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Further delineation of short-chain enoyl-CoA hydratase deficiency in the Pacific population

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SUMMARY

Short-chain enoyl-coA hydratase (SCEH) deficiency due to biallelic pathogenic *ECHS1* variants was first reported in 2014 in association with Leigh syndrome (LS) and increased S-(2-carboxypropyl)cysteine excretion. It is potentially treatable with a valine-restricted, high-energy diet and emergency regimen. Recently, Simon et al. described four Samoan children harbouring a hypomorphic allele (c.489G > A, p.Pro163=) associated with reduced levels of normally-spliced mRNA. This synonymous variant, missed on standard genomic testing, is prevalent in the Samoan population (allele frequency 0.17). Patients with LS and one *ECHS1* variant were identified in NZ and Australian genomic and clinical databases. *ECHS1* sequence data were interrogated for the c.489G > A variant and clinical data were reviewed. Thirteen patients from 10 families were identified; all had Pacific ancestry including Samoan, Māori, Cook Island Māori, and Tokelauan. All developed bilateral globus pallidi lesions, excluding one pre-symptomatic infant. Symptom onset was in early childhood, and was triggered by illness or starvation in 9/13. Four of 13 had exercise-induced dyskinesia, 9/13 optic atrophy and 6/13 nystagmus. Urine S-(2-carboxypropyl)cysteine-carnitine and other SCEH-related metabolites were normal or mildly increased. Functional studies demonstrated skipping of exon four and markedly reduced ECHS1 protein. These data provide further support for the pathogenicity of this *ECHS1* variant which is also prevalent in Māori, Cook Island Māori, and Tongan populations (allele frequency 0.14–0.24). It highlights the need to search for a

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second variant in apparent heterozygotes with an appropriate phenotype, and has implications for genetic counselling in family members who are heterozygous for the more severe *ECHS1* alleles.

Synopsis: Short-chain enoyl-CoA hydratase deficiency is a frequent cause of Leigh-like disease in Māori and wider-Pacific populations, due to the high carrier frequency of a hypomorphic *ECHS1* variant c.489G > A, p.[Pro163=, Phe139Valfs*65] that may be overlooked by standard genomic testing.

1. Introduction

Short-chain enoyl-CoA hydratase (SCEH; EC4.2.1.17) deficiency due to pathogenic biallelic variants in *ECHS1* (MIM*602292) was first described in 2014 in siblings with fatal Leigh syndrome (LS; MIM#256000) [1]. The SCEH enzyme has roles in fatty acid β-oxidation and catabolism of branched-chain amino acids, however the only known non-redundant functions of this enzyme are in the valine degradation pathway (Fig. 1) [2,3]. SCEH deficiency inhibits conversion of the highly reactive precursors methylacrylyl-CoA and acryloyl-CoA, to 3-hydroxyisobutyryl-CoA and 3-hydroxypropionyl-CoA respectively, leading to accumulation of associated metabolites including S-(2-carboxypropyl) cysteine and 2,3-dihydroxy-2-methylbutyric acid (Fig. 1). Impaired energy production occurs due to secondary inhibition of the respiratory chain and pyruvate dehydrogenase complex, presumed to be the result of toxic by-products [1,4,5].

Since the initial description of SCEH deficiency associated with LS, >85 further affected patients have been reported, with a broad phenotypic spectrum ranging from fatal neonatal lactic acidosis to paroxysmal kinesigenic dyskinesia (PKD) [6–10]. Magnetic resonance imaging (MRI) findings typically include bilateral T2-hyperintense basal ganglia lesions [6,7]. Biochemical diagnosis of SCEH deficiency is challenging, as diagnostic metabolites are not elevated in all cases [3,11]. Thus, the phenotype overlaps considerably with a large number of nuclear and mitochondrial gene-related disorders associated with LS [12]. Precise diagnosis is nevertheless essential to facilitate appropriate counselling, but also to identify treatable disorders.

Disorders of branched-chain amino acid metabolism are potentially amenable to dietary treatment. Accumulation of toxic metabolites may be attenuated by implementation of an emergency regimen to reduce catabolism of endogenous protein during illness and fasting, and by dietary restriction of amino acid precursors. Abdenur and colleagues recently described the treatment of three children with SCEH deficiency, and two with the related *HIBCH*-disorder (MIM*610690), with a valine-restricted diet [13]. Restriction of dietary valine was associated with improvement or stabilisation of clinical and neuro-imaging findings, suggesting that SCEH deficiency is a potentially treatable disorder.

In 2020, Simon et al. described four Samoan children from two unrelated families with SCEH deficiency [14]. They harboured compound heterozygous *ECHS1* variants, including a hypomorphic allele c.489G > A, p.Pro163=. This synonymous variant was associated with reduced levels of normally spliced mRNA, and was missed on standard genomic testing as it did not meet ACMG criteria for reporting, due to its high prevalence in the Samoan population with an allele frequency of 0.17. This discovery resulted in targeted testing for this variant in four patients in New Zealand who were of Pacific descent, with bilateral globus pallidi lesions and carrying another *ECHS1* pathogenic variant. All four were found to harbour the synonymous variant *in trans* with a pathogenic *ECHS1* variant.

The high carrier rate of the *ECHS1* c.489G > A allele in Samoa would suggest that this may be a common cause for bilateral basal ganglia disease in the wider Pacific region. Accordingly, this study aimed to interrogate clinical and genomic databases in Aotearoa New Zealand (NZ) and Australia, to ensure that people (particularly Māori and Pacific people) with bilateral globus pallidi lesions and previously non-diagnostic genomic testing, had been tested for this hypomorphic allele.

2. Methods

2.1. Patients

The clinical database of the National Metabolic Service in NZ was reviewed for individuals with LS or PKD, bilateral T2-hyperintense basal ganglia lesions, and one *ECHS1* variant. Additionally, clinicians in NZ (neurologists and clinical geneticists), and Australia (metabolic clinicians), were contacted to ascertain further patients meeting these criteria. Clinical data and findings of MRI scans (where performed) were reviewed. All patients or their caregivers provided informed consent for their inclusion in this study.

