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Asymptomatic ASS1 carriers with high blood citrulline levels

Hui-An Chen[1,2] | **Rai-Hseng Hsu**[1,3] | **Kai-Ling Chang**[3] | **Yi-Chen Huang**[3] | **Yun-Chen Chiang**[3] | **Ni-Chung Lee**[1,3] | **Wuh-Liang Hwu**[1,3] | **Pao-Chin Chiu**[2] | **Yin-Hsiu Chien**[1,3]

[1]Department of Pediatrics, National Taiwan University Hospital, Taipei, Taiwan

[2]Department of Pediatrics, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan

[3]Department of Medical Genetics, National Taiwan University Hospital, Taipei, Taiwan

Correspondence
Yin-Hsiu Chien, Department of Pediatrics, National Taiwan University Hospital, Taipei, Taiwan.
Email: chienyh@ntu.edu.tw

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Abstract

Introduction: Citrullinemia Type 1 (CTLN1) is an autosomal recessive disorder caused by variants in the *ASS1* gene. This study intends to clarify the etiology of false positives in newborn screening for citrullinemia.

Method: Newborns who had elevated dried-blood spot citrulline levels were enrolled, and medical records were reviewed retrospectively. Common *ASS1* variants were screened using high-resolution melting analysis.

Result: Between 2011 and 2021, 130 newborns received confirmatory testing for citrullinemia, 4 were found to be patients for CTLN1; 11 were patients with citrin deficiency; and 49 newborns were confirmed to be carrying one pathogenic *ASS1* variant. The incidence of CTLN1 was 1 in 188,380 (95% confidence interval: 1 in 73,258 to 1 in 484,416). All *ASS1* variants studied in this cohort were located in exons 11 to 15, which encode the tetrameric interface regions of the ASS1 protein. Among 10 *ASS1* carriers with elevated citrulline levels and complete sequence data, four (40%) revealed additional non-benign *ASS1* variants; in contrast, only 2 of the 26 controls (7.7%), with normal citrulline levels, had additional *ASS1* variants.

Conclusion: Heterozygote *ASS1* variants may lead to a mild elevation of blood citrulline levels: about 2–6 times the population mean. Molecular testing and family studies remain critical for precise diagnosis, genetic counseling, and management.

KEYWORDS
ASS1, carriers, citrullinemia, newborn screening, tandem mass analysis

1 | INTRODUCTION

Citrullinemia type 1 (CTLN1, OMIM# 215700), also known as ASS1 deficiency, is an inborn error disorder of the urea cycle (Quinonez & Thoene, 1993). CTLN1 is inherited recessively and is caused by variants in the *ASS1* gene that are responsible for the production of the argininosuccinate synthetase (ASS) enzyme 1 (Woo et al., 2014). Patients present with a wide spectrum of clinical symptoms ranging from fatal acute neonatal hyperammonemia to a later onset form with episodes of more subtle hyperammonemia (Kolker et al., 2015). Apart from emergent management of acute hyperammonemia, chronic management of CTLN1, emphasizes a

lifelong protein restriction diet, starting as early as possible (Häberle et al., 2019), sometimes supplemented by nitrogen scavenger therapy to prevent secondary complications (Waisbren et al., 2016). Liver transplantation has shown to improve outcomes in individuals with neonatal-onset CTLN1 and is recommended at an early age to avoid further neurological deficit (Liu et al., 2021; Vara et al., 2018). Therefore, early diagnosis, such as newborn screening (NBS), enables early interventions, including nutrition management, and timely evaluation for transplantation (Häberle et al., 2019).

NBS using citrulline as a biomarker on tandem mass spectrometry (MS/MS) has proven to be an effective method for screening CTLN1, followed by confirmatory studies with molecular genetic testing.(Posset et al., 2020; Wasim et al., 2018) During this process, we found patients with elevated citrulline levels on MS/MS that were ultimately diagnosed as carriers. The current study reviews the effectiveness of MS/MS for screening CTLN1 and compares the biochemical features of CTLN1 patients and carriers. We also propose a mechanism to explain the elevation of citrulline found in CTLN1 carriers, despite it being an autosomal recessive disease.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

Informed consent was waived, and this study was approved by the institutional review board of the National Taiwan University Hospital (NTUH-IRB; No. 202109140RIN).

