

<https://doi.org/10.1038/s41525-025-00497-1>

Mosaic X-linked adrenoleukodystrophy in males identified by newborn screening and next-generation sequencing



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Somatic mosaicism produces genetic differences between cells in an individual and is an underrecognized contributor to phenotypic variability. Precise understanding of the natural history of genetic diseases, therefore, requires detection and recognition of low-level mosaicism, which remains technically challenging, particularly for X-linked genes. Here, we identify six males with mosaic X-linked adrenoleukodystrophy (X-ALD), a neurometabolic peroxisomal disorder caused by pathogenic variants in *ABCD1* that is currently included in 44 state newborn screening (NBS) programs, and estimate the incidence of somatic mosaicism. Of 227 males from 2 laboratories performing *ABCD1* next-generation sequencing, 1.8% (4/227) had pathogenic or likely pathogenic *ABCD1* variants that were mosaic. In one mosaic male individual, allele-specific measurements across multiple tissues demonstrated *ABCD1* variant allele fractions ranging from 66 to 82%. Our findings have implications for the identification of X-ALD through NBS, and additional studies could provide insight into the pathogenesis and natural history of X-ALD.

X-linked adrenoleukodystrophy (X-ALD) is a neurometabolic disorder with an estimated prevalence of 1 in 14,000–17,000 births^{1,2}. X-ALD is caused by pathogenic loss-of-function variants in the *ABCD1* gene located on the X chromosome. *ABCD1* encodes an ATP-binding cassette that imports very long-chain fatty acids (VLCFA) into the peroxisome. Pathogenic *ABCD1* variants cause impaired peroxisomal VLCFA β -oxidation, resulting in the accumulation of VLCFA in plasma and tissues. VLCFA levels are almost universally elevated in males with pathogenic *ABCD1* variants, but there is great phenotypic variability, and biomarkers predicting

disease severity, such as extent of VLCFA elevation, have only recently been identified³. Intrafamilial phenotypic variability is common, ranging from isolated adrenal insufficiency in adulthood to a rapidly progressive inflammatory demyelinating disease in early childhood (“cerebral X-ALD”). Factors contributing to X-ALD phenotypic variability are unclear, and although peroxisomal dysfunction is central, the mechanisms by which elevated VLCFA lead to disease remain an area of active inquiry. The definitive treatment for cerebral X-ALD is hematopoietic stem cell transplantation (HSCT), which, if performed early enough, can slow or arrest

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cerebral disease progression⁴. Because of the success of HSCT, as well as hormone replacement therapy for adrenal dysfunction and a recently FDA-approved gene therapy (elivaldogene autotemcel)⁵, 44 states now include X-ALD in universal newborn screening (NBS) programs, yielding a large, pre-symptomatic cohort of male children diagnosed with X-ALD. While promising new biomarkers have been identified³, it remains challenging to predict on an individual level which of these pre-symptomatic newborn children will progress to cerebral X-ALD. We hypothesized that somatic mosaicism for pathogenic *ABCD1* variants might be present in pre-symptomatic children who were identified through NBS to be at risk for X-ALD and set out to estimate the incidence of this under-recognized factor that could potentially modify disease severity.

Results

Identification and verification of the proband with mosaic X-ALD

Our index individual (mXALD-1) was identified as having an elevated C26:0-LPC on state newborn screening, and follow-up next-generation sequencing (NGS) demonstrated the presence of a likely pathogenic missense variant (NM_000033:c.1988T>C, p.Leu663Pro) in *ABCD1* in ~82% of reads (Table 1). For hemizygous XY males, X-linked variants are expected at either 0% or 100%, so the variant allele fraction (VAF) of 82% suggested somatic mosaicism in this individual. A SNP microarray established 46, XY chromosomes, and multiplex ligation-dependent probe amplification (MLPA) analysis demonstrated the presence of a single copy of *ABCD1*. The presence of variant and reference alleles was confirmed by Sanger sequencing (Supplementary Fig. 1), and second blood samples obtained 2 years after initial sample also found a VAF of 80%, suggesting stable persistence of the *ABCD1* mutant cell line within the blood.