2.2. Biochemical analysis

Urine organic acid analysis was performed by gas chromatography–mass spectrometry (GCMS). Methylacrylyl-CoA metabolites were measured in urine by tandem mass spectrometry (MS-MS), by a published method [15].

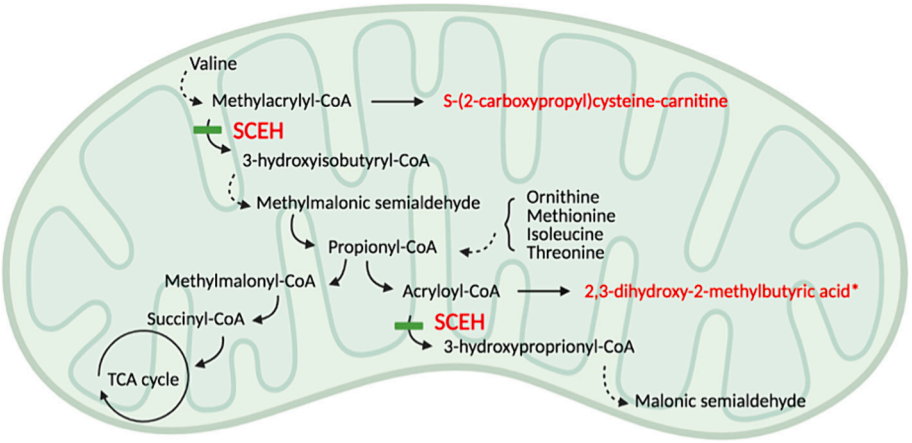

Fig. 1. Valine catabolic pathway and metabolites associated with SCEH-deficiency.
SCEH: short-chain enoyl-CoA hydratase, * Origin of 2,3DH2MB from acryloyl-CoA is indirectly inferred. Fig. 1 created with BioRender.com

2.3. Genomic sequencing and variant detection

Genomic sequencing techniques utilising gene panels, exome sequencing, or genome sequencing were performed by genomics laboratories including University of Otago, Victorian Clinical Genetics Services, Kinghorn Centre for Clinical Genomics, Children's Hospital at Westmead, Blueprint Genetics, and Molecular Vision Laboratory. Genomic data were re-analysed for *ECHS1* variants including the *ECHS1* c.489G > A allele (rs140410716). Targeted *ECHS1* sequencing was performed at Canterbury Health Laboratories to identify familial variants in at-risk family members. A Polynesian population cohort (n = 565) genotyped by low-pass sequencing followed by imputation with high-pass sequenced genomes was queried to obtain ancestry-specific allele frequency estimates for rs140410716 [16].

2.4. Molecular and protein studies

Cultured fibroblasts for four patients and two controls were grown in DMEM +10% FBS as described previously [17]. For cDNA splicing and expression studies, cells were grown with and without cycloheximide (100 ng/ml for 24 h) to inhibit nonsense-mediated decay, as previously described [17] before harvesting. Total RNA was extracted using the Illustra RNAspin Mini Isolation Kit (GE Healthcare) and synthesized into cDNA using the SuperScript III First-Strand Synthesis System (ThermoFisher Scientific) as previously described [17]. To examine the effect of the c.489G > A variant on mRNA splicing, cDNA was amplified using primers designed over the exon 2/3 boundary and exon 6 of *ECHS1* (NM_004092.4) (Forward 5' GCAGCTGGAGCTGATATCAAG 3′, Reverse 5' TTTTCTGCACACTGGATGGC 3′) and RT-PCR products cloned into the pCR™ 2.1-TOPO® using the TOPO TA cloning kit (ThermoFisher Scientific) before transformation into TOP10 competent cells (ThermoFisher Scientific) as previously described. Individual colonies thought to contain altered splice products were analysed by Sanger sequencing.

All RT-PCR products were analysed on 2% agarose (Bioline) gels using DNA molecular weight marker VIII (Sigma-Aldrich) for analysis.

For immunoblotting studies, protein lysates were prepared from cultured fibroblasts as described previously [17]. With 20 μg of each protein lysate analysed by SDS-PAGE as previously described [18], and probed with primary antibodies raised against ECHS1 (1:5000; Abcam #ab174312) and mitochondrial content control SDHA (1:10,000 Invitrogen #459200), and secondary HRP antibodies (Cytiva, mouse #GEHENA931 and rabbit #GEHENA934).

3. Results

3.1. Clinical findings

A total of thirteen patients from ten families were identified; all had Pacific ancestry including Samoan, Māori, Cook Island Māori and Tokelauan. Their clinical data are summarised in Table 1 and supplemental data S1. Of these, nine patients were identified following review of clinical and genomic databases (P1–9), including an adult male (P7) and his maternal aunt (P8) in a non-consanguineous family (Fig. 2A). Four further affected individuals were then ascertained (P10–13); two (P10–11) were younger siblings of an affected proband (P9; Fig. 2B), born to the same mother with three separate fathers of different Pacific ancestries and no known consanguinity. The remaining two (P12–13) were subsequently diagnosed by clinical genomics laboratories, using a WES-based panel (P12), or single-gene *ECHS1* sequencing in a patient for whom clinical suspicion of SCEH deficiency was high (P13).

Symptom onset was in early childhood (at age 6–18 months), excluding one pre-symptomatic infant (P11). Initial symptoms were precipitated by an identifiable catabolic event in five of these children. Four further patients had subsequent episodic neurological deterioration precipitated by intercurrent illness or fasting (at age 11 months–8.5 years). At the time of last follow up, patient ages ranged from 7 months-47 years. One patient (P6) died at 2.5 years of age.

Dystonia and paroxysmal movement disorders were present in all patients older than two years of age (n = 11). Exercise-induced dyskinesia was diagnosed in four with onset at age 2.5–4 years, and was the predominant presenting symptom in one (P4). Optic atrophy leading to progressive decline in visual acuity occurred in nine, and was first detected at 3–9 years of age (Fig. 3). Eye movement disorders were present in seven, and were the first symptoms in three. These included nystagmus in six (horizontal and rotary), oscillopsia, and saccadic overshoots with end-point nystagmus. Sensorineural hearing loss was detected in five, at 3–33 years of age. Other neurological complications included childhood-onset ataxia (n = 4), spasticity (n = 5), dysarthria (n = 2) and dysphagia (n = 2). Neurological manifestations in older children and adults were slowly progressive.

