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High incidence of null variants identified from newborn screening of X-linked adrenoleukodystrophy in Taiwan

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ABSTRACT

Background: Adrenoleukodystrophy (ALD) is an X-linked peroxisomal disorder caused by variants in the *ABCD1* gene and can lead to Addison disease, childhood cerebral ALD, or adrenomyeloneuropathy. Presymptomatic hematopoietic stem cell transplantation is the only curative treatment for the disease and requires early detection through newborn screening (NBS) and close follow-up.
Methods: An NBS program for ALD was performed by a two-tiered dried blood spot (DBS) lysophosphatidylcholine C26:0 (C26:0-LPC) concentration analysis. *ABCD1* sequencing was eventually added as a third-tier test, and whole exome sequencing was used to confirm the diagnosis of all peroxisomal diseases. Affected newborns were followed-up for adrenal insufficiency and cerebral white matter abnormalities.
Results: We identified 12 males and 10 females with *ABCD1* variants, and 3 patients with Zellweger syndrome from 320,528 newborns. Eight (36.4%) *ABCD1* variants identified in the current study were null variants, but there were no hotspots or founder effect. During a median follow-up period of 2.28 years, two (16.7%) male patients with *ABCD1* variants developed Addison's disease. Extended family screening revealed one 28-year-old asymptomatic hemizygous father of a null variant (c.678delC). Among the three with Zellweger syndrome, one died at the age of 3 months, one showed developmental delay at the age of 1 year, and one was lost to follow-up.
Conclusion: Screening for ALD has been added to the NBS program in Taiwan with a high degree of success. The screening algorithm revealed a high proportion of null variants in cases found by NBS in Taiwan, a subset of patients who may have earlier disease onset. We also demonstrate the feasibility of combining the diagnosis of ALD and other peroxisomal disorders into one screening algorithm.

1. Introduction

Adrenoleukodystrophy (ALD) is an X-linked inheritance peroxisomal disorder caused by variants in the *ABCD1* gene encoding the adrenoleukodystrophy protein (ALDP). Accumulation of very long-chain fatty acids (VLCFA) due to ALDP dysfunction leads to progressive inflammatory demyelination of the central nervous system and may cause a direct toxic effect on the adrenal glands. A broad spectrum of clinical presentations and severity have been observed, including fatal infantile cerebral ALD, adrenal insufficiency, and late-onset adrenomyeloneuropathy (AMN) [1,2]. According to previous studies, 35–50% of male patients develop cerebral ALD, and 30–40% develop AMN in their third to fourth decades [3,4]. About 80% of all affected patients suffer from adrenal insufficiency of various severity during their lifetime [5].

Currently, hematopoietic stem cell transplantation (HSCT) is the only curative management for ALD, and transplantation is preferably performed immediately after the appearance of any cerebral MRI abnormalities and before the development of neurological symptoms [6,7]. The narrow therapeutic time window exacerbates the difficulty of

Abbreviation: ALD, adrenoleukodystrophy; AMN, adrenomyeloneuropathy; VLCFA, very long-chain fatty acids; C26:0-LPC, Lysophosphatidylcholine C26:0; NBS, newborn screening; HSCT, hematopoietic stem cell transplantation; HPLC-MS/MS, High-performance liquid chromatography-tandem mass spectrometry analysis; FIA-MS/MS, flow injection-tandem mass spectrometry; DBS, dried-blood spot; ZSD, Zellweger spectrum disorder.

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accurately timing HSCT [3] and mandates careful interpretation of challenging and ambiguous MRI findings [8]. In these pre-symptomatic patients, regular interval brain magnetic resonance imaging (MRI), rated with the Loes score [9], is recommended for early detection of radiographic evidence of brain demyelination [10].

Newborn screening (NBS) for ALD was nominated for inclusion in the United States' Recommended Uniform Screening Panel (RUSP), a federal list of all genetic diseases recommended for state newborn screening (NBS) programs in 2015 [11], and was officially added in February 2016. Liquid chromatography-tandem mass spectrometry analysis of lysophosphatidylcholine C26:0 (C26:0-LPC) is a sensitive and high-throughput screening method [12,13], but false-positive and false-negative screening results can both occur. In some NBS programs, C26:0-LPC is used as the only screening marker, and those with abnormal C26:0-LPC levels were retested before confirmatory tests [14,15]. Others measured both C26:0-LPC and C24:0-LPC and referred those with positive screening results from either test [13,16]. The sequencing of the *ABCD1* gene was deemed critical for the diagnosis of ALD [17] and is included by most NBS programs for confirmatory testing.