Variations in the variant allele fraction from different tissues in a mosaic individual

To determine the tissue distribution of this variant, 6 tissues (blood, skin, buccal swab, CSF, tonsils, and urine) were obtained, and, when possible, both genomic and cell-free DNA were isolated. Using a custom droplet digital polymerase chain reaction (ddPCR) assay, we found that the VAF ranged from 66% to 82% across these samples and was lowest in the skin (Fig. 1). These results are consistent with an asymptomatic male individual with a post-zygotic mosaic variant in *ABCD1* manifesting as biochemically and molecularly confirmed X-ALD.

Discovery of additional males with mosaic X-ALD

To identify additional individuals with mosaic X-ALD, we contacted large commercial sequencing laboratories ($n = 5$) and state newborn screening programs performing NGS sequencing ($n = 7$) to find males with pathogenic or likely pathogenic (P/LP) *ABCD1* variants in which the VAF deviated from 100%. Five additional individuals were identified, for a total of six males, including our proband. The VAF was not consistently included in the clinical report; thus, the ordering healthcare provider was not always made aware that the *ABCD1* variant was mosaic. In 4 of these individuals (mXALD-2–5) clinical information was available after contact with the ordering provider (Table 1). These individuals were 6 months–6 years old, and all were identified through newborn screening. Including our proband, all 5 males are reported as asymptomatic with normal adrenal screening labs (ACTH, cortisol) and neurologic exam (though mXALD-4 was noted to have a mild speech delay). mXALD-3 and mXALD-4 had subtle differences on brain MRI, which have remained stable. mXALD-5 was born with prenatally diagnosed arthrogryposis thought to be unrelated to X-ALD, and had a normal brain MRI at 11 months of age.

Estimated incidence of mosaic X-ALD

To estimate the incidence of mosaic X-ALD in a larger cohort, we examined data from two large reference labs over a 5-year period (2017–2022). During this time, 227 males with P/LP variants in *ABCD1* were reported, four of which (4/227 or 1.8%) had VAFs that were less than 100%, consistent with mosaicism (Table 2). Clinical information was available for three individuals

(mXALD-1–3) as described above (Table 1). We chose not to include mosaic *ABCD1* variants of uncertain significance (VUS) in our estimate, since the majority of VUS are reclassified downward⁶, and the clinical indication for most individuals undergoing sequencing is a positive newborn screen, which by itself is only a screening test, and requires additional testing to identify false positives from true X-ALD cases.

Discussion

In summary, we estimate the frequency of mosaic X-ALD in a molecularly confirmed X-ALD cohort to be approximately 1.8%, and obtained clinical information for 5 mosaic X-ALD individuals. Although mosaicism in X-ALD has been previously reported^{7,8}, this is the first study to measure VAF across multiple tissues and to estimate the incidence of mosaicism in a larger cohort. The presence of relatively high VAFs (66–82%) in mXALD-1 across tissues containing all three germ layers suggests that the p.Leu663Pro variant arose very early in development, prior to gastrulation, with both mutated and non-mutated cells contributing to the inner cell mass⁹. We cannot rule out the possibility of reversion of a mutation inherited from the egg, but since the p.Leu663Pro variant in mXALD-1 was not present in his mother's blood, this would require two independent mutational events, which is unlikely.

Because some of the 227 individuals with P/LP *ABCD1* variants had clinical testing due to a known family history of X-ALD (and thus cannot have mosaic X-ALD), our estimate of 1.8% incidence of mosaicism in X-ALD may underestimate the proportion of de novo X-ALD caused by mosaicism. At the same time, our exclusion of mosaic VUS in *ABCD1* could also lead our estimate to be elevated, although we justified our decision not to include VUS in our study, as many of these variants will be downgraded, particularly in asymptomatic individuals identified on NBS. Laboratories do not routinely track or report VAF deviation from 100% in their clinical reports. VAFs between 30 and 60% are typical of heterozygous variants located on the autosomes, but caution is needed for X-linked genes, where VAFs in this range could easily be misinterpreted as heterozygous. Thus, our study suggests that mosaicism in X-ALD is underrecognized. Given the increasing number of presymptomatic males identified by expanded NBS and the increased sensitivity to mosaicism provided by NGS over Sanger sequencing, we recommend that laboratories include this information in their reports. More individuals and longer-term clinical follow-up will be required to determine if mosaic *ABCD1* variants are associated with reduced phenotypic penetrance, but this will be challenging to determine unless clinical labs routinely include the presence of mosaicism in their reporting.