2.2 | Newborn screening program for citrullinemia

NBS for CTLN1 and citrin deficiency was performed using MS/MS on dried blood spots (DBS) samples obtained 48–72 h after delivery. DBS citrulline levels were used as the target markers for both conditions. Approximately 35%–37% of newborns born in Taiwan were screened by the National Taiwan University Hospital Newborn Screening Center (Huang et al., 2006).

The cutoff for citrullinemia in patients who may need emergent management (critical cutoff) was set at 110 μM. In addition, a borderline cutoff, set at 20 μM (approximately equals to the 99.9th percentile), triggers a re-screen. Newborns who either had citrulline levels higher than the critical cutoffs or citrulline levels higher than the borderline cutoff at the 2nd screening were recalled for confirmatory testing. In June 2018, second-tier molecular testing for citrin deficiency was implemented into our newborn screening system, using 11 common variants within the Taiwanese population. Individuals with at least 1 *SLC25A13* variant identified were also recalled, even if the rechecked citrulline levels returned to normal. In this study, we only included individuals who showed persistently elevated citrulline levels, while those recalled for positive second-tier molecular screening without abnormal citrulline levels were excluded. Citrin deficiency has been discussed in a separate study.

2.3 | Study population

Newborns who received confirmatory testing for citrullinemia between 1 January 2011 and 30 September 2021, were enrolled in this study. Subjects who refused confirmatory testing or had expired were excluded. Medical records, including MS/MS data, biochemical tests (aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AlkP), total bilirubin (T. bil), direct bilirubin (D. bil), γ-glutamyl transferase (GGT), and ammonia), final diagnosis, clinical presentation, parents' citrulline levels, and genetic studies were reviewed retrospectively.

2.4 | *ASS1* gene hot spots analysis

We retrospectively analyzed *ASS1* variant hotspots for those who had persistent elevation of citrulline but no definite molecular diagnosis. High resolution melting analysis (HRM) was applied to detect the 4 most common *ASS1* variants, c.1087C>T (p.Arg363Trp), c.773+4A>C, c.847G>A (p.Glu283Lys), and c.836G>A (p.Arg279Gln), found in previous studies. DNA was extracted from one 3.2-mm punch from each DBS sample as reported previously (Chien et al., 2017) and real-time polymerase chain reaction (PCR) based HRM curve analysis was performed to detect small nucleic acid sequencing differences in PCR dissociation (melting) curves.

Real-time PCR reactions were performed with precision melt supermix (BioRad), using a 10 μl reaction volume in a 96-well reaction plate. The list of primers used for HRM and the thermal cycling protocol are available upon request. Data obtained from real-time PCR-HRM were then analyzed using Precision Melt Analysis™ Software (BioRad). Melt curve shape sensitivity for cluster detection was set to 100%; the difference in melting temperature (Tm) threshold for the cluster detection was set to 0.1 and normalized. Temperature shifted views were used for analysis. In each round, blank control, normal control, and one heterozygous positive control were included. If possible, another homozygous positive control was also included. For samples with shifted shape,

further sanger sequencing was performed to confirm the sequence variant.

2.5 | Next generation sequencing (NGS)

Initially, individual *ASS1* gene exons, including exon-intron boundaries, were analyzed by Sanger sequencing. Later, *ASS1* sequences (RefSeq: NM_054012.4), in addition to other genes leading to hyperammonemia (*CPS1*, *OTC*, *ASL*, *ARG1*, *NAGS*, *ORNT1* [*SLC25A15*], *SLC25A13*) were analyzed by targeted sequencing using a SeqCap EZ probe (Roche Nimbelgen, Basel, Switzerland) and a MiSeq sequencer (Illumina). Variants were annotated by ANNOVAR (https://wannovar.wglab.org/). The pathogenicity of the variants was classified according to the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology guidelines.

2.6 | Visualization of *ASS1* crystal structure

Intramolecular interactions were calculated and visualized using Mol* viewer (Sehnal et al., 2021) with the experimental crystal structure of *ASS1* (Karlberg et al., 2008) (PDB ID: 2NZ2).

