ARTICLE | Genetics inMedicine

Clinical, molecular, and biochemical delineation of asparagine synthetase deficiency in Saudi cohort

Essa Alharby, MSc[1], Eissa A. Faqeih, MD[2], Mohammed Saleh, MD[2], Seham Alameer, MD[3], Makki Almuntashri, MD[4], Annalisa Pastore, PhD[5], Manar A. Samman, PhD[6], Abdullah M. Alnawfal, BSc[6], Mais Hashem, BSc[7], Dimah Zaytuni, BSc[1], Ghadeer Alharbi, BSc[1], Mohammed Almannai, MD[2], Ali Alasmari, MD[2], Adel A. Mahmoud, MD[8], Ali H. Alwadei, MD[8], Lamya Jad, MD[8], Ali AlOtaibi, MD[8], Fahad Al-Hakami, PhD[3], Wafaa Eyaid, MD[9,10], Fowzan S. Alkuraya, MD[7,11], Majid Alfadhel, MD[9,10], Roy W. A. Peake, PhD[12] and Naif A. M. Almontashiri, PhD[1,13]

Purpose: Asparagine synthetase deficiency (ASNSD) is a rare neurometabolic disease. Patients may not demonstrate low asparagine levels, which highlights the advantage of molecular over biochemical testing in the initial work-up of ASNSD. We aimed to further delineate the ASNSD variant and phenotypic spectrum and determine the value of biochemical testing as a frontline investigation in ASNSD.

Methods: We retrospectively collected the clinical and molecular information on 13 families with ASNSD from the major metabolic clinics in Saudi Arabia.

Results: The major phenotypes included congenital microcephaly (100%), facial dysmorphism (100%), global developmental delay (100%), brain abnormalities (100%), spasticity (86%), and infantile-onset seizures (93%). Additional unreported phenotypes included umbilical hernia, osteopenia, eczema, lung hypoplasia, and hearing loss. Overall, seven homozygous variants accounted for ASNSD. The p.Tyr398Cys and p.Asn75Ile variants accounted for 54% of the cases. The clinical sensitivity and specificity of the proposed biochemical analysis of cerebrospinal fluid (CSF) for the detection of patients with ASNSD were 83% and 98%, respectively.

Conclusion: Our study describes the largest reported ASNSD cohort with clinical, molecular, and biochemical characterization. Taking into consideration the suboptimal sensitivity of biochemical screening, the delineation of the phenotype variant spectrum is of diagnostic utility for accurate diagnosis, prognosis, counseling, and carrier screening.

Genetics in Medicine (2020) 22:2071–2080; https://doi.org/10.1038/s41436-020-0919-x

Key words: asparagine synthetase deficiency; cerebral atrophy; congenital microcephaly; dysmorphism; amino acid analysis

INTRODUCTION

Asparagine synthetase deficiency (ASNSD; OMIM615574) is an ultrarare and lethal neurodegenerative autosomal recessive disease caused by homozygous or compound heterozygous variants in the asparagine synthetase (*ASNS*; MIM108370) gene. It is a neurometabolic disorder with significant clinical homogeneity characterized by severe congenital microcephaly, severe developmental delay, axial hypotonia followed by progressive hypertonia, spastic quadriplegia, intractable seizures and hyperreflexia, dysmorphic features, feeding difficulties, and cortical blindness.[1] Brain magnetic resonance image (MRI) findings include progressive cerebral atrophy, simplified gyral pattern, delayed myelination, pontine hypoplasia, thin corpus callosum, and ventriculomegaly.

Human asparagine synthetase is an enzyme of 561 residues. It belongs to the family of glutamine amidotransferases type II (GATase) asparagine synthase B type that catalyze the adenosine triphosphate (ATP)–dependent conversion of aspartate to asparagine.[2] This enzyme is a homodimer, with each monomer containing both a glutaminase domain (residues 29–160) and an asparagine synthetase domain (residues 381–547). The enzymatic conversion of aspartate

[1]Center for Genetics and Inherited Diseases, Taibah University, Almadinah Almunwarah, Saudi Arabia; [2]Section of Medical Genetics, Children's Specialist Hospital, King Fahad Medical City, Riyadh, Saudi Arabia; [3]King Abdulaziz Medical City/King Saud bin Abdulaziz University for Health Sciences, Jeddah, Saudi Arabia; [4]Department of Radiology, King Abdulaziz Medical City, King Saud bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia; [5]National Institute for Medical Research, The Ridgeway, Mill Hill, London, UK; [6]Molecular Pathology, Pathology and Clinical Laboratory Medicine Administration, King Fahad Medical City, Riyadh, Saudi Arabia; [7]Department of Genetics, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia; [8]Pediatric Neurology Department, National Neuroscience Institute, King Fahad Medical City, Riyadh, Saudi Arabia; [9]Medical Genomics Research Department, King Abdullah International Medical Research Center (KAIMRC), King Saud Bin Abdulaziz University for Health Sciences, Ministry of National Guard–Health Affairs (MNGHA), Riyadh, Saudi Arabia; [10]Division of Genetics, Department of Pediatrics, King Abdullah Specialized Children's Hospital (KASCH), King Abdulaziz Medical City, Ministry of National Guard–Health Affairs (MNGHA), Riyadh, Saudi Arabia; [11]College of Medicine, Alfaisal University, Riyadh, Saudi Arabia; [12]Department of Laboratory Medicine, Boston Children's Hospital, Boston, MA, USA; [13]Faculty of Applied Medical Sciences, Taibah University, Almadinah Almunwarah, Saudi Arabia. Correspondence: Naif A. M. Almontashiri (nmontashri@taibahu.edu.sa)
These authors contributed equally: Essa Alharby, Eissa A. Faqeih, Mohammed Saleh, Seham Alameer

Submitted 1 May 2020; revised 17 July 2020; accepted: 20 July 2020
Published online: 3 August 2020

to asparagine occurs via three separate reactions: (1) activation of aspartate by formation of an aspartyl-AMP intermediate; (2) hydrolysis of glutamine to glutamate and ammonia, and (3) breakdown of the aspartyl-AMP intermediate via nucleophilic attack by ammonia.[3]