Our study adds to the growing body of literature detecting mosaicism in non-neoplastic, developmental diseases^{10–12}. X-linked mosaicism is rare and has only been published in two other X-linked conditions, to our knowledge (hypophosphatasia¹³ and VEXAS¹⁴). Our 1.8% estimate of mosaicism in X-ALD is consistent with other similar studies. A recent study examining autosomal dominant polycystic kidney disease (ADPKD) found that approximately 1% of all cases and up to 10% of genetically unsolved cases were mosaic, and these individuals generally had less severe disease¹⁵. We feel this is an important question that requires exploration in X-ALD, as accurate risk calculation could have direct implications for risk counseling as well as screening and management recommendations for newborns with X-ALD. Additionally, the distribution and burden of pathogenic variants across different tissues may be an independent phenotypic modifier in X-ALD, similar to engineered tissue-specific mouse mutants. In mosaic individuals with elevated tissue-specific mutation burden, stratifying the clinical impact of peroxisomal dysfunction across vulnerable tissues—such as the CNS, adrenal cortex, and testis—may yield critical insights into disease pathogenesis. Given that X-ALD pathology manifests with striking tissue selectivity despite systemic VLCFA accumulation, studying mosaicism offers a unique opportunity to dissect how localized peroxisomal deficits contribute to early cellular dysfunction and disease progression¹⁶. Although there are standards for reporting mosaicism in cancer¹⁷, no such standards currently exist for non-neoplastic conditions. We recommend that sequencing laboratories continue to report mosaicism when found and

Table 1 | Comparison of ABCD1 mosaicism in five male individuals identified through newborn screening

	mXALD-1	mXALD-2	mXALD-3	mXALD-4	mXALD-5
Initial VLCFA testing	NBS Tier 1: C26:0-LPC (μmol/L)	0.52 (cutoff ≥ 0.22)	0.48 [Ref Range < 0.2]	0.48 [RR 0.25–0.38; cutoff ≥ 0.42]	0.306 (cutoff < 0.18)
	NBS Tier 2: C26:0-LPC (μmol/L)	0.36 (cutoff ≥ 0.12)	0.37 [Ref Range < 0.2]	0.17 [cutoff > 0.15; RR 0.04–0.09]	
	Confirmatory C26:0 (μmol/L)	0.7 (RR 0.05–0.41)	2.09 [RR ≤ 1.3 nmol/mL]	1.19 [RR ≤ 1.3 nmol/mL]	0.800 (0.23 ± 0.09)
	Confirmatory C26:0/C22:0 ratio	1.4 (RR 0.6–1.1)	0.039 [RR ≤ 0.023]	0.02 (RR not provided)	0.04 (0.01 ± 0.004)
	Confirmatory C24:0/C22:0 ratio	0.05 (RR < 0.02)	1.23 [RR ≤ 1.39 ratio]	0.93 (RR not provided)	1.256 (0.84 ± 0.10)
Variant analysis	Tissue tested	blood	buccal	blood	blood
	Mosaic Variant Identified	c.1988 T > C, p.Leu663Pro	c.1817 C > T, p.Ser606Leu	c.1826 A > G, p.Glu609Gly	c.1850 G > A, p.Arg617His
	% VAF – Lab #A (NGS coverage)	82% (77x)	60% (285x)	unknown (not reported by lab)	unknown
	% VAF – Lab #B (NGS coverage)	80% (117x)	n/a	unknown (not reported by lab)	n/a
	Next-generation sequencing (±Sanger)	NGS (+Sanger)	NGS (+Sanger)	NGS (+Sanger)	NGS (+Sanger)
	Variant Classification	Likely pathogenic	Pathogenic	Likely Pathogenic	Pathogenic
	Age at time of testing	11.5 weeks old	unknown	4 months	unknown
Verification	Confirmed XY? (i.e. karyotype or SNP)	YES	YES	YES	NO
	Maternal ABCD1 testing negative?	YES	YES	YES	YES
	Negative family history?	YES	YES	YES	unknown
	Sibling testing?	YES	n/a	n/a	unknown
	Confirm 1 ABCD1 copy (i.e. MLPA)	YES	YES	unknown	YES
Clinical Information	Current age (2023)	4.0 years	2.5 years	5 years	6 years
	Clinical symptoms	pre/asymptomatic	pre/asymptomatic	pre/asymptomatic	pre/asymptomatic
	Age of most recent MRI results	4.0 years	2.5 years	5 years	6 years
	Most recent MRI results (LOES score)	normal (LOES 0)	normal (LOES 0*)	normal (LOES 2**)	normal (LOES 1***)