Of three patients who underwent formal neuro-psychometric evaluation, two were found to have a moderate intellectual disability at age 6–13 years. One had cognitive ability in the average range at 9.5 years, with mild impairments in attention and executive function. Intellectual disability was suspected in three further adult patients yet to undergo formal evaluation.

No patients were diagnosed with cardiomyopathy, and echocardiography was unremarkable in six patients who were evaluated at age 15 months-32 years.

3.2. Neuro-imaging findings

MR brain imaging was available for review in ten patients with age of imaging ranging from one to 33 years. All symptomatic individuals had bilateral globus pallidi lesions on MRI brain. Globus pallidi lesions were characterised by T2-hyperintensity and T1-hypointensity, with diffusion restriction and areas of FLAIR hyperintensity (Fig. 4). MR spectroscopy revealed a small or absent lactate peak. Bilateral globus pallidi were involved symmetrically with sparing or minor abnormality of other deep grey matter structures. Milder T2 hyperintense lesions were noted involving the caudate heads (n = 3), substantia nigra, thalami, dentate nuclei, dorsal brainstem tracts, dorso-lateral putamina, and peri-aqueductal, and peri-ventricular white matter (n = 1).

Serial imaging was available for six patients and revealed progression of globus pallidi lesions including cystic change (n = 2), volume loss (n = 1), and calcifications (n = 1). New T2-hyperintense basal ganglia lesions were identified on repeat MRI in only one individual (P5), who was found to have new dorsal putamina lesions at 4.5 years of age. Mild generalised cerebral atrophy was observed in one patient (P12) at 28 years of age.

3.3. Biochemical findings

Metabolic acidosis was present at the time of initial presenting illness in five patients (age 9 months-3 years) and was severe (pH <7) in two (P6, P9), and associated with significant ketosis in two (P1, P9). Lactate in plasma was mildly elevated in three (range 3.1–3.7 mmol/L) at the time of initial presentation. Lactate in cerebrospinal fluid (CSF), when measured (n = 4), was normal (range 1.2–1.6 mmol/L).

Urine organic acids were normal (n = 4) or showed non-specific abnormalities which included; ketosis, increased lactate excretion, mild elevations of 3-methylglutaconate (n = 3) and/or 3-hydroxyisovalerate (n = 3). Significantly increased *erythro*-2,3-dihydroxy-2-methylbutyrate was detected in one individual. Previously detected organic acid abnormalities resolved in follow-up samples in two patients.

Urine S-(2-carboxypropyl)cysteine-carnitine was normal or mildly increased relative to OHC4-carnitine (Fig. 5) [15]. Other SCEH deficiency-related metabolites were statistically increased in the overall cohort but levels overlapped considerably with controls and were lower than other SCEH deficient patients without the p.Pro163 = allele. (Supplemental Fig. 2).

Table 1
Clinical data summary.

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13
Family (if applicable)							F1 (proband)	F1	F2 (proband)	F2	F2		
ECHS1 allele 1	c.817 A > G p. Lys273Glu	c.833C > T p. Ala278Val	c.299 T > C p. Ile100Thr	c.607G > A p. Ala203Thr	c.299 T > C p. Ile100Thr	c.744 T > G p.Phe248Leu	c.817 A > G p.Lys273Glu	c.817 A > G p. Lys273Glu	c.299 T > C p.Ile100Thr	c.299 T > C p.Ile100Thr	c.299 T > C p. Ile100Thr	c.817 A > G p. Lys273Glu	c.827 T > C p. (Met276Thr)
ECHS1 allele 2						c.[489G > A,415_514del] p.[Pro163=,Phe139Valfs*65]							
Ethnicity of parent harbouring allele 2	Māori	Unknown	Samoa	Samoa	Māori	Samoa	Māori	Māori	Tokelau	Cook Island Māori	Māori	Māori	Māori
Age at diagnosis	6y	20 m	13y	8y	30y	N/A	26y	47y	5y	15 m	Birth	28y	2y
Age at last follow-up	8y	3y	14y	10y	31y	Deceased at 2.5y	27y	48y	6y	2y	7 m	29y	2y
Early childhood onset	+	+	+	+	+	+	+	+	+	+	+	+	+
Globus pallidi T2 hyper-intensity on MRI	+	+	+	+	+	+	+	+	+	+	N/D	+	+
Catabolic trigger	+	+	+	+	+	+			+	+			+
Dystonia	+	+	+	+	+	+	+	+	+			+	+
(exercise-induced dyskinesia)	(+)		(+)	(+)					(+)				
Optic atrophy	+		+		+	+	+	+	+			+	+
Ocular motility abnormality	+		+	+					+	+	+	+	
Sensorineural hearing loss	+						+	+	+			+	
Intellectual impairment	+		+		+		+	+					
Spasticity		+	+				+	+					+
Ataxia			+					+				+	+
Dysarthria							+					+	
Dysphagia							+	+					
Episodes of acidosis or hypoglycaemia (age)	KA (9 m)					Profound MA (12 m)			Severe KA (13 m) Fasting HG (5y)	Mild MA (18 m)			
Other neurological features	Seizures	GDD					Headache Psychiatric symptoms			Mild GDD			GDD

N/A = not available, N/D = not done, MA = metabolic acidosis, KA = ketoacidosis, HG = hypoglycaemia, GDD = Global developmental delay.