ALD has been included in our universal NBS program since 2016. We verified all positive C26:0-LPC dried-blood spot (DBS) samples using two different methods to decrease false-positive results. Molecular genetic testing of *ABCD1* sequencing was also added later, using the initial DBS, to facilitate confirmatory testing. Here, we report our screening results from 320,528 newborns.

2. Material and methods

2.1. Study population

Newborn Screening for ALD was launched on Nov. 1st, 2016, at the National Taiwan University Hospital (NTUH) Newborn Screening Center [18], which is responsible for screening 30–35% newborns in Taiwan. Second-tier molecular tests and confirmatory testing were also performed by NTUH. Information including serial C26:0-LPC data, biochemical exams, physical examination, diagnosis, clinical presentation, genotypes, and results of family segregation analysis of all patients found via NBS was collected in this study. Informed consent was waived and this study was approved by the institutional review board of the NTUH (NTUH-IRB; No. 202203077RIN).

2.2. Analysis of C26:0-LPC and ABCD1 sequencing

DBS samples were obtained from newborns 48–72 h after delivery. DBS C26:0-LPC level was firstly analyzed with the NeoBase™ 2 non-derivatized flow injection-tandem mass spectrometry (FIA-MS/MS) kit (PerkinElmer, Turku, Finland). Mass spectra was acquired with a TQD equipment (Waters, Milford, MA, USA), and data were analyzed using MassLynx™ and NeoLynx™ Software. Samples suspicious of C26:0-LPC elevation were verified with high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) (LCMS-8500, Shimadzu, Kyoto, Japan) using the same DBS. A Waters XBridge BEH C18 XP column (2.1 mm × 50 mm, particle size 2.5 μm) was used for separation (Waters, Milford, MA, USA). DNA was extracted from one 3.2-mm punch of the original for next generation sequencing (NGS). *ABCD1* sequences (RefSeq: NM_000033) were analyzed by targeted sequencing using a SeqCap EZ probe (Roche Nimbelgen, Basel, Switzerland) and a MiSeq sequencer (Illumina, San Diego, CA, USA). For whole exome sequencing (WES), library preparation was carried out with an Illumina TruSeq® library preparation kit (Illumina, Inc., San Diego, CA, USA), and sequencing was conducted on a NovaSeq6000 machine (Illumina, Inc.). Sequences were aligned to the human reference genome build (hg38) followed by variant calling according to the GATK 4.0 best practice pipeline. Variants were annotated by ANNOVAR (https://wannovar.wglab.org/). The pathogenicity of variants was classified according to the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology guidelines [19].

2.3. Algorithm of NBS

During the first period of the study, the cut-off value of C26:0-LPC for the first screen was 0.3 μM for first-tier FIA-MS/MS, defined by the 97th percentile of normal newborns, and 0.4 μM for the second-tier HPLC-MS/MS. Newborns with a positive first-screen HPLC-MS/MS result were requested to receive a second screen with a cut-off value of 0.4 μM by HPLC-MS/MS. Those who had a positive second screen were referred for confirmatory testing, which included molecular analysis, VLCFA analysis, general biochemistry (aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, sodium (Na), potassium (K), chloride (Cl), ACTH, and cortisol), and extended family screening if feasible.

After Aug. 1st, 2019, a third-tier *ABCD1* sequencing was added, using the initial DBS obtained for the first screen (Fig. 1). Newborns with *ABCD1* variants identified by the third-tier sequencing, including pathogenic variants, likely pathogenic variants, or variants with unknown significance (VUS), were referred for confirmatory testing regardless of the second-screen C26:0-LPC results. Furthermore, those who showed persistently elevated C26:0-LPC levels on the second screen but had a negative report on *ABCD1* variants were also referred. The results of third-tier *ABCD1* sequencing were available at the time of confirmatory testing and aided clinical decisions. Alternatively, WES was recommended for any newborns who tested negative for *ABCD1* variants but had persistently elevated second-screen C26:0-LPC levels to exclude Zellweger spectrum disorder (ZSD).

In Taiwan, a compulsory social insurance program, the National Health Insurance Program, covers nearly all non-elective health services. These services encompass all confirmatory biochemical studies for every individual recalled by our NBS program, and the follow-up biochemical and imaging studies. Genetic analysis for asymptomatic family members is not covered. Males with *ABCD1* variants identified in the current study were followed by adrenal function tests every 3–6 months after diagnosis and brain MRI every 6–12 months after 1 year of age. Adrenal insufficiency was suspected if any patients presented with increased morning plasma ACTH levels for two consecutive visits, followed by an ACTH stimulation test to confirm the diagnosis.

