansion in *FGF14* causes the autosomal-dominant adult-onset ataxia
- SCA50/ATX-FGF14. *Am J Hum Genet* **110**: 105–119. doi:10.1016/j.ajhg.2022.11.015
- Rajan-Babu IS, Dolzhenko E, Eberle MA, Friedman JM. 2024. Sequence composition changes in short tandem repeats: heterogeneity, detection, mechanisms and clinical implications. *Nat Rev Genet* **25**: 476–499. doi:10.1038/s41576-024-00696-z
- R Core Team. 2024. *R: a language and environment for statistical computing*. Foundation for Statistical Computing, Vienna. <https://www.R-project.org/>.
- Read JL, Davies KC, Thompson GC, Delatycki MB, Lockhart PJ. 2023. Challenges facing repeat expansion identification, characterisation, and the pathway to discovery. *Emerg Top Life Sci* **7**: 339–348. doi:10.1042/ETLS20230019
- Rehm HL. 2017. Evolving health care through personal genomics. *Nat Rev Genet* **18**: 259–267. doi:10.1038/nrg.2016.162
- Ren J, Gu B, Chaisson MJP. 2023. Vamos: variable-number tandem repeats annotation using efficient motif sets. *Genome Biol* **24**: 175. doi:10.1186/s13059-023-03010-y
- Rexach J, Lee H, Martinez-Agosto JA, Németh AH, Fogel BL. 2019. Clinical application of next-generation sequencing to the practice of neurology. *Lancet Neurol* **18**: 492–503. doi:10.1016/S1474-4422(19)30033-X
- Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, et al. 2015. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* **17**: 405–424. doi:10.1038/gim.2015.30
- Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP. 2011. Integrative genomics viewer. *Nat Biotechnol* **29**: 24–26. doi:10.1038/nbt.1754
- Rosenbohm A, Pott H, Thomsen M, Rafehi H, Kaya S, Szymczak S, Volk AE, Mueller K, Silveira I, Weishaupt JH, et al. 2022. Familial cerebellar ataxia and amyotrophic lateral sclerosis/frontotemporal dementia with *DAB1* and *C9ORF72* repeat expansions: an 18-year study. *Mov Disord* **37**: 2427–2439. doi:10.1002/mds.29221
- Ruano L, Melo C, Silva MC, Coutinho P. 2014. The global epidemiology of hereditary ataxia and spastic paraplegia: a systematic review of prevalence studies. *Neuroepidemiology* **42**: 174–183. doi:10.1159/000358801
- Rudaks LI, Yeow D, Ng K, Deveson IW, Kennerson ML, Kumar KR. 2024. An update on the adult-onset hereditary cerebellar ataxias: novel genetic causes and new diagnostic approaches. *The Cerebellum* **23**: 2152–2168. doi:10.1007/s12311-024-01703-z
- Sadedin SP, Ellis JA, Masters SL, Oshlack A. 2018. Ximmer: a system for improving accuracy and consistency of CNV calling from exome data. *GigaScience* **7**: giv112. doi:10.1093/gigascience/giy112
- Schlötterer C, Tautz D. 1992. Slippage synthesis of simple sequence DNA. *Nucleic Acids Res* **20**: 211–215. doi:10.1093/nar/20.2.211
- Scriba CK, Stevanovski I, Chintalaphani SR, Gamaarachchi H, Ghaoui R, Ghia D, Henderson RD, Jordan N, Winkler A, Lamont PJ, et al. 2023. *RFC1* in an Australasian neurological disease cohort: extending the genetic heterogeneity and implications for diagnostics. *Brain Commun* **5**: fcad208. doi:10.1093/braincomms/fcad208
- Sigurpalsdóttir BD, Stefansson OA, Holley G, Beyter D, Zink F, Hardarson M, Sverrisson S, Kristinsdóttir N, Magnúsdóttir DN, Magnússon O, et al. 2024. A comparison of methods for detecting DNA methylation from long-read sequencing of human genomes. *Genome Biol* **25**: 69. doi:10.1186/s13059-024-03207-9
- Stevanovski I, Chintalaphani SR, Gamaarachchi H, Ferguson JM, Pineda SS, Scriba CK, Tchan M, Fung V, Ng K, Cortese A, et al. 2022. Comprehensive genetic diagnosis of tandem repeat expansion disorders with programmable targeted nanopore sequencing. *Sci Adv* **8**: eabm5386. doi:10.1126/sciadv.abm5386
- Sullivan R, Chen S, Saunders CT, Yau WY, Goh YY, O’Connor E, Dominik-N, Deforie VG, Morsy H, Cortese A, et al. 2024. *RFC1* repeat expansion analysis from whole genome sequencing data simplifies screening and increases diagnostic rates. medRxiv doi:10.1101/2024.02.28.24303510
- Tankard RM, Bennett MF, Degorski P, Delatycki MB, Lockhart PJ, Bahlo M. 2018. Detecting expansions of tandem repeats in cohorts sequenced with short-read sequencing data. *Am J Hum Genet* **103**: 858–873. doi:10.1016/j.ajhg.2018.10.015
- Turro E, Astle WJ, Megy K, Gräf S, Greene D, Shamardina O, Allen HL, Sanchis-Juan A, Frontini M, Thys C, et al. 2020. Whole-genome sequencing of patients with rare diseases in a national health system. *Nature* **583**: 96–102. doi:10.1038/s41586-020-2434-2
- Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP, Pizzuti A, Reiner O, Richards S, Victoria MF, Zhang FP, et al. 1991. Identification of a gene (*FMR-1*) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* **65**: 905–914. doi:10.1016/0092-8674(91)90397-H

Wilke C, Pellerin D, Mengel D, Traschütz A, Danzi MC, Dicaire MJ, Neumann M, Lerche H, Bender B, Houlden H, et al. 2023. GAA-FGF14 ataxia (SCA27B): phenotypic profile, natural history progression and 4-aminopyridine treatment response. *Brain* **146**: 4144–4157. doi:10.1093/brain/awad157

Willems T, Gymrek M, Highnam G, Mittelman D, The 1000 Genomes Project Consortium, Erlich Y. 2014. The landscape of human STR variation. *Genome Res* **24**: 1894–1904. doi:10.1101/gr.177774.114

Zhou Y, Yuan Y, Liu Z, Zeng S, Chen Z, Shen L, Jiang H, Xia K, Tang B, Wang J. 2019. Genetic and clinical analyses of spinocerebellar ataxia type 8 in mainland China. *J Neurol* **266**: 2979–2986. doi:10.1007/s00415-019-09519-2

Received June 11, 2024; accepted in revised form November 21, 2024.