ASNS is highly expressed in both the adult and developing embryonic brain, particularly in the cortical plate and ventricular layers of the cerebral cortex.[4] Asparagine is a nonessential amino acid, required for cell proliferation and early embryonic brain development.[5,6] Studies on cultured fibroblasts from ASNSD patients demonstrated reduced cellular proliferation when cultured in asparagine-restricted growth medium compared with fibroblasts from unaffected relatives. This would suggest that de novo synthesis of asparagine is impaired, rendering asparagine an essential amino acid in those patients.[6,7] Thus, the dependence of the brain on the de novo synthesis of asparagine, and subsequent cerebral deficiency of this amino acid, largely explains the neurological sequelae associated with this disorder. As such, demonstration of reduced asparagine levels in body fluids, and in particular, cerebrospinal fluid (CSF), has been traditionally used in the identification of patients with ASNSD. However, there are significant challenges associated with biochemical screening for ASNSD, with poor reliability and sensitivity often cited as limitations. Several patients have been reported with unremarkable plasma and/or CSF asparagine levels, limiting the use of biochemical testing as a frontline screening investigation.[4,7–10] In contrast, molecular testing offers a more definitive diagnostic screen for patients presenting with ASNSD-like phenotype, particularly in cases where biochemical data is equivocal. To date, 36 ASNSD families have been reported globally, 12 of whom were Saudi families with homozygous variants reported by our group.[9,11–13] Given the high rate of consanguinity in Saudi Arabia, it is expected that there is a significant number of additional unreported cases of ASNSD with known or novel variants and clinical features. In the absence of robust biochemical testing, it is imperative that molecular testing is used to identify these individuals. In turn, this will provide data to enable the delineation of the phenotype and direct genetic testing, variants interpretation, and clinical diagnosis of this rare disorder.

The main objectives of this study were to collect and analyze ASNSD patients' data from large genetic and metabolic centers across the country and characterize them at the clinical, biochemical, and molecular levels.

MATERIALS AND METHODS

Ethics statement

Informed consent for genetic testing and participation in research was obtained from all study participants. The study was approved by the Institutional Review Board (IRB) at Taibah University (MLT-2019-07) and King Fahad Medical City (19–512).

Human subjects

A retrospective review and collation of clinical, biochemical, and molecular data was performed across four large genetic metabolic centers in Saudi Arabia for 13 unpublished Saudi families with an established diagnosis of ASNSD. Informed consent for genetic testing and participation in research was obtained from all study participants. The study was approved by the Institutional Review Board (IRB) at Taibah University (MLT-2019-07) and King Fahad Medical City (19–512).

MRI studies

A neuroradiologist retrospectively reviewed the MRI studies for 13 patients (with the exception of patient F13) and the findings were recorded. Microcephaly was evaluated on MRI and correlated with clinical head circumference measurement. Brain atrophy was evaluated based on the imaging findings of cortical volume loss, reduced white matter bulk, thinning of the corpus callosum, and ventriculomegaly. Myelination was assessed considering the age at the time of imaging. Additionally, MRI images were evaluated for migration and organization malformations of cortical development.

Molecular testing and variants curation

Exome sequencing (ES) and Sanger confirmation for the affected cases were performed in CAP-accredited, in-house, or commercial molecular laboratories as described previously.[11,14] Sequence variants were classified and reported by board-certified geneticists following Human Genome Variation Society (HGVS) nomenclature and American College of Medical Genetics and Genomics (ACMG) guidelines.[15]

Structural modeling of missense variants

Molecular modeling of asparagine synthetase was performed using the automated homology modeling server SWISS-MODEL (http://swissmodel.expasy.org/SWISS-MODEL.html) based on the structure of the *E. coli* ortholog (PDB: 1CT9; UniProtKB: P22106) as the template.[16,17] The sequences were first aligned with the ClustalX program to optimize insertions/deletions.[18] The model was visualized and analyzed with the Molmol program.

Statistical analysis

Statistical analysis of biochemical data was performed using Prism version 7.04 (GraphPad Software, San Diego, CA). Plasma and CSF asparagine and glutamine levels were measuring by conventional amino acid methods. We further interrogated CSF data by adjusting asparagine concentration for glutamine concentration and expressing as a ratio. Median CSF asparagine concentrations were compared between ASNSD patients and pediatric controls ($n = 637$) by Mann–Whitney U test. Similarly, comparison of median values for the CSF glutamine:asparagine ratio was also performed by Mann–Whitney U test.

RESULTS

Known clinical features

Consanguinity was reported in all families. Our study included 13 ASNSD families (31 patients) with median age of 30 months at last follow-up (age range: 0.26–84 months) (Table 1). There were 15 patients with molecular confirmation, of whom 14 had detailed clinical characterization. There were 17 additional relatives with similar history but without detailed clinical characterization and molecular confirmation due to inability to collect DNA samples as a result of early deaths in 76% (13/17) of those individuals (Fig. 1).

Congenital or prenatal-onset microcephaly, severe developmental delay, and facial dysmorphism were present in all ASNSD patients for whom clinical information was available (Fig. 2a). Other features included infantile seizures 93% (13/14), progressive spasticity in 86% (12/14), abnormal EEG (for those who have undergone EEG) in 91% (10/11), hyperekplexia (including the probable hyperekplexia cases[19]) in 36% (5/14), and cortical blindness in 21% (3/14) of the patients. Brain MRI, which was performed for all patients except F13, showed brain atrophy in 100% (13/13) and delayed myelination in 77% (10/13) (Fig. 2b, c). The main cortical developmental finding was simplified gyration and sulcation, present in 38% of the patients (5/13). The cortical developmental malformation was found in only one patient, characterized by focal cortical thickening associated with simplified gyri and sulci (Fig. 2b). Therefore, the penetrance of the brain abnormalities, after excluding patient F13, was 100% (13/13) of the patients who had brain MRI. Infantile or childhood mortality was reported in 71% (22/31) (median age: 21.5 months; age range: 0.26–72 months) of all patients with or without molecular confirmations. An updated review of the clinical features of the current and published cases of ASNSD was conducted to show the total percentage contribution of the individual features to the ASNSD phenotype (Table 2 and S1). Congenital microcephaly, severe developmental delay, brain atrophy, spastic quadriplegia, seizure, and abnormal EEG were reported in most of the patients.