3 | RESULTS

3.1 | Allocation of the study subjects

Between 1 January 2011 and 30 September 2021, 753,520 newborns were screened using MS/MS by NTUH-NBSC. Within our study period, there were 3 newborns whose parents refused to receive confirmatory testing. Their initial citrulline levels were 26.4, 21.62, and 37.35 μM, respectively. One newborn, with an initial citrulline level of 31.1 μM, expired before the clinical visit. He was born extremely preterm (birth body weight 626 g), which was presumably the major cause of mortality. During this period, there have been no reported missed patients with CTLN1. A total 130 newborns received confirmatory testing due to persistently elevated citrulline, of which 4 patients with CTLN1 and 11 patients with citrin deficiency were identified (Figure 1). The incidence of CTLN1 was 1 in 188,380 (95% confidence interval: 1 in 73,258 to 1 in 484,416). The incidence of citrin deficiency in this study (1 in 68,502) was underestimated, since we did not include those identified through second-tier molecular screening. The cause

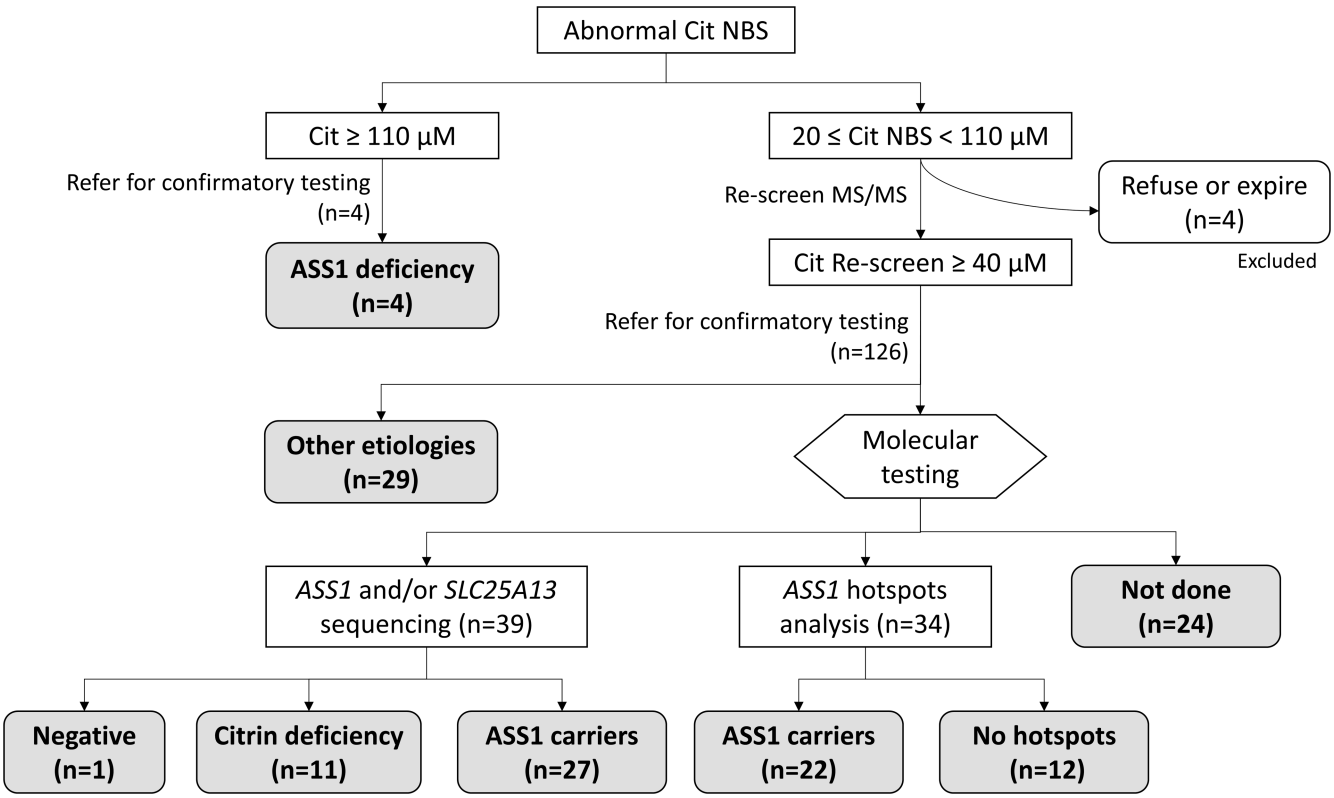

FIGURE 1 Diagnostic algorithm for citrullinemia type I found by newborn screening. Cit, Citrulline; NBS, newborn screening; NGS, next generation sequencing.