Note: NBS newborn screening C26:0-LPC, C26:0, C24:0, C22:0 = very long-chain fatty acids. RR reference range. NGS next-generation sequencing. "Sanger" Sanger sequencing. Clinical "cutoff" for abnormal values and/or RR provided, as reported by clinical sequencing laboratories. Labs 1 and 2 indicate samples were sent for verification at a second lab, but are not necessarily obtained from identical labs or the labs listed in Table 2. *Areas of non-enhancing T2 hyperintensity. T1 hypointensity in the periventricular white matter along the frontal horns. **mild hyperintensity involving the bilateral parietal occipital periventricular white matter on axial FLAIR images, score 1. Tiny foci of FLAIR hyperintensities noted involving the central part of the bilateral parietal occipital white matter, score 1. ***Suspected minor T2/FLAIR hyperintensities. †Did not initially identify mosaic ABCD1 variant, whole genome sequencing was sent at 1-day old for non-ALD indications, and resulted prior to knowledge of abnormal NBS. The lab was later able to identify the presence of a variant, but only after Sanger sequencing of ABCD1 was performed through a second lab. **** Non-ALD related clinical features include arthrogryposis.

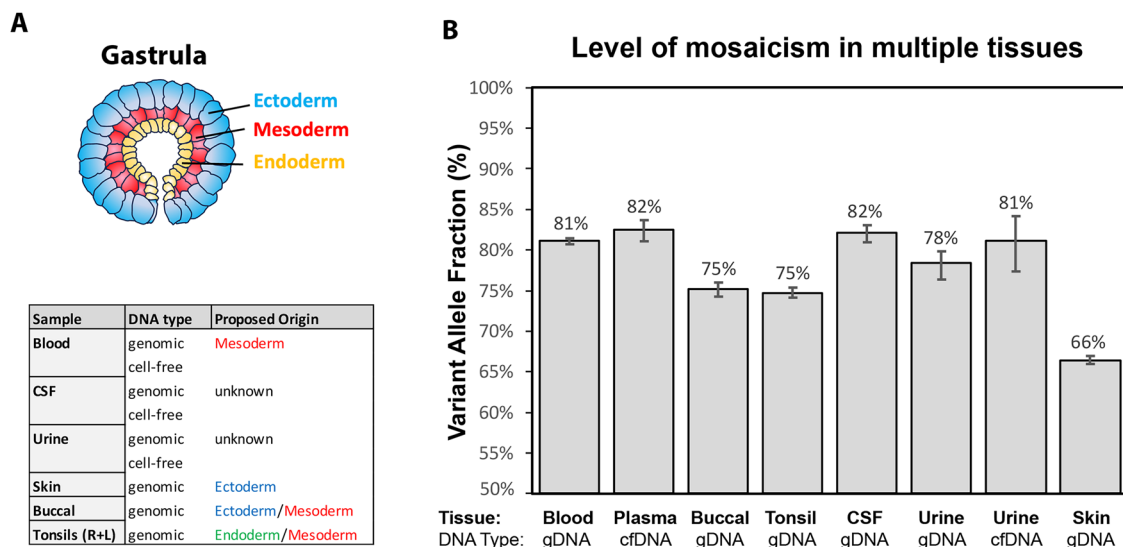


Fig. 1 | Comparison of levels of *ABCD1* 1988T > C mosaicism in multiple tissues of the proband (mXALD-1). **A** Various tissue samples were obtained, and the proposed germ layer of origin. **B** The graph shows the comparison of the variant allele fraction (VAF) from genomic DNA in six different tissues, including blood, CSF (pellet), urine (pellet), tonsil, skin, and buccal sample, and cell-free DNA

derived from plasma and urine. Representative data presented as individual values of $n = 4$ replicates. Error bars represent the min/max “Total Error” of the mutant reads based on four merged replicates as provided by Quantasoft software. VAF is calculated by mutant concentration divided by total concentration. The figure was created using Microsoft Excel and Adobe Photoshop software.

consider including the variant allele fraction and testing of other tissues when clinically indicated. Our study adds to the knowledge of X-ALD and mosaicism within common genetic conditions and opens the door for further studies determining the role of mosaicism in human disease.