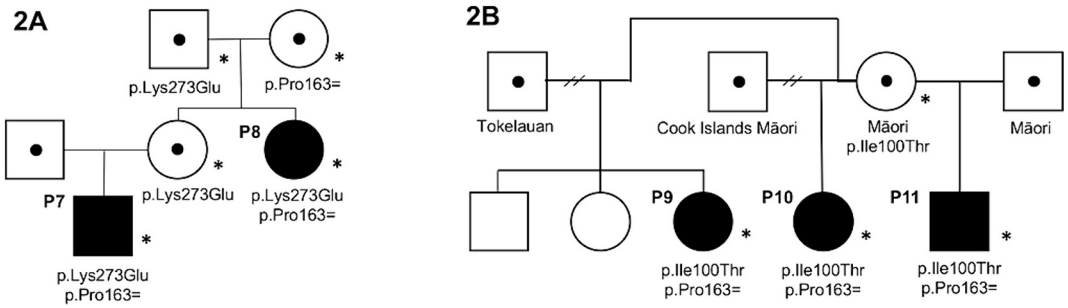

Fig. 2. A) Pedigrees of family F1 (P7,8), and (B) family F2 (P9–11).
*Indicates genotype confirmed.

3.4. Molecular and functional genomics findings

All 13 patients were heterozygous for the *ECHS1*: c.489G > A allele, *in trans* with one of six (likely) pathogenic variants, of which all but c.744 T > G p.Phe248Leu and c.827 T > C p.(Met276Thr) were previously reported (Table 1) [2,14,19–23]. Of the previously reported variants, c.817 A > G p.Lys273Glu was identified in three apparently unrelated families and c.607G > A p.Ala203Thr in two. The missense variants were all rare, with no homozygotes reported in gnomAD v4, and the most frequent of the previously reported variants, c.817 A > G p.Lys273Glu, found at a minor allele frequency (MAF) of 0.00002788 (45 heterozygotes from 1,614,022 alleles tested). The novel c.744 T > G p.Phe248Leu variant was absent in gnomAD v4, and c.827 T > C p.(Met276Thr) was heterozygous in 8 individuals. *In silico* tools predicted all missense variants to have a deleterious effect. While the phenotype and biochemistry findings were consistent with the reported spectrum of ECHS1-disease, further evidence was sought to support the pathogenicity of these variants, in the absence of clearly diagnostic elevations of methylacrylyl and acryloyl metabolites.

Fibroblasts were cultured from individuals (P4–7) harbouring the c.607G > A p.Ala203Thr, c.299 T > C p.Ile100Thr, c.744 T > G p.Phe248Leu, and c.817 A > G p.Lys273Glu variants *in trans* with the c.489G > A allele. cDNA studies targeting exons 3 to 6 identified a smaller 301 bp band in addition to the expected sized band of 401 bp in all four patients (Fig. 6A).

Sanger sequencing of the 401 bp product (grown without cycloheximide) showed that the c.489G > A variant was heterozygous, however the mutant allele was at a proportion lower than expected (Fig. 6B). The presence of two species within the larger band as well as an additional smaller band suggested that there were two consequences of the c.489G > A variant, indicative of a hypomorphic effect. To functionally validate the exon 4 skipping event associated with the c.489G > A as proposed by previous studies [14,19], cloning and sanger sequencing of the smaller band was undertaken. This confirmed that the c.489G > A variant resulted in both the synonymous p.Pro163 = and a premature termination product due to the exon 4 skip, c.415_514del; p.Phe139Valfs*65 (Fig. 6B). This smaller band was present both with and without cycloheximide, suggesting it undergoes partial but not complete degradation *via* nonsense-mediated decay. Immunoblot analysis showed that ECHS1 protein levels were absent or markedly reduced in all four individuals as compared with controls (Fig. 6C).

Genome sequence data of individuals of Polynesian ancestry were interrogated to determine the prevalence of the *ECHS1* c.489G > A allele in these populations. The MAF was 0.20 in individuals of Samoan ancestry (254 chromosomes examined), 0.14 in Māori (680 chromosomes), 0.20 in Cook Island Māori (90 chromosomes), and 0.24 in people of Tongan ancestry (104 chromosomes).

4. Discussion

Simon et al. identified a common, synonymous *ECHS1* variant in four patients with SCEH deficiency, and demonstrated that this unlikely candidate variant was associated with reduced levels of normally spliced mRNA [14]. Appreciation of the high carrier frequency of this hypomorphic variant in the Samoan population prompted a search for undiagnosed patients, with previously non-diagnostic genomic testing in NZ and Australia. The subsequent rapid identification of thirteen new patients (12 in NZ) with this hitherto extremely rare disease was striking, considering the small population of NZ (4,699,755, Census data 2018), with 16.5% reporting Māori ancestry, and 8% reporting Pacific ancestry. Of note, seven patients were born in NZ in the preceding ten years, suggesting a population incidence of at least 1/85,000. As all patients had Māori or Pacific ancestries, the estimated incidence in these groups is significantly higher (approximately 1/34,000 in Māori).

The clinical phenotype in our cohort reflects the spectrum of severity reported in SCEH deficiency. It includes one individual with a severe, fatal disease course resembling classical LS, and one with an apparently isolated paroxysmal movement disorder. However our cohort also demonstrates a recognisable clinical picture with several consistent findings, including characteristic globus pallidi lesions, and disease onset in late infancy associated with a catabolic trigger. Other noteworthy findings included the absence of extra-neurological organ manifestations, and a slowly progressive course in affected adults.

Biochemical abnormalities in our cohort were variably present, and included non-specific features such as 3-methylglutaconic aciduria and episodes of ketoacidosis or lactic acidosis. Diagnostic methylacrylyl-CoA metabolites were detectable but overlapped significantly with ketotic controls (Fig. 5). While we were able to demonstrate statistically significant increases in other metabolites associated with SCEH deficiency in the overall cohort, there was considerable overlap with controls (Supplemental Fig. 2). The absence of clearly diagnostic abnormalities resulted in the diagnosis being dismissed in three individuals who were known to harbour a heterozygous *ECHS1* variant following initial genomic testing. Therefore diagnosis of SCEH deficiency is dependent on recognition of the clinical, imaging and biochemical phenotype, and detection of pathogenic biallelic *ECHS1* variants by molecular genetic analysis.