of the observed elevated citrulline was categorized as 'others' for 29 newborns, whose citrulline levels returned to normal during follow up or with an explainable cause such as liver failure, prematurity, and hyperphenylalaninemia.

For those who had mild elevated citrulline levels, without an identifiable cause, we sequenced the *ASS1* gene and the genes associated with urea cycle disorders if possible. We found 22 *ASS1* carriers and another 5 cases of double heterozygous carriers of *ASS1* and *SLC25A13* (referred as double carriers). One newborn had a negative *ASS1* gene sequencing result, and their citrulline levels later returned to normal; this individual was grouped into 'others'. We checked for 4 common *ASS1* variants in 34 samples out of the remaining 58 newborns with undetermined etiology and found 22 additional *ASS1* carriers. These included three cases with unexplainable shifted HRM curve; they were later confirmed with heterozygous c.773C>A (p.Ala258Glu) (n = 2) and heterozygous c.848delA (p.Glu283Glyfs*13) (n = 1) using Sanger sequencing. The pathogenic or likely pathogenic variants found in CTLN1 patients and *ASS1* carriers are listed in Table 1. The most common variant found in this cohort was c.1087C>T (p.Arg363Trp), accounting for 47.4% of all alleles, followed by c.773+4A>C (19.3%), c.847G>A (p.Glu283Lys), and c.836G>A (p.Arg279Gln). The variants identified in the carriers in this study are visualized on the crystal structure showing their relative location on an *ASS1* monomer (Figure 2). All *ASS1* variants found on the carriers in this cohort were located in exons 11 to 15, which encode the tetrameric interface regions.

3.2 | Citrulline levels of newborns with different *ASS1* variants

Citrulline levels were compared among carriers with the 4 variants (Figure 3). The group with the highest median citrulline levels consisted of carriers with variant c.1087C>T, especially on plasma levels upon confirmatory tests (median citrulline 85.8 μM; SD 17.9 μM; normal <40 μM). Notably, the intronic variant c.773+4A>C had the lowest median citrulline levels (median 57.9 μM; SD 25.3 μM) in the confirmatory plasma levels, although there was no statistically significant difference compared to the corresponding levels in the c.1087C>T group ($p = 0.068$). In fact, two out of the 7 cases with c.773+4A>C had normal plasma citrulline levels in the confirmatory tests. Furthermore, all carriers' plasma citrulline levels were lower than those in CTLN1 patients, all of whom had plasma citrulline levels of over 200 μM in confirmatory tests. Interestingly, in this study, patients with citrin deficiency had only slightly elevated DBS citrulline levels during the first screening exam, but these levels typically increased dramatically on follow up, with some patients exhibiting plasma citrulline levels higher than 500 μM.

TABLE 1 The frequency of *ASS1* variants on patients and carriers found by NBS

	CTLN1 (*n* = 4)	*ASS1* carrier (*n* = 49)	Genetic testing		Total	%
			by NGS	by hotspot		
c.688+2delT	1				1	1.8
c.773C>A (p.Ala258Glu)		2		2	2	3.5
c.773+4A>C	4	7	4	3	11	19.3
c.787G>A (p.Val263Met)	1				1	1.8
c.836G>A (p.Arg279Gln)		4	2	2	4	7.0
c.847G>A (p.Glu283Lys)		4	3	1	4	7.0
c.848delA (p.Glu283Glyfs*13)		1		1	1	1.8
c.880G>A (p.His294Tyr)		1	1		1	1.8
c.910C>T (p.Arg304Trp)		1	1		1	1.8
c.919C>T (p.Arg307Cys)	1				1	1.8
c.965A>G (p.Tyr322Cys)		1	1		1	1.8
c.970G>A (p.Gly324Ser)		1	1		1	1.8
c.1087C>T (p.Arg363Trp)	1	26	13	13	27	47.4
c.1128_1134delinsG (p.Ser376_Asn378delinsArg)		1	1		1	1.8
Total	8	49	27	22	57	

Note: Version: NM_054012.4 (ASS1).