Methods

Human subjects

mXALD-1 was enrolled in the study using the X-ALD Metabolomics IRB (Study #2497), which was approved (effective 10/22/2020) by the Institutional Review Board (IRB) at Seattle Children’s Hospital. mXALD-2, mXALD-3, mXALD-4, and mXALD-5 were recruited to participate in the study at their respective institutions by providing limited clinical data only, and written informed consent for publication was provided by parents or legal guardians on behalf of their children <18 years of age, as all included participants were minors. Assent was obtained from minors whenever possible. All human participants or samples are in compliance with all relevant ethical regulations, including the Declaration of Helsinki.

Sample processing

All samples were collected in conjunction with a planned surgical procedure requiring sedation. Whole blood, urine, and cerebrospinal fluid (CSF) samples were collected in cell-free DNA BCT tubes (“Streck tubes,” Streck Omaha, NE) and centrifuged at 1600g for 15 min. The resulting supernatant was centrifuged for an additional 15 min at 16,000g at 4 °C to remove remaining gDNA “pellet” debris. The supernatant was separated into aliquots and stored at −80 °C. “Pellet” gDNA was similarly aliquoted and frozen at −80 °C. cfDNA extraction was performed with QIAamp MinElute ccfDNA Kit (Qiagen, Venlo, Netherlands). Genomic DNA (gDNA) was extracted from tonsils, skin, and gDNA “pellets” of urine, CSF, and blood, using PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Buccal swabs were extracted by prepIT-L2P from DNA GenoTek, using the Oragene Saliva Protocol modified for a 2 ml sample. All DNA samples were quantified with Qubit fluorometry (Thermo Fisher Scientific, Waltham, MA, USA). cfDNA was further analyzed on an Agilent 2200 TapeStation with High Sensitivity D1000 ScreenTape (Agilent, Santa Clara, CA, USA).

Digital droplet PCR (ddPCR)

Primers, Affinity Plus® Iowa Black™ FQ Probes, and positive control gene block were designed for the patient-specific variant *ABCD1* c.1988T>C

(p.Leu663Pro) following previously used assay design guidelines¹⁸ and purchased from IDT (Integrative DNA Technologies, Coralville, IA). The reverse primer used in our assay is a previously published sequence known to avoid amplifying notorious *ABCD1* pseudogenes³. Primers were designed based on published sequences in order to avoid known *ABCD1* pseudogenes (F-ATTGCCCTGCTCTCCATCAC, R-TGCTGCTGCCGGGCCCGC) and amplified a 121 bp fragment surrounding the known variant. 20 ng of DNA was used per ddPCR reaction for male patients and male controls in order to match the amount of coverage that 10 ng input achieves in non-sex chromosome assays. All controls and samples were run in quadruplicate. The variant allele fraction (VAF) was calculated as the mutant concentration divided by the mutant concentration plus wild-type concentration. Controls included a no-template control (NTC) (VAF 0%), WT cfDNA (VAF 0%), WT gDNA (VAF 0%), *ABCD1* L663P gblock (VAF 100%), and a second patient with a different, non-mosaic *ABCD1* variant (1553 T > C) (VAF 0%).

Data compilation

mXALD-1 was identified through clinical care at our institution. To identify other mosaic individuals (mXALD-2 through mXALD-4), contact was made with multiple commercial *ABCD1* sequencing laboratories and state NBS labs from across the United States. Some labs that were approached that used NGS-sequencing platforms were able to provide aggregate de-identified information about *ABCD1* variants that were able to be extracted from large internal databases after performing a query. The requested data included all pathogenic and likely pathogenic *ABCD1* variants identified from male individuals from each laboratory, and if any of those pathogenic and likely pathogenic *ABCD1* variants that had been reported in clinical reports had actually been mosaic. If mosaic variants were identified, we requested any additional information on confirmatory Sanger sequencing. Communication with the clinical provider was made only if they responded to the lab’s inquiry on our behalf. Where clinical information was made available, we have included that in Table 1.