Additional clinical features in this study

In addition to the known features associated with ASNSD, we have described some of the following previously unreported phenotypes in patients with ASNSD (Table 1).

Umbilical hernia

Found in two patients from different families (F2 and 8) with different *ASNS* variants. Patient F2 had small reducible umbilical hernia not associated with any skin changes or signs of intestinal obstruction. Abdominal ultrasound in the second patient revealed an anterior abdominal wall defect with evidence of gas-containing bowel loop herniation (Fig. 2d).

Osteopenia

Described in two siblings (F11), with additional feature of pectus carnatum in the index case. Skeletal survey showed generalized reduction of bone density and contractures of the elbow and ankle joins, bilateral deformity of the foot, abnormal deformities and bowing of the distal left and anterior right femurs (Fig. 2e–g).

Eczema

Found in two patients from different families (F8 and 10) with different *ASNS* variants. However, the eczema was mild and distributed over the cheeks, dorsum of both hands and feet. It was treated by application of topical steroids.

High arched palate

Observed in two patients from different families (F10 and 11) with different *ASNS* variants.

Lung hypoplasia

Described in one neonate (F13) who had nonreassuring fetal heart monitoring at the time of labor prompting emergency C-section at 36 + 4 weeks of gestation. Reported abnormalities included congenital microcephaly, abnormal brain MRI, dysmorphic features, lung hypoplasia, hypotension, severe edema, and hypoalbuminemia. Despite increasing mechanical ventilation due to frequent desaturation attacks, he died on day 8 of life with respiratory failure.

Lactic acidosis

Observed in one family (F6), likely attributed to the presence of a homozygous loss of function (LOF) variant in the *CLPB* gene (NM_030813.5: c.654dupA; p.Gln219fs). A homozygous LOF variant in *CLPB* gene has been previously reported in four newborn siblings with congenital microcephaly, severe encephalopathy, 3-methylglutaconic aciduria, and lactic acidosis.[20] Thus, he had dual molecular diagnosis.

Plasma and CSF amino acids analysis

A summary of biochemical data in patients with ASNSD is provided in Table S2. Plasma asparagine (and other relevant amino acids concentrations), from this and previous studies, were collected for 21 patients with ASNSD. The results are summarized in Fig. S1a. Of 21 patients, 9 had decreased plasma asparagine concentrations compared with local population reference intervals, and a further 3 patients had low/normal values. From the data it may be inferred that the sensitivity of plasma asparagine as an indicator of ASNSD, even in fasting samples, is limited. Unsurprisingly, CSF asparagine levels represent a more reliable marker of ASNS deficiency. In the ASNS patients ($n = 8$) where CSF data were available, the majority (75%) had low or low/normal concentrations. Comparison of median CSF asparagine concentration (0.5; 95% Confidence Level (CL): 0.0–5.0) with 637 unaffected controls (7.1; 95% CL: 6.8–7.6) demonstrated statistical significance ($p < 0.0001$) at the 0.01 level. Fig. S1b shows CSF asparagine concentrations in six patients expressed as a function of the glutamine:asparagine ratio. Similarly, comparison of the median CSF glutamine:asparagine ratios in affected patients (544; 95% CL: 128–922)

Table 1 The demographic, clinical features and molecular results of the Saudi asparagine synthetase deficiency (ASNSD) families included in this study.

Patient ID	cDNA change	AA change	Gender	Age at last follow-up	Outcome (age at death)	Birth weight (kg)	Dysmorphic features	Congenital microcephaly	Developmental delay	Seizure	Spasticity	Axial hypotonia	Abnormal EEG	Abnormal brain MRI	Hyper-eklexia	Cortical blindness	Additional features	Family history (outcome)
F1	c.1193A>G	p. Tyr398Cys	Male	6 years	Deceased (6 years)	NA	+ (Receding forehead, short neck, large feet and ears)	+ (HC: 28 cm)	+ (Severe)	+	+	+	+	+	+ (Probable)	-	-	+ (1 sibling; alive at age 3 months)
F2	c.1193A>G	p. Tyr398Cys	Male	1.4 years	Deceased (1.7 years)	NA	+ (Sloped forehead, proptotic eyes)	+ (HC: 28 cm)	+ (Severe)	+	+	+	+	+	+ (Probable)	-	+ (Umbilical hernia)	+ (3 cousins; died at unknown age)
F3	c.1137 +1G>A	p.?	Male	2.6 years	Deceased (3.6 years)	2.4	+ (Sloped forehead, large ears)	+ (HC: 30 cm)	+ (Severe)	+	+	-	+	+	+	+	-	+ (1 sibling; died at age 3 months)
F4	c.1211G>A	p. Arg404His	Male	22 days	Deceased (22 days)	2.4	+ (Large hands, micrognathia)	+ (HC: 29 cm)	+ (Severe)	+	+	+	NA	+	+	-	-	+ (2 siblings; died at age 4 months)
F5	c.1193A>G	p. Tyr398Cys	Female	4 years	Deceased (4 years)	NA	+ (Sloped forehead, micrognathia)	+	+ (Severe)	+	-	+	+	+	-	-	-	+ (1 sibling; died at age 2 years)
F6	c.1193A>G	p. Tyr398Cys	Male	1.6 years	Alive	NA	+ (Receding forehead, micrognathia, large ears)	+	+ (Severe)	+	+	+	NA	+	+ (Probable)	+	+ (Persistent lactic acidosis [see the heading "Additional clinical features in this study"])	+ (1 sibling; died at age 3.5 years)
F7	c.224A>T	p.Asn75Ile	Female	2.6 years	Deceased (2.6 years)	3.5	+ (Simple ears)	+ (HC: 33 cm)	+ (Severe)	+	+	-	-	+	-	-	+ (Sensorineural hearing loss)	+ (1 sibling; died at age 3 years)
F8	c.224A>T	p.Asn75Ile	Male	3 years	Alive	NA	+	+	+ (Severe)	+	+	-	+	+	-	+	+ (Umbilical hernia, eczema)	+ (4 paternal cousins; all died between age 1 and 3 years)
F9	c.224A>T	p.Asn75Ile	Female	2.6 years	Alive	2	+ (Sloped forehead, prominent ears)	+ (HC: 28.5 cm)	+ (Severe)	+	+	-	+	+	-	-	-	-
F10	c.1424C>A	p. Thr475Asn	Male	2.2 years	Alive	NA	+ (Sloped forehead, long eye lashes)	+	+ (Severe)	+	+	+	+	+	-	-	+ (High arched palate, eczema, undescended testis)	+ (1 sibling; alive at age 5 years)
F11-1	c.1424C>A	p. Thr475Asn	Female	2.9 years	Alive	NA	+ (Frontal bossing, low hair line, prominent ears, bulbous nose, high arched palate)	+ (HC: 39 cm)	+ (Severe)	+	+	+	+	+	-	-	+ (Osteopenia, high arched palate)	+ (Sibling and cousin; alive)
F11-2	c.1424C>A	p. Thr475Asn	Female	7 years	Alive	NA	+ (Triangular face, low set, large anteriorly rotated ears, down slanting eyes, almond shaped eyes)	+ (HC: 40 cm)	+ (Severe)	+	+	+	+	+	-	-	+ (Osteopenia, pectus carinatum)	+ (Sibling and cousin; alive)
F12	c.674-1G>A	p.?	Male	3 months	Deceased (3 months)	2.3	+ (Sloping forehead, low set ears, micrognathia)	+ (HC: 45 cm)	+ (Severe)	+	+	+	+	+	-	-	-	+ (1 sibling; aborted)
F13	c.1219C>T	p.Arg407*	Female	8 days	Deceased (8 days)	2.3	+ (Micrognathia, depressed nasal bridge and low set ears)	+ (HC: 28 cm)	+ (Severe)	-	-	-	NA	NA	-	-	+ (Lung hypoplasia, scleroderma pitting edema)	? (Sibling died neonatally of unknown cause)