2.4. Statistical analysis

The incidence rate was calculated for males and females with *ABCD1* variants, respectively, and the 95% confidence interval was calculated using Poisson distribution. Mann-Whitney U was performed to compare C26:0-LPC levels on initial NBS between males and females with *ABCD1* variants, and Zellweger patients, as well as test the difference of C26:0-LPC levels between males with null *ABCD1* variants against those with missense variants on initial screening and second screening. *P*-value <0.05 denoted the presence of a statistically significant difference.

3. Results

3.1. Incidence of ALD

During the first period of the study, 181,342 newborns were screened. The algorithm for ALD screening changed several times during this period; only the diagnostic outcome was recorded. Among the 181,342, 17 were referred for confirmatory testing, of whom 5 males and 4 females with *ABCD1* variants and 2 patients with ZSD were diagnosed. During the second period of the study, after the implementation of third-tier *ABCD1* sequencing, 139,186 newborns were screened; 26 were positive for the first screen (0.0187%). Fig. 1 demonstrates the algorithm that we applied during this period. Of these 26 newborns, *ABCD1* variants were found in 13 (50%); 7 males and 6 females with *ABCD1* variants were diagnosed. Of the remaining 13 (50%)

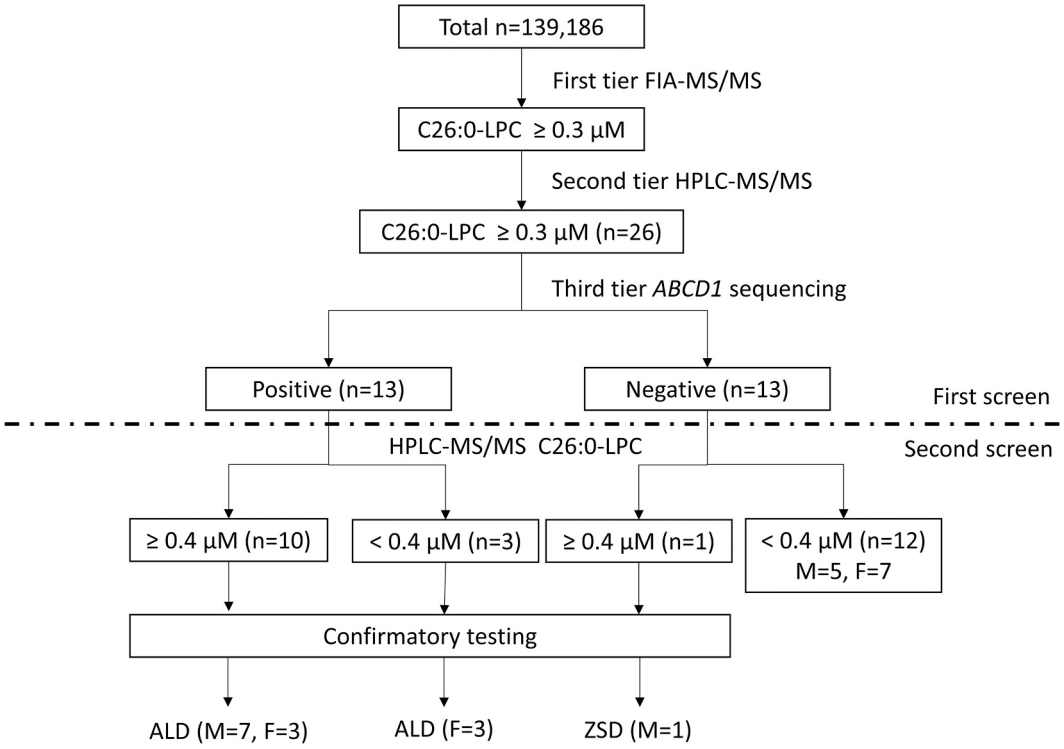

Fig. 1. Algorithmic workflow including molecular testing for adrenoleukodystrophy newborn screening. The cutoff for first screen was 0.3 μM, and the second screen cutoff was 0.4 μM. Newborns positive for the first screening, including the first and second tier C26:0-LPC analysis using different methods, were subjected to both third-tier molecular testing for *ABCD1* sequencing and a second screen since Aug. 1st, 2019. Those with a negative *ABCD1* sequencing but positive second screen result were subjected to whole exome sequencing to clarify the etiology.

newborns who tested negative for *ABCD1* variants, one showed persistent elevated C26:0-LPC level on the second screen. This male infant was referred for a WES study and was diagnosed with ZSD. Of the 12 newborns (5 males and 7 females) who had elevated C26:0-LPC levels on the first screen and normal levels on the second screen, none presented with associated symptoms or had abnormal biochemical data at the time of referral; follow-up for these newborns was not arranged. Therefore, the positive predictive value (PPV) was 53.8% (14/26) for the first screen and 100% for those who entered the confirmatory testing.