Abbreviations: CTLN1: type 1 citrullinemia; NBS: newborn screening; NGS: next generation sequencing.

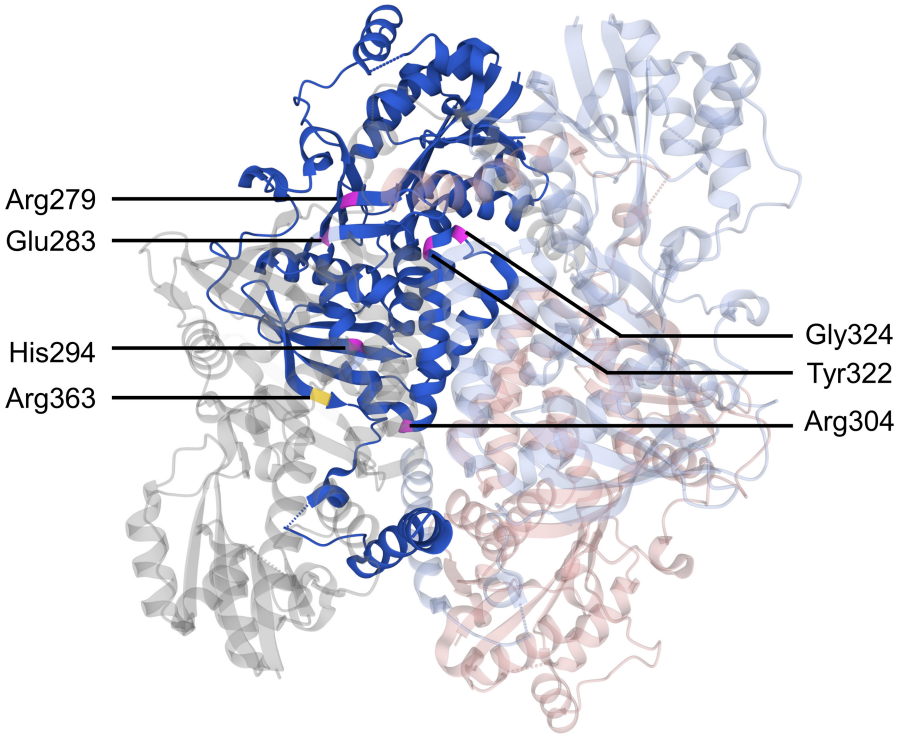

FIGURE 2 *ASS1* carrier variants visualized on the crystal structure of ASS1 tetramers. *ASS1* variants are visualized on the crystal structure of a single monomer (represented in solid blue) of the ASS1 homotetramer. The most commonly affected residue Arg363 found on carriers (yellow), and the other affected residues, Arg279, Glu283, His294, Arg304, Tyr322, and Gly324 (all pink) are all located on the monomer-monomer interface of the ASS1 tetramer. Adapted from PDB ID: 2NZ2 (Karlberg et al., 2008) created with Mol* D. (Sehnal et al., 2021), and RCSB PDB (https://www.rcsb.org/3d-view/2nz2).