The hypomorphic allele c.489G > A was present in all 13 individuals, but was initially missed by genomic testing in nine of them. Two of the six missense variants identified were classified as pathogenic (p.Lys273Glu) or likely pathogenic (p.Ala278Val) according to the ACMG criteria, due to previous reports of pathogenicity for both the same amino acid change, or an alternate change, respectively [2,14,19–23]. A further two variants (p.Ile100Thr, p.Ala203Thr) are both listed as variants of uncertain significance in ClinVar, with one (p.Ala203Thr) previously published [24]. The remaining two missense variants were previously unreported. Cellular studies were undertaken in representative individuals harbouring four of these six variants, the results of which supported the pathogenicity of the p.Ala203Thr, p.Ile100Thr, p.Lys273Glu and the novel p.Phe248Leu variants, in combination with the hypomorphic c.489G > A variant. According to the ACMG criteria, the c.744 T > G; p.Phe248Leu variant should be classified as likely

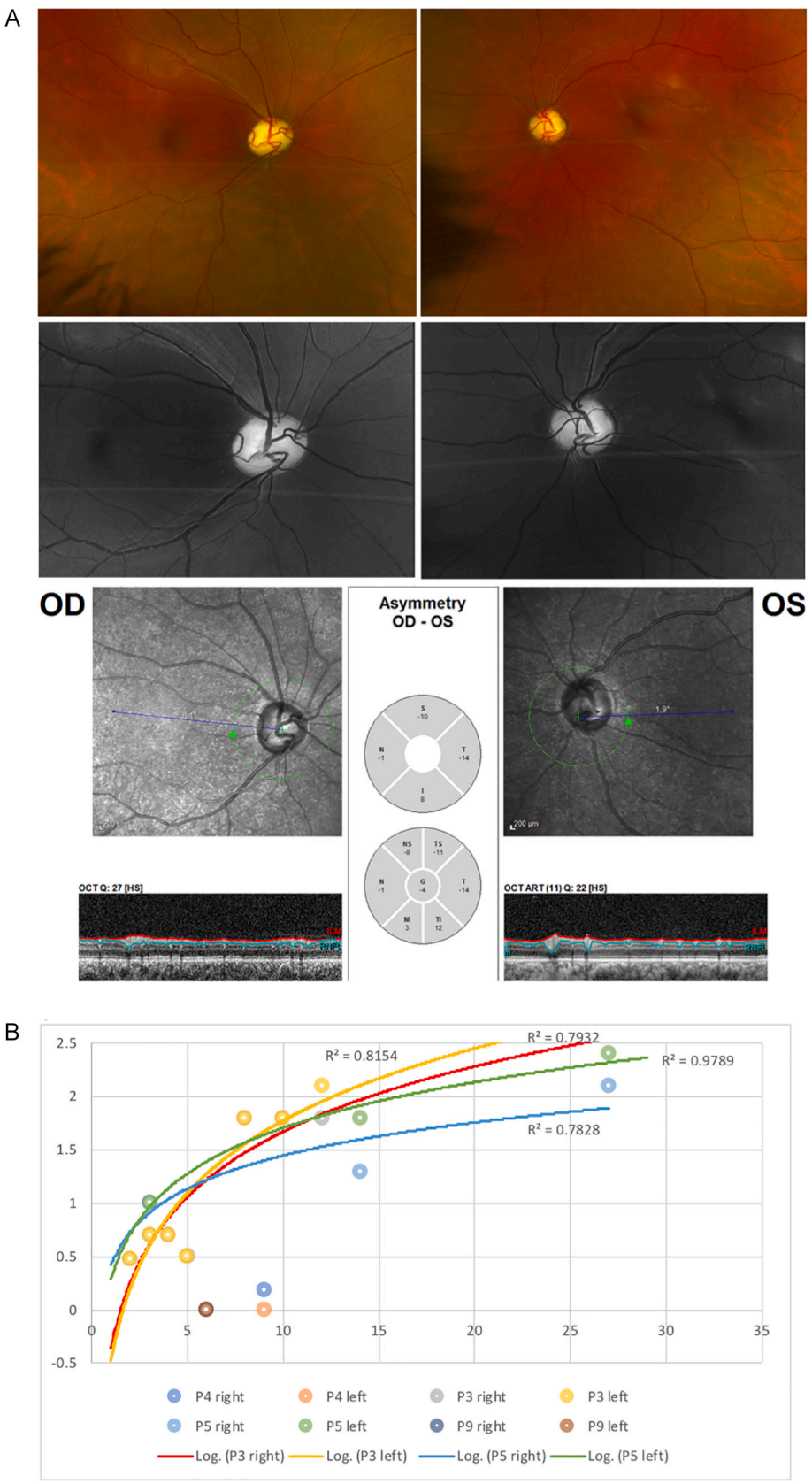

Fig. 3. Ophthalmic phenotype.
Fig. 3A: Retinal photography (colour above, red free below) and optical coherence tomography of right and left optic nerve heads in P5, demonstrating optic disk pallor and thinning of the retinal nerve fibre layer. Fig. 3B showing visual acuity expressed as the logarithm of visual acuity (logMAR, x axis) over time (in years, y axis) and logarithmic regression of visual acuity (R [2]) in right and left eyes for P3, P4, P5 and P9. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