A total of 320,528 newborns were screened during the whole study period, and 12 males and 10 females with *ABCD1* variants, and 3 patients with ZSD were identified. The incidence of ALD in male newborns was 1 in 13,825 (95% confidence interval (CI): 1/7914–1/26,755), while the incidence of females with *ABCD1* variants was 1 in 15,463 (95% confidence interval (CI): 1/8408–1/32,246). Extended family screening discovered a 28-year-old male with a null *ABCD1* variant (c.678delC) after the ALD diagnosis of his daughter but has remained asymptomatic to date. Another male infant carrying *ABCD1* variant c.438del revealed a family history of two deceased grand-uncles with suspicious symptoms; one died at an early age due to progressive disability, while the other lost his ability to walk as a teenager. One male infant with *ABCD1* variants (*ABCD1* variant c.1007A > C) detected by NBS was related to another female infant with *ABCD1* variants also detected by NBS, but his family did not take part in family screening. Family screening was not performed for 2 of the 12 males and 7 of the 10 females with *ABCD1* variants.

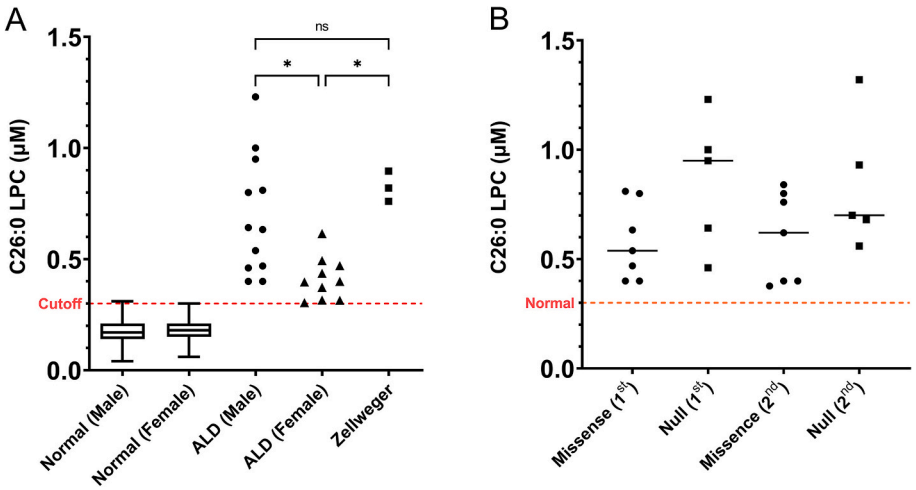

Fig. 2. C26:0-LPC levels in newborn screening reported in this study. (A) First screen C26:0-LPC levels for normal male newborns, normal female newborns, males with *ABCD1* variants (n = 12), females with *ABCD1* variants (n = 10), and Zellweger spectrum disorders (n = 3) are shown here. Significant differences were noted between males and emales with *ABCD1* variants (p = 0.0026) and between Zellweger spectrum disorders and females with *ABCD1* variants (p = 0.007). (B) Comparison of C26:0-LPC levels between male newborns with null (n = 5) or missense (n = 7) variants on the first and second screening. The boxes are the interquartile ranges (IQR, 25th to 75th percentile), and the whiskers are the ranges between 1.5 IQR of the lower quartile and 1.5 IQR of the higher quartile; the vertical bar denotes the median. *: $p < 0.05$.

3.2. Biochemical and molecular manifestations

Patients with ZSD had the highest C26:0-LPC levels (median 0.82 μm), followed by males with *ABCD1* variants (median 0.69 μm); both had C26:0-LPC levels significantly higher than the females with *ABCD1* variants (median 0.41 μm), with p values 0.007 and 0.026, respectively (Fig. 2A). No significant difference was found between ZSD patients and males with *ABCD1* variants. Among all the 18 *ABCD1* variants identified in the current study, 8 (44.4%) were novel, and 8 (44.4%) were null variants (Table 1). All identified variants were pathogenic or likely pathogenic, but no variant hotspots were recognized because only 4 variants occurred twice. Of the 8 *ABCD1* null variants identified in the current study, there were 3 nonsense variants, 4 frameshift variants, and one 2-exon deletion. Each null variant occurred only once in the current study; eight of the 22 (36.4%) confirmed patients had null variants. C26:0-LPC levels at both the first and second screen tended to be higher in males with null variants than those with missense variants, although they did not achieve significance ($p = 0.10$ and 0.25, respectively) (Fig. 2B). We have discovered no correlation between variants and their C26:0-LPC levels among females with *ABCD1* variants.