Probable indicates documented semiology suggesting startle baby with abnormal EEG (patient may have both seizures and hyperekplexia).[19] "+" and "−" indicate the presence or absence of that clinical feature. NA indicates data are not available or study not performed and therefore we excluded that case from the denominator when calculating the penetrance of that particular feature. See Supplemental information for additional neurological features and studies.

cDNA complementary DNA, *AA* amino acid, *EEG* electroencephalogram, *HC* head circumference, *MRI* magnetic resonance image.

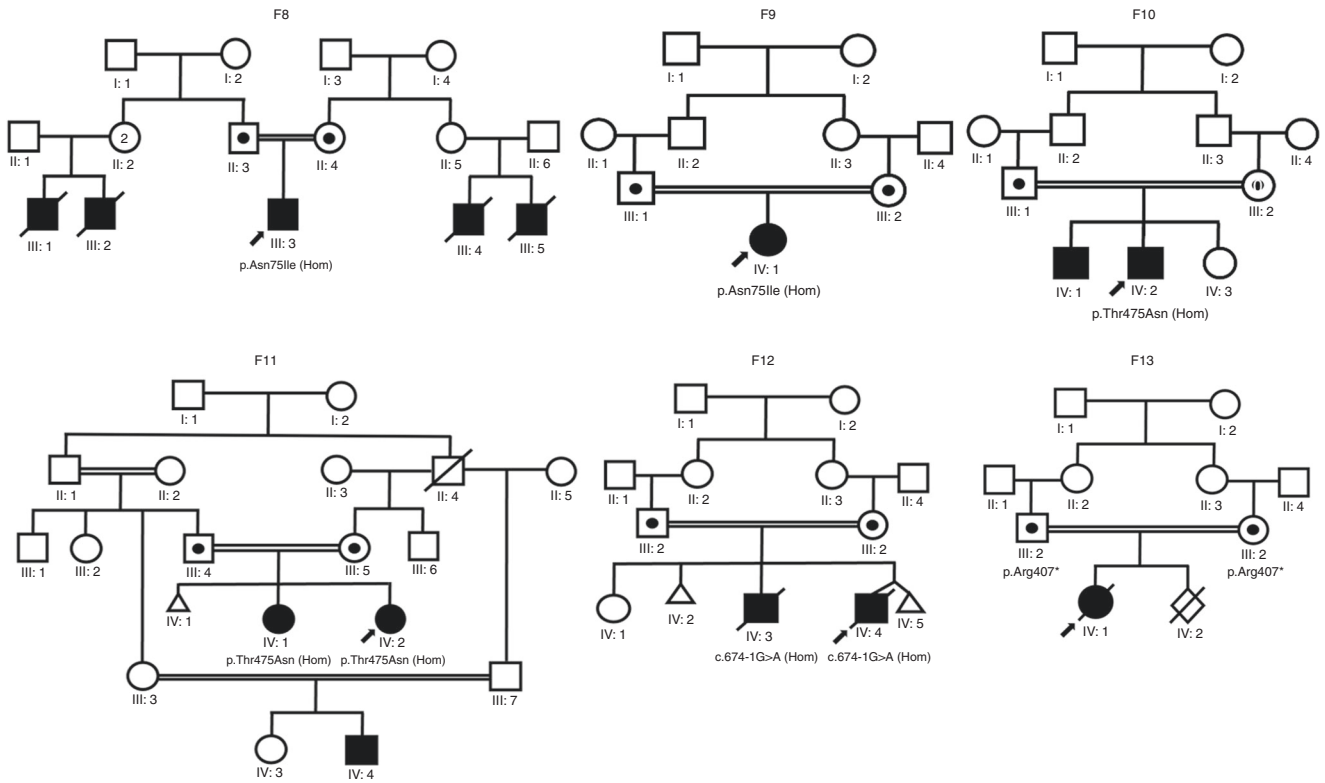

Fig. 1 Pedigrees of 13 asparagine synthetase deficiency (ASNSD) families with remarkable history and mortality of ASNSD. F13 had molecular autopsy by proxy testing of parents due to lack of available DNA from either deceased child.

versus controls (median: 71; 95% CL: 67–75) also demonstrated statistical significance ($p < 0.0001$) at the 0.01 level. These results indicate that the majority of ASNS patient values are clustered in a small region bordered by the 5th and 95th percentiles for asparagine (1.7 mcmol/L) and glutamine: asparagine ratio (477), respectively (Fig. S1b). If we used these criteria as screen positive cut-off values, this translates to a diagnostic sensitivity of 83% (95% CL: 36–99) and specificity of 98% (95% CL: 96–99) for ASNS deficiency.