Identifying the frequency of mosaicism

Two large commercial labs identified all males who had pathogenic or likely pathogenic (P/LP) *ABCD1* variants identified and reported out on clinical sequencing. The majority of these patients were infant males who were referred for *ABCD1* sequencing after presenting with elevations of C26:0-

Table 2 | Frequency of mosaicism detected in male individuals with Pathogenic or Likely Pathogenic *ABCD1* variants identified through next-generation sequencing

		Mosaic males with P/LP <i>ABCD1</i> variants	All males with P/LP <i>ABCD1</i> variants*
<i>ABCD1</i> SEQUENCING	Lab #1	2	114
	Lab #2	2	113
	Total	4	227
		% mosaic cases	4/227 = 1.8%

Two large commercial labs identified all males who had pathogenic or likely pathogenic (P/LP) *ABCD1* variants identified and reported out on clinical sequencing. Four males with P or LP *ABCD1* variants had actually had mosaic variants identified. For each lab, the number of males with mosaic P/LP *ABCD1* variants was divided by the total number of males with identified P/LP *ABCD1* variants to estimate the frequency of mosaicism. It should be noted that this includes both de novo and known familial variants, as the indication for sequencing was not provided. Variants of uncertain significance were excluded. Variants were verified to confirm 4 independent variants. Three of the four variants are included in Table 1. For the remaining variant, we obtained the level of mosaicism, but the ordering provider was not reachable to provide clinical information.

*familial + de novo.

LPC on newborn screening. Some of these males were older individuals who had a clinical suspicion for X-ALD either because of positive family members or because of symptomatology. We asked each lab if they were able to identify if any of the males with P or LP *ABCD1* variants had actually had mosaic variants identified. For each lab, the number of males with mosaic P/LP *ABCD1* variants was divided by the total number of males with identified P/LP *ABCD1* variants. Both commercial labs used NGS sequencing, which had been verified by Sanger sequencing. Variants of uncertain significance were excluded, as the clinical relevance of these variants is more challenging to interpret. The four mosaic variants identified were unique from one another, indicating four separate individuals. Three of the four variants are included in Table 1. For the remaining variant, we obtained the level of mosaicism, but the ordering provider was not reachable to provide clinical information.

Data availability

Information on the pathogenicity of reported *ABCD1* variants is publicly available on “The *ABCD1* Variant Registry” (<https://adrenoleukodystrophy.info/mutations-and-variants-in-abcd1>). Clinical data are available from the authors on reasonable request.

Received: 18 September 2024; Accepted: 22 April 2025;
Published online: 09 May 2025

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Acknowledgements

We would like to thank the newborn screening laboratories and commercial sequencing laboratories that provided internal data. We would like to thank the individuals and their families for their participation in this study.

Author contributions

A.C.K. designed the study, initiated this work, supervised the conduct of the study, and drafted the paper. A.C.K., A.S., and M.P.P. collected and analyzed patient samples. D.M.J. designed ddPCR assay. A.C.K., D.M.J., M.P., and N.A. performed and analyzed molecular experiments. R.B., M.M., I.S., and P.K. analyzed and interpreted genetic data. E.B., L.O.G., F.S.E., B.E.R., D.W., A.R., E.S., D.V., E.C., K.W., E.S., B.E.H., R.T., T.C.L., and A.S. recruited and evaluated the study participants. J.T.B. supervised the study. All authors revised the paper.

Competing interests

Authors P.K. and M.M.M. are employees of GeneDx, LLC, but declare no financial or non-financial competing interests. A.R. is an employee at Natera but declares no financial or non-financial competing interests. F.S.E. is PI of an in vivo gene therapy trial in Canavan disease (Sponsor: ASPA

Therapeutics), Co-PI of ex vivo lentiviral gene therapy trial in cerebral adrenoleukodystrophy (Sponsor: bluebird bio), Co-PI of PI of in vivo gene therapy trial in GM2 (Former Sponsor: Sio Therapeutics), Site-PI of Alexander Disease trial of ASOs (Sponsor: Ionis Pharmaceuticals), Consultant to Atlas Venture, Acadia Pharmaceuticals, Leal Therapeutics, Vigil, Takeda Therapeutics, Sanofi, SwanBio Therapeutics and UpToDate, founder of SwanBio Therapeutics, and is a NINDS Advisory Council Member but declares no financial or non-financial competing interests. The remaining authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41525-025-00497-1>.

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