3.3. Follow-up

During a median follow-up period of 2.28 years (range 3 months to 5 years), two of the 12 (16.7%) males with *ABCD1* variants developed Addison's disease. Elevation of ACTH was found around 1 year of age for both patients. Both patients demonstrated poor response to ACTH stimulation test and were diagnosed with primary adrenal insufficiency. Neither patient had electrolyte imbalance, hyperpigmentation, or other associated symptoms. Corticosteroid replacement therapy was prescribed after diagnosis. Among the other subjects, three older patients (aged 4, 2, and 1) received brain MRIs during this study period. One subject had a suspicious white matter lesion at the age of 1 year and 10 months. However, a follow-up scan 4 months later showed no progression of the lesion. No patients show abnormal neurological symptoms or signs, and nor did any patients receive HSCT.

We encountered two asymptomatic males with *ABCD1* variants during this period: one (Fig. 3 A-C) was diagnosed through another NBS program, while the other (Fig. 3 D-E) was diagnosed through family screening. During follow-up of patient 1, a suspicious T2 hyperintensity at right parietal white matter was noticed at 1 year and 10 months old (Fig. 3B), indicating high risk of ALD progression. As a result, an MRI follow-up was scheduled 3 months later, which showed no progression of MRI findings (Fig. 3C); thus, HSCT has not yet been arranged. Patient 2 had normal MRI scans for the first 4 years of life (Fig. 3D). When he was 4 years and 2 months old, a faint and ambiguous T2-hyperintense lesion in the splenium was found (Fig. 3E). A follow-up scan 6 months later revealed rapid progression of splenium T2-hyperintensity (Fig. 3F), and HSCT was performed at the age of 5 with poor outcomes.

3.4. Zellweger spectrum disorders

Among the three ZSD patients, one carried *PEX1* compound heterozygous variants (c.2709_2710del, and c.2391_2392del) and expired at 3 months old. The second patient had homozygous *HSD17B4* pathogenic variant [c.1041 T > A (p.Tyr347Ter)]. He had hypotonia and developmental delay at 1 year and 2 months old and is currently under experimental therapy. Another male newborn with *PEX1* compound heterozygous variants c.2966 T > C (p.Ile989Thr) and c.3125 T > C (p.Phe1042Ser). He had only mild head lag at the age of 2 months and may have the mild form of ZSD (Heimler syndrome-1). He has since been lost to follow-up.

4. Discussion

4.1. Incidence of ALD and performance of the screening

In the current study, we report the incidence of ALD from NBS in Taiwan as 1:13,825 in the male population, which is similar to the incidence rate previously reported by the California screening program (1 in 14,390) [14] and the New York screening program (1 in 14,700) [20], although different numbers have reported from several smaller programs (Table 2) [13,21]. The incidences derived from the NBS programs are also close to the estimated incidence rate of 1:14,000 [22]. Since the algorithm employed by each screening program differs, we have mainly compared our results to those found by the California and New York screening program, as all three programs arranged second screening using LC-MS/MS for abnormal C26:0-LPC levels found by FIA-MS/MS. Our program and the New York program shared similar PPV, while the California program had a lower PPV of 68%, with females outnumbering males in both referrals and confirmed cases. The difference may be caused by the referral of all abnormal first-screen newborns for confirmation in California. An additional reason may be due to the higher cut-off in the second HPLC-MS/MS tier that we have set; California's second tier's cut-off is 0.22 uM compared to 0.4 uM in Taiwan [14]. While *ABCD1* sequencing was performed for all newborns, patient referral did not take the results into consideration.

In our screening program, due to the high accessibility of medical facilities in Taiwan, including local hospitals and clinics, we were able to refer suspicious newborns for a local second screen while

Table 1
ABCD1 variants found by newborn screening.

ABCD1 variant	Exon	Male	C26:0-LPC (μm)[†]	Female	C26:0-LPC (μm)[†]	Null variant	Novel variant	ACMG interpretation
c.253dup (p.Arg85fs)*	1			1	0.36	+		Pathogenic
c.341 T > C (p.Leu114Pro)	1	1	0.76	1	0.31			Pathogenic
c.438del (Phe146Leufx*52)*	1	1	0.93			+	+	Likely pathogenic
c.487C > T (p.Arg163Cys)	1			1	0.48			Likely pathogenic
c.565C > T (p.Arg189Trp)	1	1	0.62	1	0.31			Pathogenic
c.584A > T (p.Gln195Leu)	1	1	0.38				+	Likely pathogenic
c.678delC (p.Tyr227Thrfs*109)*	1			1	0.40	+		Pathogenic
c.1007A > C (p.Lys336Thr)	2	1	0.84	1	0.65		+	Likely pathogenic
c.1111G > T (p.Glu371Ter)*	3			1	0.66	+	+	Likely pathogenic
c.1195_1196del (p.Ile399Ter)*	3	1	0.70			+	+	Likely pathogenic
c.1252C > T (p.Arg418Trp)	4	1	0.80	1	0.41			Likely pathogenic
c.1390C > T (p.Arg464Ter)*	4	1	1.32			+		Pathogenic
c.1415_1416del (p.Gln472fs)*	5	1	0.68			+		Pathogenic
c.1439C > G (p.Pro480Arg)	5	1	0.40				+	Likely pathogenic
c.1736 T > C (p.Ile579Thr)	7	1	0.40				+	Likely pathogenic
Exon 8 & exon 9 deletion*	8, 9	1	0.56			+		Pathogenic
c.1928 T > A (p.Ile643Asn)	9			1	0.27		+	Likely pathogenic