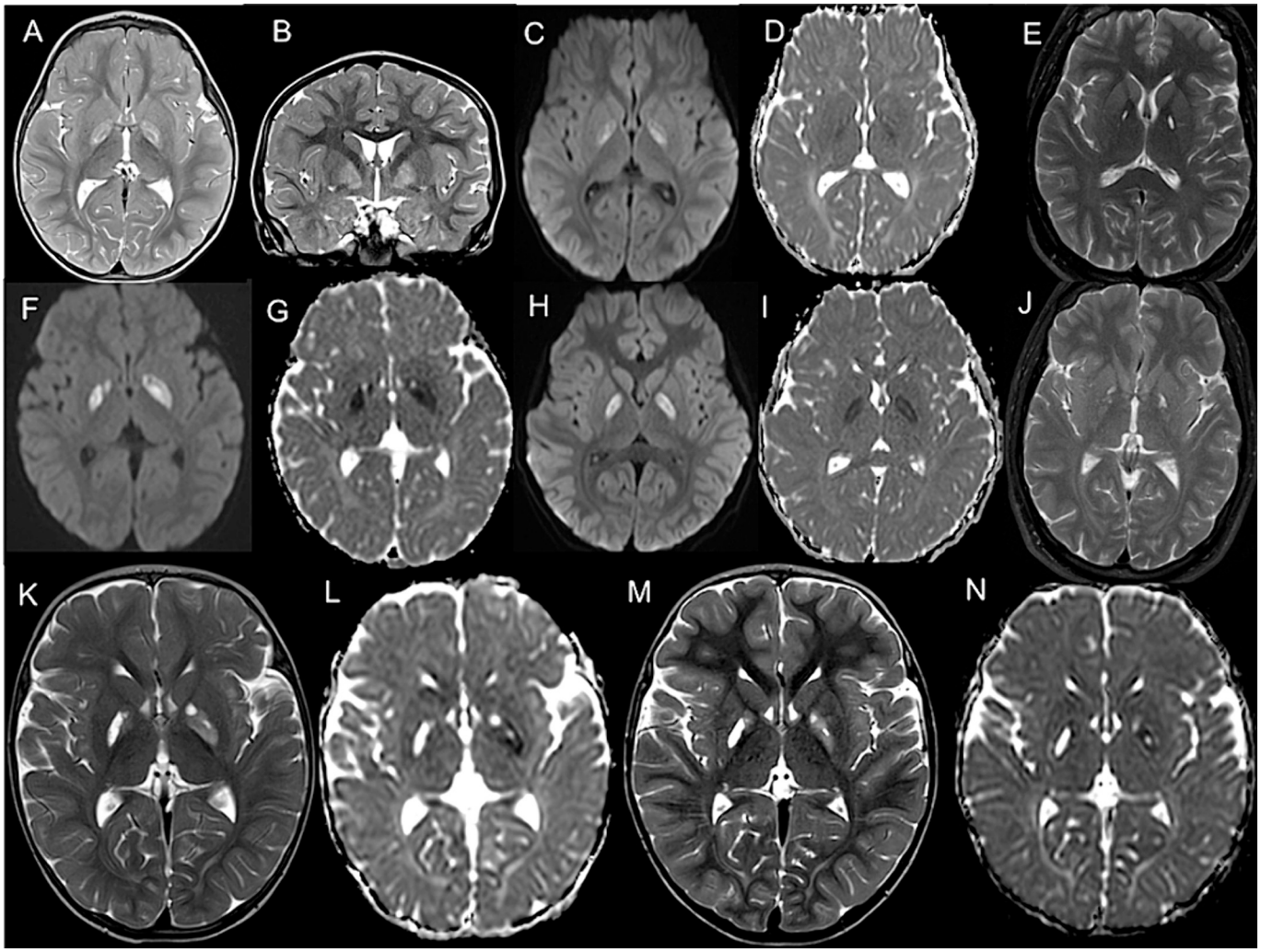

Fig. 4. Neuro-imaging.
MRI showing symmetrical T2-hyperintense globus pallidi lesions in; P10 at two years of age, T2-weighted A) axial and B) coronal images, showing linear foci of higher T2 signal, C) diffusion-weighted image (DWI), and D) apparent diffusion-weighted coefficient (ADC) map; E) P8 at 32 years of age, T2 image showing globus pallidi volume loss and small foci of high signal; P13 at two years of age F) DWI and G) ADC map; P4 at five years of age H) DWI and I) ADC map; P7 at 23 years of age, T2 image showing globus pallidi volume loss and small foci of high signal; P2 at 15 months of age K) T2 and L) ADC map, and at three years of age M) T2 and L) ADC map, demonstrating interval reduction in volume, and absence of restricted diffusion in areas of highest T2 signal.

pathogenic (detected in an individual with a highly suggestive phenotype, absent in gnomAD v4, deleterious *in silico* predictions, with *in vitro* studies supporting pathogenicity).

While a pathogenic classification can be easily applied to these missense variants with the ACMG criteria when supported by these cellular studies, classification of the hypomorphic c.489G > A variant remains challenging. Amendments to the original ACMG criteria, which did not address non-canonical silent splicing variants such as the c.489G > A variant, have been published in guidelines from the ClinGen SVI Splicing Subgroup in 2023, allowing the application of a PVS1_Strong (RNA) criterion to be applied for this hypomorphic variant [23,25]. However, no specific guidelines are able to address its high carrier frequencies in population databases (MAF of 0.01052 East Asians in gnomAD v4 and 0.17 in Pacific populations, this paper and Simon *et al*) [14], nor its occurrence in clinically unaffected homozygous individuals (with 4 listed in gnomAD v4, and 34 in a Samoan population database of apparently healthy individuals) [14], which both attract significant benign weight.

It is well documented that the classification of hypomorphic variants remains problematic for many genetic conditions and that there is a need for clear guidelines to resolve their often conflicting characteristics, especially when these variants are common but only cause disease when *in trans* with a fully penetrant pathogenic allele (as seen in these patients), but not when in homozygosity [25–27]. Based on current ACMG guidelines, it is unsurprising that this variant is classified as having conflicting interpretations in ClinVar (Variation ID: 385034). However, with this current publication and prior publications, there are now 17 unrelated families harbouring this c.489G > A *in trans* with a pathogenic missense variant [14,19]. This, along with multiple independently conducted functional studies, support a classification of likely pathogenic/pathogenic [14,19].