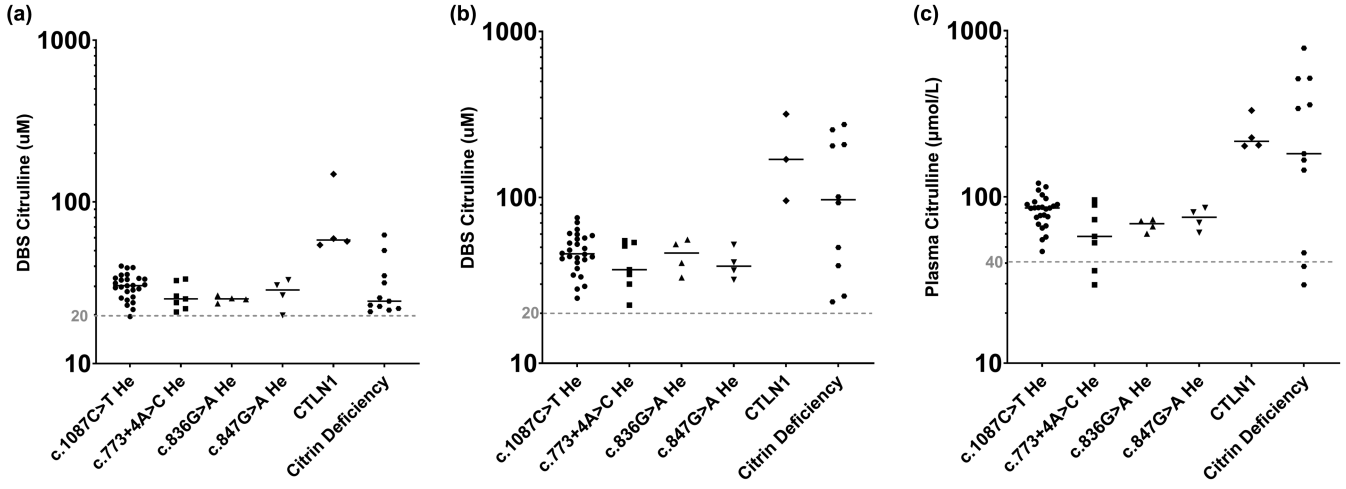

FIGURE 3 Comparison of citrulline levels between CTLN1 patients, citrin deficiency, and ASS1 carriers. Citrulline levels of newborns carrying one of the 4 ASS1 variants (c.1087C>T, c.773+4A>C, c,836G>a, c.847G>a) compared to confirmed newborns with CTLN1 and citrin deficiency. Citrulline levels were measured in newborns at first screening DBS (a), re-screen DBS (b), and confirmatory plasma samples (c). The Y axis was shown in log scale. CTLN1, citrullinemia type I.

3.3 | Parents of the carriers

To understand how citrulline levels affect carriers, citrulline levels of the carriers' parents, at least one of whom is also presumably a carrier, were checked (Table 2). Both parents' levels were checked in 14 families (including the parents of one pair of twins); only one parent was checked in four families. Elevation of citrulline were seen in 10 (71%) of the 14 completely-studied families (the highest level was 109.53 µM; normal <32.7 µM). The remaining four families exhibited citrulline levels within 10% of the upper limit. Nine out of the ten families with abnormal parental citrulline levels had only one parent showing an elevated citrulline level. Elevation of citrulline were also seen in 3 of the 4 single-parent-studied families (the highest level was 109 µM by plasma amino acid analysis; normal <47 µM). Citrulline elevation was seen in both genders. Since infants of nursing mothers may be affected by the mothers' citrulline status, we also checked the status of nursing (Table 2). The newborns of nursing mothers with elevated citrulline (Case 2, 5, and 6) also tend to have higher citrulline levels on the retest cit. However, some

TABLE 2 Citrulline levels of *ASS1* carriers and their parents

No	*ASS1* variant	NBS cit	Retest cit	ABNL parent	Father	Mother	Test	Diet
1	c.970G>A (p.Gly324Ser)	20.26	31.47	NA	—	42.2	Plasma AA	FM
2	c.1087C>T (p.Arg363Trp)	30.27	56.07	Mother	—	*86.4*	Plasma AA	BM
3	c.1087C>T (p.Arg363Trp)	33.8	40.52	Mother	—	*51.49*	MS/MS	Mixed
4	c.1087C>T (p.Arg363Trp)	23.82	43.99	Father	*109*	—	Plasma AA	Mixed
5	c.836G>A (p.Arg279Gln)	26.37	32.84	Mother	41	*83.3*	Plasma AA	BM
6	c.1087C>T (p.Arg363Trp)	31.41	46.01	Mother	34.9	*65.9*	MS/MS	BM
7	c.1087C>T (p.Arg363Trp)	39.12	49.22	Mother	29	*44.8*	MS/MS	Mixed
8	c.773+4A>C	21.87	53.47	Mother	40.3	*51.1*	Plasma AA	Mixed
9	c.773+4A>C	26.17	30	Mother	32.81	*109.53*	MS/MS	Mixed
10	c.965A>G (p.Tyr322Cys)	26.48	44.16	Mother	25.83	*36.78*	MS/MS	Mixed
11	c.773C>A (p.Ala258Glu)	29.38	41.47	Both	*39.3*	*53.1*	MS/MS	Mixed
12	c.1087C>T (p.Arg363Trp)	33.64	59.82	Father	*55.22*	23.29	MS/MS	BM
13	c.1087C>T (p.Arg363Trp)	28.5	64.06	Father	*43.52*	19.9	MS/MS	FM
14	c.910C>T (p.Arg304Trp)	25.6	43.41	Father	*37.39*	32.9	MS/MS	FM
15	c.773+4A>C	25.13	22.4	Nil	35.71	20.35	MS/MS	FM
16	c.773+4A>C	23.85	34.34	Nil	31.24	26.03	MS/MS	BM
17	c.847G>A (p.Glu283Lys)	30.56	36.34	Nil	33.91	22.46	MS/MS	BM
18	c.1087C>T (p.Arg363Trp)	32.83	53.01	Nil	30.8	42.4	Plasma AA	Mixed