Version: NM_000033.4(ABCD1).
[†] The result of C26:0-LPC from the first-screen LC-MS/MS study.

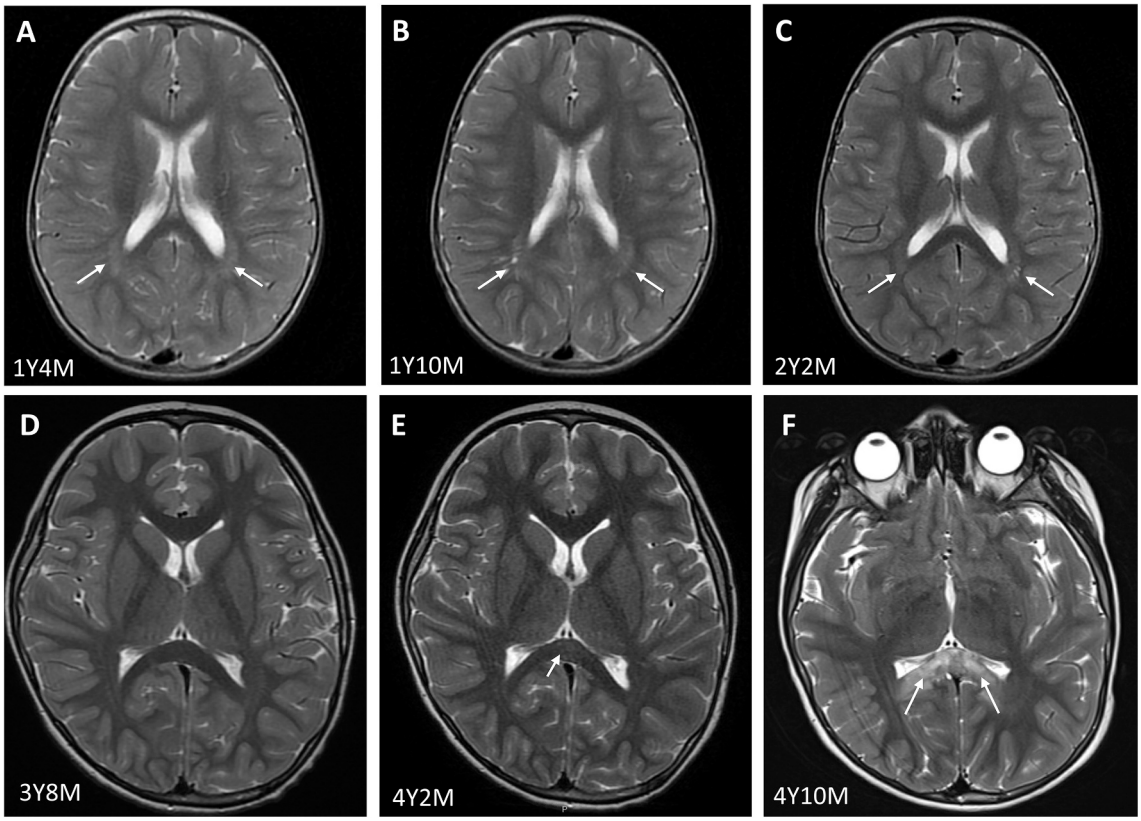

Fig. 3. Brain MRI images of two adrenoleukodystrophy patients. Serial MRI scans for patient 1(A-C). Normal appearance of MRI at 1 year and 4 months old (A). Follow-up MRI at the age of 1 year 10 months showed a suspicious T2 hyperintensity at right parietal white matter (B). however, 3 months later there was no progression of MRI findings (C). Serial MRI scans for patient 2 (D—F). Normal appearance of MRI at 3 year and 8 months old (C). Follow-up MRI performed rat the age of 4 yeas 2 months was initially reported as normal, but the T2 abnormality at the splenium was already present (E). Follow-up scans revealed rapid progression of splenium T2-hyperintensity (F).