The finding of absent or markedly reduced ECHS1 protein in these individuals suggests that these missense variants lead to instability and degradation of the ECHS1 protein. Absent ECHS1 protein could be expected to result in a severe, neonatal-onset phenotype, however it is important to note that Western blot is not a quantitative assay. The phenotype in our cohort is similar to the patients described by Simon et al. who harboured the hypomorphic allele, and who had residual SCEH enzyme activity and detectable ECHS1 protein [14]. Thus some residual SCEH activity would be expected in our patients in view of their phenotype and genotype. It is a limitation of our study that SCEH enzymology was not performed in our cohort, however this was not

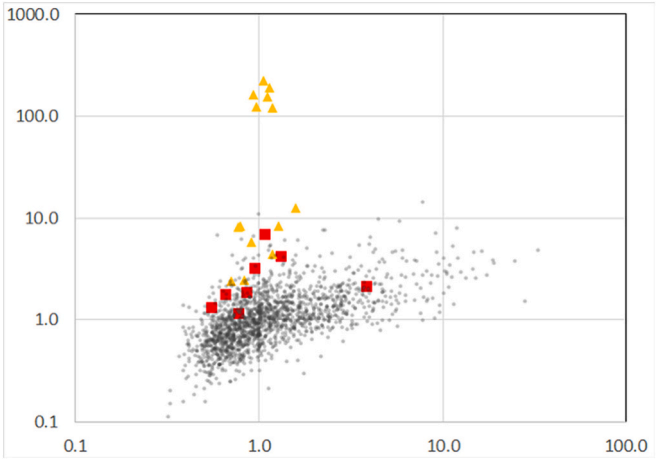

Fig. 5. Levels of urine S-(2-carboxypropyl)cysteine-carnitine (y-axis) *vs* OHC4 carnitine (x-axis).
Results are expressed as multiples of median on a log-log scale. Red squares are SCEH deficient Pacific Islanders (8 samples from 4 subjects), yellow triangles are other, non p.Pro163 = SCEH deficient subjects (14 samples from 6 subjects), grey dots are controls ($n = 1753$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

considered necessary for diagnosis.

The novel c.827 T > C p.(Met276Thr) variant was identified in P13 subsequent to these cellular studies. This variant was identified in an individual with a highly suggestive phenotype, was absent in databases of healthy controls, *in silico* tools predicted pathogenicity, and it was *in trans* with the hypomorphic allele. However, while we consider this variant to be likely pathogenic, without formal recognition of c.489G > A as a pathogenic allele, it remains a variant of uncertain significance [23].

The c.489G > A allele was detected in individuals with diverse Pacific ancestries, and in several apparently unrelated Māori families. In three non-consanguineous kindreds, affected family members included half-siblings and second or third degree relatives. Thus pedigrees were not typical of autosomal recessive inheritance, and alternative modes of inheritance were initially considered. This represented an additional challenge in recognising this recessive disorder, and suggested a high prevalence of this variant in wider Pacific populations, beyond Samoa. This was confirmed in a database of healthy individuals of Māori, Samoan, Tongan, and Cook Island ancestry. The high allele frequency was remarkably constant in these populations, consistent with close ancestral relationships between these peoples. Interestingly, the c.489G > A allele also occurs at an increased frequency in East Asians (MAF 0.01), compared to the global MAF of 0.0004 in gnomAD v4, and has recently been detected in five individuals with SCEH deficiency in Japan [19]. However their ancestry was not reported.

It is noteworthy that no individuals in our cohort were found to be homozygous for the hypomorphic c.489G > A allele. Simon et al. reported an asymptomatic homozygous adult with residual SCEH enzyme activity comparable to heterozygous carriers. While detailed phenotyping and long-term outcomes in homozygous adults have not yet been reported, it seems likely that homozygosity for this variant is clinically benign. This at least partly explains the discrepancy between the very high carrier frequency in Pacific populations, compared to the relatively low incidence of SCEH deficiency. It is also likely that further affected, compound heterozygous patients remain undiagnosed.