Citrulline level 99.9th percentile of general adult population: MS 32.27 μM; plasma 47 μM/L. Italicized values indicate abnormal citrulline levels.

Abbreviations: ABNL, abnormal; BM, breast milk; CIT, citrulline; FM, formula milk; NA, not available; MS/MS, tandem mass spectrometry; NBS, newborn screening; Plasma AA, plasma amino acids analysis.

newborns, such as Case 12, were breast fed from a normal mother; in this case the cause for the elevation was likely genetically inherited from the father. The parent with the highest citrulline level was found to be a double carrier mother, and she gave birth to one double carrier newborn. All parents denied history of metabolic decompensation, failure to thrive, or developmental delay.

3.4 | Polymorphisms found in *ASS1* carriers

We searched all *ASS1* sequence variants in 10 newborns with persistent elevation of citrulline levels using NGS raw sequence data. We found additional likely-pathogenic variants or variants of unknown significance (VUS) in 4 (40%) out of the 10 subjects. In comparison, only 2 out of 26 (7.7%) normal controls, who had normal citrulline levels, had additional VUSs of the *ASS1* gene (χ^2 test, $p = 0.020$), and none of them had likely pathogenic or pathogenic variants.

4 | DISCUSSION

In the present study, we demonstrated that the presence of a few *ASS1* variants explained the large number of false-positive cases in newborn screening for CTLN1. Newborns and their carrier parents, seen in a previous report (Sugawara et al., 2018) and in our study, exhibited mild, but persistent, elevation of citrulline levels: about 2 to 6 times the population mean (mean 15 μM; SD 3.6 μM). Since none of those carriers, including the adults, showed any symptoms, we can confirm that it is a benign condition. DBS citrulline levels are employed as a biomarker for newborn screening of both CTLN1 and citrin deficiency. Patients with citrin deficiency may present with only slightly elevated citrulline initially, but they may develop highly elevated citrulline levels later with increased likelihood of serious illness if not properly managed. Therefore, the initial cutoff for re-screen was set at 20 μM, a relatively conservative cutoff to reduce false negatives. When we adjusted our screening algorithm in 2018 to include a second-tier molecular test for citrin deficiency, we increased the cutoff for re-screening from 20 μM to 40 μM (mean + 10 SD) to decrease the detection of false-positive patients who carry only one *ASS1* variant without increasing the false-negative results.

Citrulline itself is minimally toxic but the elevation of citrulline in *ASS1* carriers is unexpected. Most metabolic diseases are inherited as recessive disorders because enzymes are highly active biomolecules, and 50% of normal quality of an enzyme is often sufficient for adequate

biological function. Thus, we explored potential mechanisms to explain why carriers of a number of *ASS1* variants have an elevation of blood citrulline levels.