simultaneously performing third-tier *ABCD1* sequencing analysis on the initial DBS sample. Both second-screen C26:0-LPC level and *ABCD1* sequencing results were taken into consideration prior to issuing referral notice for confirmatory testing. After third-tier implementation, of the 26 newborns who tested with elevated C26:0-LPC levels at the first screening, only 14 newborns were eventually referred for confirmatory testing either due to positive *ABCD1* sequencing results or persistently elevated C26:0-LPC. All 14 infants were diagnosed with ALD or ZSD. Correspondingly, the PPV for our confirmatory testing, which was usually done in designated medical centers, reaches 100%. To date, none of the 12 patients who did not receive confirmatory have been diagnosed with ALD or other peroxisomal disorders. The New York program also had the *ABCD1* sequencing data available before the clinic visits and reported a similar confirmation rate of 32 out of 33 referrals [20].

None of our screened individuals has developed neurological symptoms or signs yet. Many of our subjects have not yet received MRI examinations due to their young age. None of the individuals who received MRIs showed evidence of abnormal brain imaging. These findings are consistent with other screening programs where a low prevalence of cerebral ALD is universally reported, ranging from 0% to 7%. Our cohort diagnosed two patients with Addison's disease, and early corticosteroid supplementation was given before any associated symptoms or adrenal crisis developed. We believe our ALD NBS algorithm demonstrates good performance, provides the ability to detect early onset adrenal insufficiency and cerebral involvement, and enables prompt treatment. Extended family screening has also discovered relatives at risk, allowing for earlier recognition and intervention.

To date, there have been no reported false-negative cases. However, considering the variable clinical manifestation and a broad range of onset age, there may still be false-negatives who have not yet developed associated symptoms. Moreover, heterozygous females with *ABCD1* variants were more likely to be missed due to normal biochemical results.

We do not include ZSD as a target of our NBS program, since there is currently no known effective management for patients with ZSD. However, we try to document all patients that are found through our NBS program. As such, we do not have complete information regarding missed cases.

4.2. Higher percentage of severe variants

Eight of the 22 ALD patients identified in the current study carry null variants. Null variants were reported in only 1/6 ALD individuals in North Carolina [13], 1/12 in Minnesota [23], and 2/15 in Illinois [21]. Therefore, the high proportion of null variants was a specific finding in the current study. As no variant occurred more than two times, no variant hotspot or founder variant was found in our study population. The ABCD1 Variant Database (https://adrenoleukodystrophy.info/) includes 3401 cases and reported that 29.7% of the cases had null variants, including 9.9% nonsense pathogenic variants, 17.2% frameshift pathogenic variants and 2.6% of one or more exons deletion [24]. Therefore, our program's proportion of null variants was closer to this clinical database than other screening programs. The availability of sequencing results and a second screen before confirmation may explain the differences.

Furthermore, none of our identified variants in our study was classified as VUS, while 147/353 (41.6%) *ABCD1* variants in the California program were reported as VUS [14]. The C26:0-LPC cutoff for the first screen (0.3 μM) in our algorithm was set higher than that used by the California program (0.15–0.22 μM). The higher cutoff may explain the

Table 2
Summary of results reported from current adrenoleukodystrophy newborn screening programs.

Population	Study size	PPV[†]	Diagnosis				Incidence		Overall[‡]	Manifestation		Variants		Screen marker	2nd-tier molecular	Reference
			M-ALD	F-ALD	ZSD	AGS	M-ALD	F-ALD		ccALD	Addison	VUS	Null			
NTUH	320,528	100%[§]	12	10	3	0	1/13,825	1/15,463	1/14,569	0	2/12	0/18	8/22	C26:0-LPC	Y[§]	Current study
North Carolina	52,301	8/12 (67%)	3	3	1	1	1/8333	1/9100	1/8717	0	2/3	0/6	1/6	C24:0-LPC, C26:0-LPC	N	Lee et al. [13]
Minnesota	67,835	Positive 100%; Borderline 6.8%	9	5	0	0	1/3878	1/6584	1/4845	*NA*	*NA*	1/12	1/12	C26:0-LPC	N	Wiens et al. [23]
Illinois	276,000	Positive 67%; Borderline 6.8%	7	10[††]	3	0	*NA*	*NA*	1/16,200	*NA*	*NA*	8/15	2/15	C26:0-LPC	N	Burton et al. [21]
California	1,854,631	240/355 (68%)	95	110	23	*NA*	1/14,390	1/9593	1/15,455	5 (7%)	14 (20%)	147/353	*NA*	C26:0-LPC	Y	Matteson et al. [14]
New York	365,000	32/33	13	14[‡‡]	4	1	1/14,700	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	C26:0-LPC	Y	Kemper et al. [20]
Pennsylvania	542,554	48/51	21	23	4	0	1/13,000	1/11,000	1/12,330	0/21	4/21	21/44	11/44	C26:0-LPC	Y	Priestley et al. [38]

PPV: positive predictive value; M-ALD: males with *ABCD1* variants; F-ALD: females with *ABCD1* variants; ZSD: Zellweger spectrum disorder; AGS: Aicardi-Goutières Syndrome; ccALD: childhood cerebral adrenoleukodystrophy; VUS: variants of unknown significance; C26:0-LPC: Lysophosphatidylcholine C26:0; *NA*: not available.