Molecular testing and genotype–phenotype correlation

Most of the index cases underwent chromosomal analysis (karyotype and chromosomal microarray [CMA]) and ES performed stepwise as genetically indicated. We detected seven homozygous variants in the *ASNS* gene (Table S3). Three variants were LOF variants (two splicing and one nonsense) and four were missense variants. All of the variants were previously detected in Saudi ASNSD patients, with the exception of the p.Thr475Asn and c.674-1G>A variants, which are novel in this study (Fig. **3a**). All variants are clustered in the highly conserved asparagine synthetase domain of ASNS protein, except for the p.Asn75Ile variant, which is located in the glutamine aminotransferase type II domain (Fig. **3c**).

The p.Tyr398Cys and p.Asn75Ile variants account for ASNSD in 31% (4/13) and 23% (3/13) of the families in this study, respectively. These variants also account for 32% (8/25) and 24% (6/25) of the total Saudi families, respectively, including those reported (Fig. **3a**). Haplotype analysis of the overlapping region of homozygosity (ROH) in patients with the p.Tyr398Cys variant supported its founder nature (data not shown). The ROH data were not available for the other variants.

Molecular modeling data suggest that the variants occurred at buried or semiburied residues distant from the dimer interface (Fig. **3b** and Supplemental Information). Asp54 is a semiexposed residue close to the surface. The conserved Asn75 is positioned in the interface between the glutaminase domain and the asparagine synthetase domain, and forms an H-bond with the conserved Arg27, which is predicted to have a stabilizing effect on the protein fold. A variant changing Tyr398 to cysteine could potentially introduce a reactive thiol residue, able to cross-link to form intra- or intermolecular disulfide bonds with other cysteine residues. Arg404 is close to the AMP molecule of the active site and is predicted to have a role in the functionality of the active site itself. Trp452 is deeply buried in a narrow pocket. Finally, Thr475 is positioned in the loop where it may have a stabilizing role in the confirmation of AMP.

According to ACMG criteria, these missense variants are classified as pathogenic or likely pathogenic based on their complete segregation with this single-gene disease, conservation across species, low-allele frequency in controls, biochemical effect, and predicted impact by molecular modeling (Table **1** and Table S2–4).

The pathogenic homozygous LOF variants (p.Arg407*, c.674-1G>A and c.1137+1G>A) were detected in four

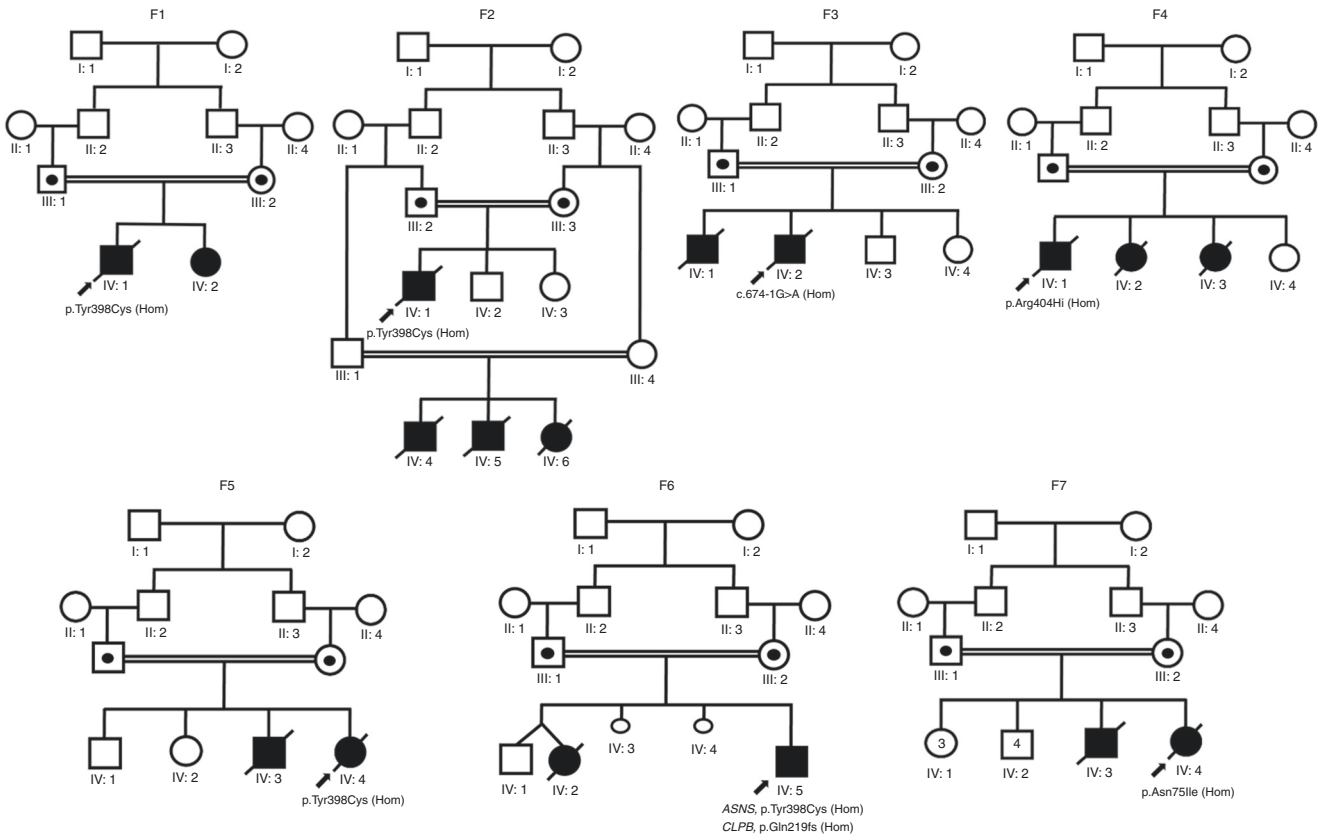

Fig. 1 Continued.

patients; three died at the age of 3 months or earlier, and the other died at the age of 3.6 years (Table 1). The p.Thr475Asn variant is likely a milder variant, detected in four patients (F10 and 11) who are still alive at ages 2.2, 5, 1.8, and 7 years, respectively. This prediction is supported by molecular modeling studies, which showed that this substitution resulted in relatively milder perturbation of the conformation of the loop.