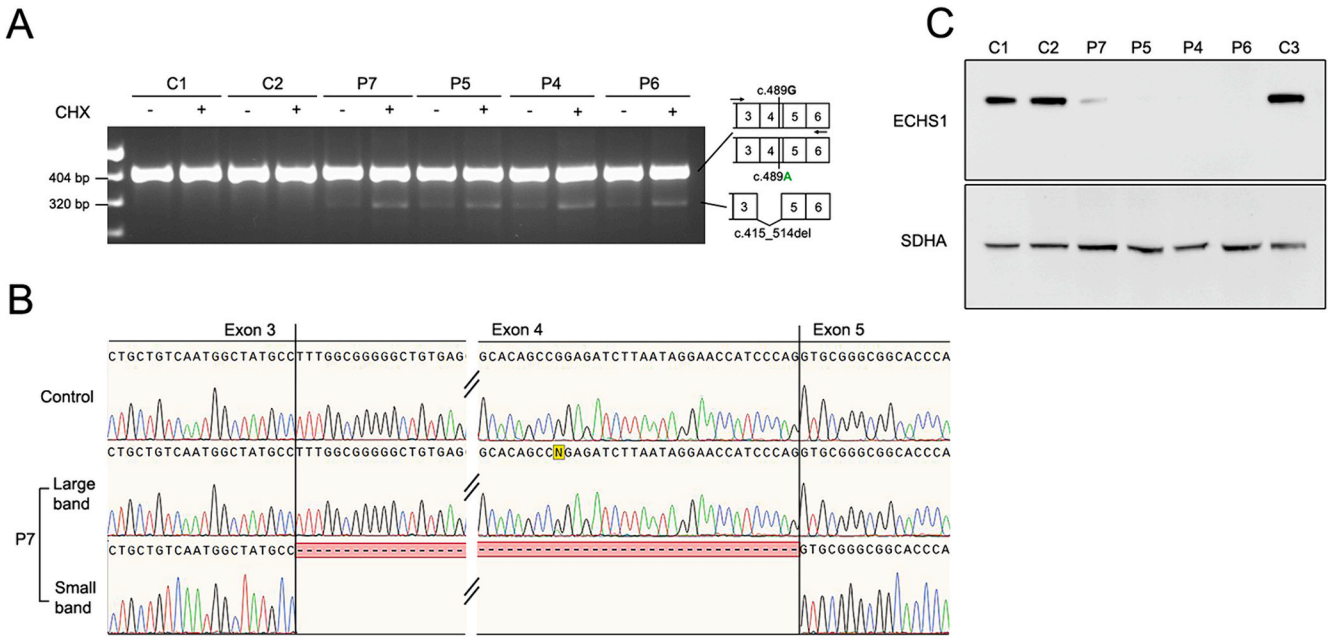

Fig. 6. cDNA and protein studies in control and patient fibroblasts showing altered splicing and reduced ECHS1 protein expression in patients.
A) Gel electrophoresis of PCR products from controls (C1, 2) and patients (P4–7) containing exons 3 to 6 of *ECHS1* shows a fragment of the expected size (401 bp) in both controls and patients in the presence (+) and absence (−) of cycloheximide (CHX) to inhibit nonsense mediated decay (NMD). An additional smaller 301 bp band was only present in patient cells and more prominent with the addition of CHX due to incomplete NMD. Schematic diagram (right) shows the different RT-PCR products amplified in patients with boxes representing exons and arrows indicating primer sites. Size markers from DNA molecular weight marker VIII (Sigma-Aldrich) with 404 bp and 320 bp are indicated (left). B) Sanger sequencing of the large 401 bp band from control and P7 (-CHX) shows the c.489G > A variant (highlighted in yellow) as heterozygous but with allelic imbalance in the patient. Sanger sequencing of the cloned small 301 bp band from P7 (+CHX) shows the skipping of exon 4 (c.415_514del; p.Phe139Valfs*65) indicated in red. Exon boundaries indicated as is the internal clipping of exon 4 with diagonal lines C) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of ECHS1 protein levels from control (C1–3) and patient (P4–7) fibroblasts. Complex II subunit SDHA was used as a loading control for mitochondrial content. Missense allele genotype in patients: P4 c.607G > A p.Ala203Thr, P5 c.299 T > C p.Ile100Thr, P6 c.744 T > G p.Phe248Leu, and P7 c.817 A > G p.Lys273Glu. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The phenotype, or absence thereof, associated with the c.489G > A variant in homozygosity, has important implications for genetic counselling in affected families and the wider Pacific community. Cascade testing in affected families would likely identify large numbers of c.489G > A carriers. A very low risk of affected offspring in carriers for the hypomorphic variant could be predicted, due to the low prevalence of other pathogenic *ECHS1* variants, and the likely benign outcome of homozygosity. Therefore, in populations with a high carrier frequency for the hypomorphic allele, we have suggested cascade testing only for the other *ECHS1* pathogenic variants. However, evaluation of further homozygous individuals would provide additional reassurance supporting this approach.

Despite advances in genomic testing, a molecular diagnosis is reached in <50% of patients with suspected mitochondrial disease including LS [28,29]. This cohort demonstrates the diagnostic odyssey frequently experienced by individuals with mitochondrial disease, and reinforces the importance of pursuing a second variant in apparent heterozygotes with the appropriate phenotype. The utility of a specific molecular diagnosis is well described, and the identification of potentially treatable disorders such as SCEH deficiency is of particular importance [13,29]. However the optimum management for SCEH deficiency is not well established, and the benefits of dietary treatment in older children and adults are not clear [13,30]. The outcome of treatment in our cohort, with dietary restriction of valine and provision of an emergency regimen during illness and fasting to prevent catabolism, will be reported in a further publication.

Genomic testing techniques typically identify large numbers of variants, necessitating the use of variant-filtering algorithms to narrow the analysis. Such algorithms are likely to dismiss common, synonymous variants, especially those observed in healthy homozygotes, unless the variant has been previously reported in association with disease [14]. Thus, correct interpretation of genomic data relies on databases of variants detected in the context of diagnostic testing, and variants detected in control populations of healthy individuals. However, the under-representation of indigenous and ethnic minority populations in such databases may limit their utility in some populations, thereby increasing the likelihood of non-diagnostic or uncertain results of genetic testing [31–33]. The reduced diagnostic power of genomic testing in this setting, in addition to inequitable access to testing, is likely to compound existing health inequities faced by these communities [32,33]. It is therefore imperative that high-quality databases of population-specific genomic variation are developed, with appropriate community participation and governance to ensure that such initiatives reflect the values and priorities of the population [32–34].

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Ethics approval

This study was conducted in accordance with the revised Declaration of Helsinki. Informed consent for diagnostic or research investigations from the respective responsible human ethics institutional review boards (P6 under HREC/16/MH/251 and P4, 5 and 7 under HREC/RCH/34228).

A patient consent statement

Informed consent was obtained from all patients or their legal caregivers, including written consent wherever possible.

Documentation of approval from the Institutional Committee for Care and Use of Laboratory Animals (or comparable committee)

This study does not contain any studies with animal subjects.

CRediT authorship contribution statement

Isaac Bernhardt: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Conceptualization. **Leah E. Frajman:** Writing – review & editing, Investigation, Formal analysis. **Bryony Ryder:** Writing – review & editing, Investigation, Conceptualization. **Erik Andersen:** Writing – review & editing, Investigation, Conceptualization. **Callum Wilson:** Writing – review & editing, Investigation, Conceptualization. **Colina McKeown:** Writing – review & editing, Investigation. **Tim Anderson:** Writing – review & editing, Investigation. **David Coman:** Writing – review & editing, Investigation. **Andrea L. Vincent:** Writing – review & editing, Visualization, Investigation. **Christina Buchanan:** Writing – review & editing, Investigation. **Richard Roxburgh:** Writing – review & editing, Investigation. **James Pitt:** Writing – review & editing, Visualization, Investigation, Conceptualization. **Mark De Hora:** Writing – review & editing, Investigation. **John Christodoulou:** Writing – review & editing, Project administration, Conceptualization. **David R. Thorburn:** Writing – review & editing, Project administration, Conceptualization. **Francessa Wilson:** Writing – review & editing, Visualization, Investigation. **Kylie M. Drake:** Writing – review & editing, Investigation. **Megan Leask:** Writing – review & editing, Investigation. **Anne-Marie Yardley:** Writing – review & editing, Investigation. **Tony Merriman:** Writing – review & editing, Supervision, Investigation. **Stephen Robertson:** Writing – review & editing, Project administration, Investigation, Conceptualization. **Alison G. Compton:** Writing – review & editing, Visualization, Supervision, Project administration, Investigation, Conceptualization. **Emma Glamuzina:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data archiving is not mandated but data will be made available on reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgme.2024.108508.

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