† PPV is defined as the total numbers of patients at confirmatory diagnosis.

‡ The overall incidence for males and females with *ABCD1* variants, without ZSD or other diagnosis.

§ After implementation of third tier molecular screening on Aug. 1st, 2019.

†† Including 13 females with *ABCD1* variants and 1 Klinefelter syndrome with heterozygous *ABCD1* variant.

‡‡ One homozygous female.

lower proportion of females with *ABCD1* variants (10/22, 45.5%) compared to the California program (53.7%) because they have significantly lower C26:0-LPC levels compared to males (Fig. 2.). Although the identification of females with *ABCD1* variants can trigger extended family screening, prenatal diagnosis and family planning, disclosing the carrier status also has drawbacks, including discrimination within the family and poses no immediate benefit to the female babies [25].

4.3. Phenotype prediction

The association between genotype and phenotype is elusive, as different clinical presentations and phenotypes can be observed among siblings and twins sharing the same variant [26,27]. Among heterozygous females with *ABCD1* variants, cerebral ALD or Addison's disease are rarely seen, but up to 80% of females with *ABCD1* variants may manifest AMN associated symptoms [5,28–30]. Hemizygous males with severe variants have been shown to develop cerebral ALD more frequently than those with less detrimental variants [31]. Although a direct correlation between genotype and phenotype has not been established, and the prognostic value of C26:0-LPC level to predict ALD disease severity is unknown [32], overproduction of C26:0-LPC has been correlated with the development of neurodegeneration [33]. Inflammation has been suggested as an important factor for ALD pathogenesis [34], and increased inflammatory and low anti-inflammatory cytokines levels were found in symptomatic ALD patients compared with asymptomatic hemizygous individuals [35]. While not directly correlated with ALD, lysophosphatidylcholine is known as a strong pro-inflammatory mediator [36]. In our study, we identified a high proportion of null variants in our population. Furthermore, C26:0-LPC tended to be higher in these null variants, suggesting that these identified male infants may be more likely to develop symptoms during childhood. Access to this information allows for patient-centered genetic counseling and increases attentiveness to disease progression on follow-up. However, we did notice a male family member with a null *ABCD1* variant c.678delC, who is 28-year-old currently, and has remained asymptomatic. Therefore, further long-term follow-up for these individuals is necessary.

4.4. Challenging imaging diagnosis

Since 90 % of cerebral ALD patients develop their first imaging signs or symptoms between the ages of 3 and 12 years; the current guideline recommends an initial MRI scan to be performed between 12 and 18 months, with a more intensive screening by every 6 months during the risky period from 3 to 12 years old [10]. Treatment of ALD is recommended at the first sign of ALD on MRI and preferably prior to the development of clinical symptoms [6]. However, early findings of ALD on MRI may be faint and result in delayed recognition of disease progression, as in patient 2, but overinterpretation may cause unnecessary anxiety as shown in patient 1. It is recently reported that neurofilament light chain can be a biomarker for monitoring neurodegeneration in X-linked ALD [37], and we are optimistic that biomarkers can be applied for monitoring pre-symptomatic ALD patients detected by NBS.

5. Conclusion

Screening for ALD has been added to the NBS program in Taiwan with a high degree of success. The screening algorithm revealed a high proportion of null variants in cases found by NBS of ALD in Taiwan. We predict that severe loss-of-function variants might lead to an earlier age of disease onset, but further long-term follow-up studies are still needed. Secondly, we combined the diagnosis of ALD and other peroxisomal disorders into one screening algorithm and found that individuals with Zellweger syndrome had a slightly higher median C26:0-LPC level compared to males with *ABCD1* variants and both were significantly higher than C26:0-LPC level in heterozygous females with *ABCD1* variants.

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CRediT authorship contribution statement

Hui-An Chen: Formal analysis. **Rai-Hseng Hsu:** Validation. **Pin-Wen Chen:** Investigation. **Ni-Chung Lee:** Data curation. **Pao-Chin Chiu:** Data curation. **Wuh-Liang Hwu:** Conceptualization, Methodology. **Yin-Hsiu Chien:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors of this manuscript declare no relationships with any companies, whose products or services may be related to the subject matter of the article.

Data availability

Data will be made available on request.

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