DISCUSSION

Asparagine synthetase deficiency is reported in 36 families. Of the reported families, we contributed all of the Saudi ASNSD families (12/36). This is the largest study of this rare and neurodegenerative disease. In this study, we reported an additional 13 Saudi ASNSD families to expand the total number of ASNSD families to 49, of whom 25 are Saudi families. The p.Tyr398Cys and p.Asn75Ile variants account for ASNSD in 56% of the Saudi families. More importantly, we have contributed to expansion of the clinical phenotype, established the *ASNS* variants landscape, and determined the utility of biochemical testing as a frontline investigation in ASNSD.

ASNSD is a clinically homogeneous neurological disease.[1] In our study, congenital microcephaly, global developmental delay, facial dysmorphism, and brain abnormalities were present in all ASNSD patients for whom clinical information and imaging data were available. The majority of the patients had infantile seizures, progressive spasticity, and abnormal EEG. Recently, diaphragmatic eventration, a rare additional feature of ASNSD, was reported in siblings with ASNSD.[21] We also reported lissencephaly (four families), strabismus (two families), and bilateral microphthalmos (one family) in association with ASNSD.[13] In this study, we have observed additional clinical features of umbilical hernia, osteopenia, eczema, high arched palate, lung hypoplasia, and bilateral hearing loss. Some of these features could be incidental and not related to ASNSD. However, most of these features are present in more than one family and were associated with different *ASNS* variants. Additionally, chromosomal analysis and ES in these patients revealed no additional variants in other known or candidate genes to account for the additional phenotypes. Taken together, the additional clinical features may represent a phenotypic expansion of ASNSD. Future studies are warranted to investigate the association of the additional phenotypes with ASNSD.

A total of 35 ASNSD-causing variants have been reported (Table S4). The majority of these variants are missense variants, clustered in the asparagine synthetase domain (Fig. 3c). Of the ASNSD-causing variants, we previously reported seven homozygous variants in 12 families. In this study, in addition to other reported variants, we described two novel homozygous variants that are classified as pathogenic according to the ACMG guidelines given the available clinical, segregation, frequency, molecular, biochemical, and