The first possibility is a dominant-negative effect. The crystal structure of human ASS forms a functional tetramer, which consists of two identical dimers. Interestingly, almost every variant appearing in our cohort was located within exon 11 to exon 15, which encode the dimeric or tetrameric interaction regions (Figure 1). For example, in our study, the most common location of the variant, p.Arg363, was on exon 14. Therefore the variant p.Arg363Tyr likely affected the oligomerization of the ASS1 protein (Diez-Fernandez et al., 2017), which affecting the other normal counter partner; this caused an elevation of blood citrulline levels in carriers. The effect of the intronic variant c.773+4 was unpredictable; carriers with c.773+4 could have normal plasma citrulline levels (Figure 3c).

The parents' sub-study indirectly supports this dominant-negative effect. Ten out of the 14 completely-studied families showed elevated parental citrulline levels; this was observed in a single parent in 9 families and in both parents in 1 family. However, due to the retrospective study design, we did not have information about the 4 CTLN1 parents, and we did not conduct DNA sequencing in parents. Therefore, the correlation between citrulline levels and adult carriers lacks solid evidence. Nevertheless, we were able to directly demonstrate a relationship between newborns with mild elevation of citrulline and the occurrence of *ASS1* variants, supporting the role of *ASS1* variants in elevated citrulline levels.

Another confounding factor is diet. Ingestion of citrulline-rich foods has been clearly correlated with elevated plasma citrulline levels (Mandel et al., 2005); therefore elevated citrulline levels may occur in adults due to their diet and not due to *ASS1* variants. Unfortunately, we did not collect information regarding diet from parents. Infants of nursing mothers with elevated blood citrulline may also have elevated citrulline levels, as demonstrated in Case 5 and Case 6 (Table 2). However, as demonstrated in Case 12, it was the father, and not the nursing mother, who was found to have elevated citrulline. Overall, diet and nursing may contribute individually, or in combination, to the phenomenon of mild elevation of citrulline in *ASS1* carriers. Further structured studies may elucidate the exact mechanism.

In addition, the presence of additional variants of the *ASS1* gene, either in-cis or in-trans, may modify the activity of the assembled ASS1 protein. We have demonstrated that additional potential disease-associating variants on *ASS1* were present in 40% of the *ASS1* carriers, who had persistently elevated citrulline levels, while only 7.7% of individuals with normal citrulline levels had VUS polymorphisms. In addition, only exons and intron-exon boundaries were sequenced. Non-coding sequence variants in trans with the known pathogenic *ASS1* variants may provide an alternative explanation for the mildly elevated citrulline levels. Copy number variant calling was not performed in our targeted NGS panel study, and therefore, these carriers were not checked for exonic deletion or duplications. Other modifier genes and epigenetic regulators could either affect the function of ASS1 or alter the final blood levels of citrulline; this aspect is beyond the scope of the current study.

In conclusion, heterozygous carriers of *ASS1* may present with elevated citrulline levels: about 2 to 6 times the population mean. While the exact pathophysiology of elevated citrulline levels in *ASS1* carriers remains unclear, all carriers and their parents in this cohort remained symptom free without treatment. Complete molecular diagnosis, including *ASS1*, *SLC25A13*, and other UCD-related genes, for the etiology of elevation of citrulline levels is recommended for precise genetic counseling and management.

AUTHOR CONTRIBUTIONS

Hui-An Chen: Methodology, Data analysis, Writing - Original Draft; **Rai-Hseng Hsu**: Data acquisition; **Kai-Ling Chang**: Data analysis; **Yi-Chen Huang**: Data analysis; **Yun-Chen Chiang**: Data acquisition; **Ni-Chung Lee**: Data interpretation, Investigation; **Wuh-Liang Hwu**: Conceptualization, Supervision; **Pao-Chin Chiu**: Data acquisition, Investigation; **Yin-Hsiu Chien**: Conceptualization, Writing - Review & Editing, Supervision. All authors have reviewed critically and approved the final manuscript.

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CONFLICT OF 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.

ETHICAL APPROVAL

Informed consent was waived, and this study was approved by the institutional review board of the National Taiwan University Hospital (NTUH-IRB; No. 202109140RIN).

DATA AVAILABILITY STATEMENT

Data available on request from the authors

ORCID

Hui-An Chen https://orcid.org/0000-0003-2103-9062
Ni-Chung Lee https://orcid.org/0000-0002-5011-7499
Yin-Hsiu Chien https://orcid.org/0000-0001-8802-5728

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