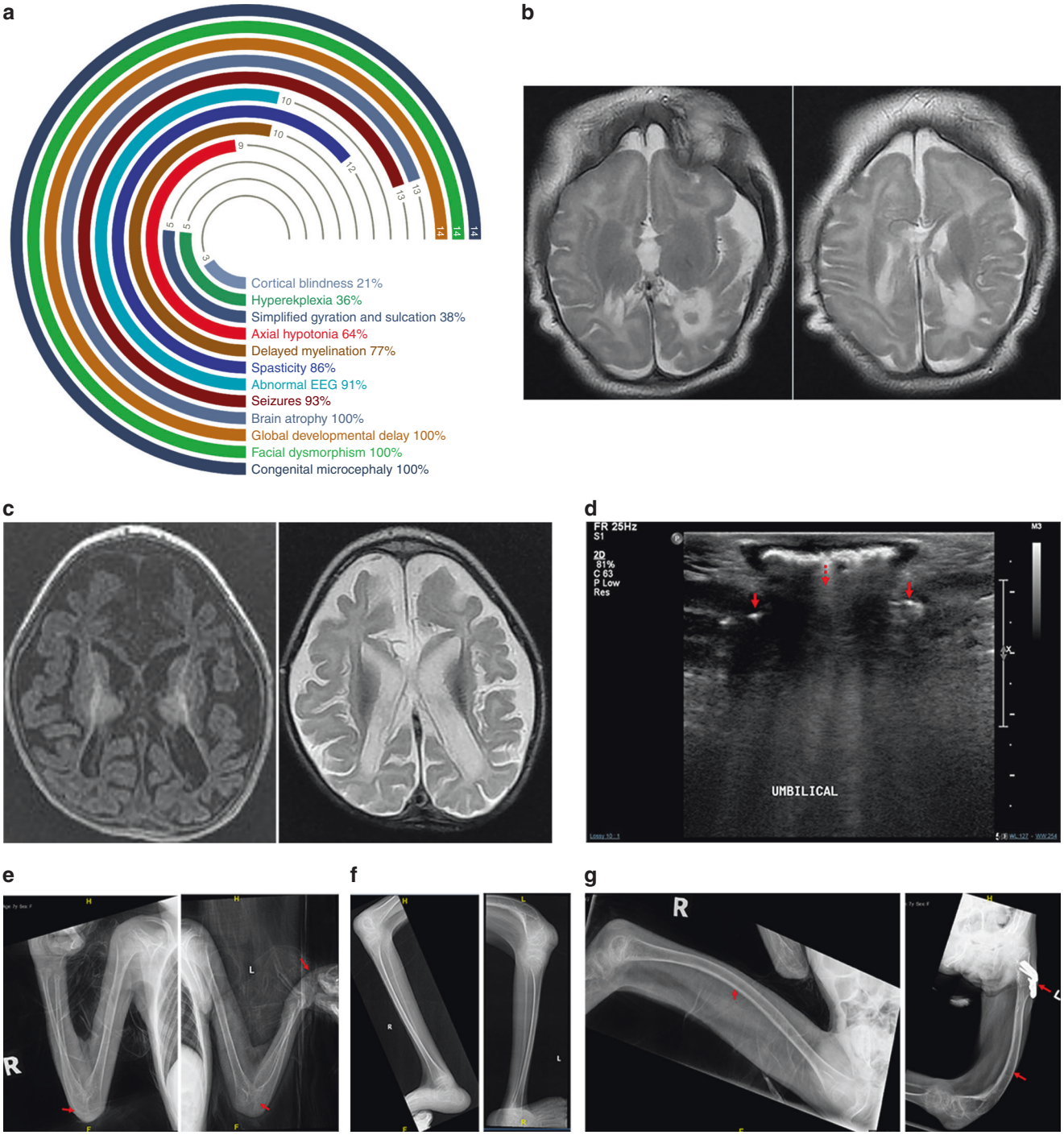

Fig. 2 Clinical features of the asparagine synthetase deficiency (ASNSD) patients. (**a**) Percentage representation of the main clinical features of ASNSD in this study. (**b**) Axial T2 magnetic resonance image (MRI) (patient F1) at the age of 7 months shows microcephaly, frontal cortical thickening, and simplified gyri and sulci. There is T2 hyperintense signal in the white matter related to delayed myelination. (**c**) Axial T1WI and T2WI (patient F2), respectively, at the age of 14 months show microcephaly, severe brain atrophy, and delayed myelination. (**d**) Ultrasound image of the umbilical region demonstrated (patient F2). Wide neck hernia with 2.5-cm defect in the mid anterior abdominal wall muscles (solid arrows). Small bowel loop herniated in the hernia sac with no complications (dotted arrow). Skeletal survey (patient F11-2) of the (**e**) upper extremities showed generalized osteopenia, fixed contracture, deformity of the elbow and wrist joints with dislocation of the left distal radioulnar joint; (**f**) legs showed generalized osteopenia, long bone thinning with contractures and deformities of the knee and ankle joints; and (**g**) femurs showed anterior bowing of the right femur. There are a plate and screws transfixing the proximal left femur associated with abnormal deformity and bowing of the distal femur. Periosteal reaction is noted at the posterior cortex of distal femur that could be related to healing fracture.

Table 2 Summary of the clinical features of all reported asparagine synthetase deficiency (ASNSD) patients, including those reported in this study.

Number of patients	63
Number of families	49
Sex (male:female)	34:28
Consanguinity	34/48 (71%)
Neonatal onset	60/63 (95%)
Severe developmental delay	61/63 (97%)
Congenital and progressive microcephaly	61/63 (97%)
Abnormal EEG	43/60 (72%)
Spastic quadriplegia	41/63 (65%)
Axial hypotonia	34/63 (54%)
Seizure	51/63 (81%)
Cortical blindness	16/63 (25%)
Hyperekplexia	17/63 (27%)
Brain atrophy	51/57 (89%)

prediction data. Of the nine Saudi *ASNS* variants, eight variants have never been reported in families from outside the Arabian Peninsula. Consistently, the p.Tyr398Cys variant, which accounts for 32% of ASNSD in the Saudi population, has been previously reported in an Emirati family.[8] Indeed, haplotype analysis supported the founder nature of the p.Tyr398Cys variant. It is noteworthy that point variants, small deletions, and duplications account for 97% of all ASNSD cases (Fig. **3c** and Table S4). Therefore, gene sequencing–based assays are recommended to investigate cases with ASNSD-like phenotype.

In inborn errors of metabolism, biallelic LOF variants resulting in absent or truncated enzymes are usually associated with more severe clinical phenotype and poorer outcome compared with the missense variants. There is insufficient genotype–phenotype correlation in ASNSD, especially in nonconsanguineous families where biallelic LOF variants are never reported.[1] However, we previously reported the homozygous LOF p.Arg407* variant in an ASNSD patient who died at 6 weeks of age, and had family history of four maternal aunts who died at ages 4, 5, 3, and 6 weeks, respectively.[9] Another homozygous LOF variant, p.Lys66fs, was reported in a Yemeni patient who died at age 3 months from respiratory distress (ACMG poster, https://epostersonline.com/acmg2016/node/1304). In our study, we reported three LOF variants, one of which is p.Arg407*, in three patients: two died at ages 3 months, and the patient with the p.Arg407* variant died of respiratory failure at age 8 days. In contrast, the p.Thr475Asn variant is present in four patients who are still alive at ages 2.2, 5, 1.8, and 7 years, respectively. Therefore, the neonatal or prenatal lethality due to biallelic LOF variants in *ASNS* might be underdiagnosed and might explain the underrepresentation of the LOF variants compared with the missense variants in ASNSD patients (Fig. **3c**).

Supplementation of the deficient amino acid has proven effective in amino acid synthesis deficiencies (e.g., serine biosynthesis disorders and glutamine synthetase deficiency).[21–23] However, our attempts to correct low cerebral asparagine levels through asparagine supplementation in two siblings with the p.Tyr398Cys variant resulted in increasing frequency and duration of seizures.[12] In contrast, a recent study described asparagine supplementation in two siblings with ASNSD and low CSF asparagine levels. The results showed that supplementation was well tolerated, and halted disease progression in both patients. One of the patients also demonstrated improvements in cognition and nonverbal communication.[10] However, one of the improved patients suffered from congenital microcephaly, which means that microcephaly has started in utero and is irreversible if treatment is initiated postnatally. Therefore, with the small sample size of only two patients and our previous study that does not support a beneficial but rather an adverse outcome of asparagine supplementation, it is very difficult at this stage to conclude that asparagine supplementation is beneficial for this disease. Functional animal studies and clinical trials of sufficiently large sample size are warranted to evaluate the efficacy of asparagine supplementation.

Our study also demonstrated the limitations associated with biochemical screening for ASNSD. The limitations of plasma asparagine analysis are well recognized, particularly the difficulties associated with obtaining fasting samples. Although CSF asparagine analysis is more useful, a significant number of ASNSD patients present with asparagine levels within the reference intervals.[4,9,10] There may be more utility in expressing CSF asparagine as a ratio with other amino acids, such as glutamine. Using this approach, we determined the sensitivity of CSF analysis to be approximately 83%, which is not ideal for a screening marker. Additionally, the invasiveness of obtaining spinal fluid is another important factor to be considered. Rather, direct assessment of ASNS enzyme activity as a biochemical indicator of deficiency may have a role in both screening and response to treatment in patients with suspected or reestablished disease. In contrast to biochemical screening, molecular analysis provides a definitive, noninvasive, cost-effective alternative as a frontline investigation.

Further studies are warranted to uncover the mechanism by which the variants in *ASNS* gene fully explain the phenotype associated with ASNSD. Prenatal-onset microcephaly is well established in ASNSD; therefore asparagine supplementation in families with history of ASNSD warrants further experimental investigations as the case for serine deficiency to rescue or mitigate the congenital central nervous system (CNS) and other extraneurological features of the disease.[4,9,24] Gene therapy–based approaches can be considered as alternative means of treatment to asparagine supplementation for those with milder variants, such as the p.Thr475Asn variant, to account for and rescue the known and possibly unknown functions of the ASNS enzyme.

A limitation of this study is that it is a retrospective observational study; therefore, the selection and information biases are high. Another limitation is the lack of detailed

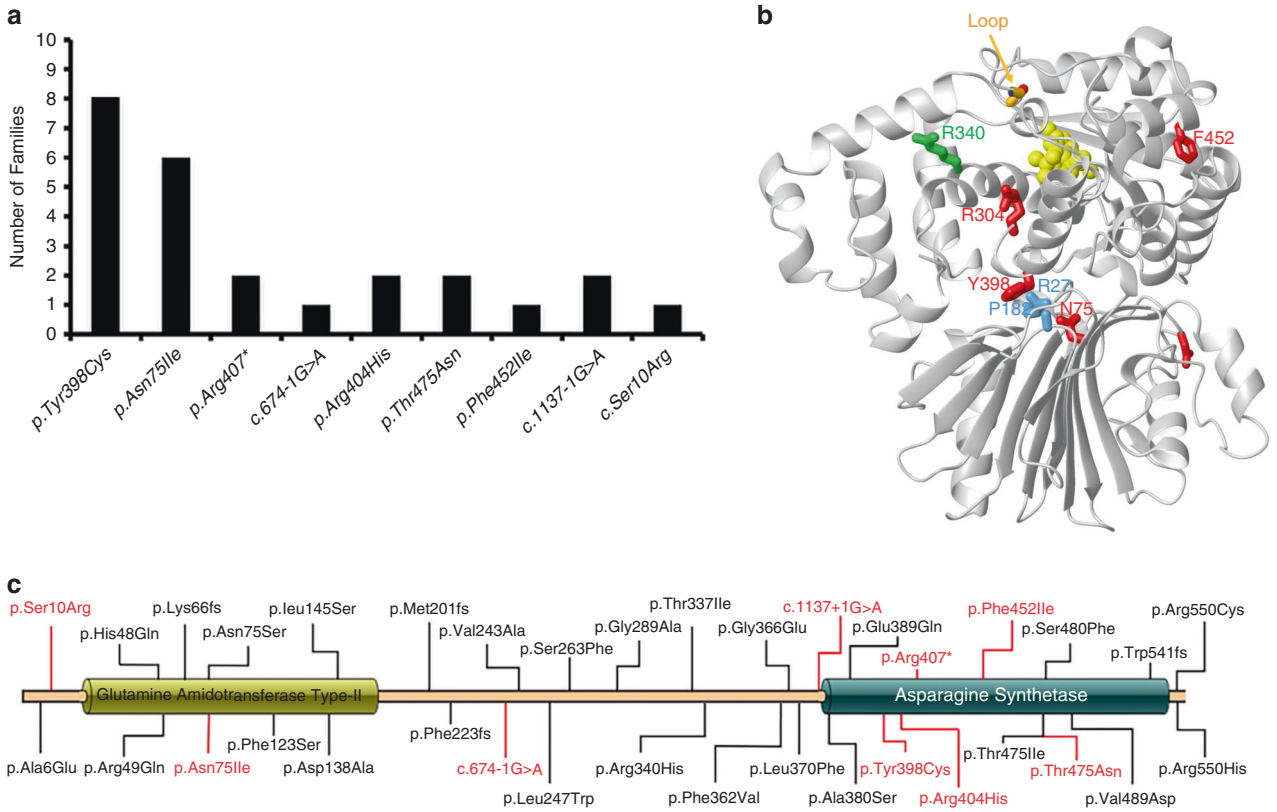

Fig. 3 Variants landscape and predicted structural impact. (**a**) Variants detected to date in Saudi asparagine synthetase deficiency (ASNSD) families. (**b**) Mapping the clinically important variants on the surface of asparagine synthetase. The side chains of the *ASNS* variants p.Asn75Ile, p.Tyr398Cys, p.Arg404His, and p.Phe452Ile are depicted in red; those of the residues that form interactions with these residues are shown in light blue. The side chain of Arg340 previously reported for a variant to an alanine is shown in green. The position of the loop, which should host Thr475, is shown in orange. AMP is shown in green in full atoms representation. (**c**) Schematic diagram of the functional domains of ASNS protein (the longest isoform, 561 AAs) along with the corresponding position of all reported variants. The red-colored variants represent the Saudi-specific variants.

clinical features for 16 of the relatives. This is mainly because of the high mortality and early infantile death rate among our ASNSD patients (71% [22/31]). In many cases, the family history of similar disease and early deaths triggered the first visit for the index cases to the clinic. An additional limitation is the lack of biochemical data for most of the patients in this study due to the early deaths before the genetic results or the rejection of the CSF sampling by the families. However, to mitigate this issue, we analyzed the results after we combined our data with the published biochemical data for the ASNSD patients.

In conclusion, this is the largest ASNSD study to date, which expands the number of published ASNSD patients, clinical features, biochemical utility, and variant spectrum associated with ASNSD. The findings presented in this study have several clinical values. First, knowing the full spectrum of the disease, including the rare clinical features, helps to narrow the differential diagnosis and to follow an efficient pathway for the diagnosis of patients with ASNSD presentations. Second, it provides the *ASNS* variant landscape for particular populations such as the Saudi population, with the supporting clinical, genetic, and functional data for accurate variant classification. This is critical to shorten the diagnostic odyssey and reach accurate and timely diagnosis, and to provide the proper genetic counseling for reproductive options to avoid the recurrence of this lethal genetic disease. For the Saudi population, these findings should be of great value to the comprehensive premarital screening program to be launched in the country in the near future.

SUPPLEMENTARY INFORMATION

The online version of this article (https://doi.org/10.1038/s41436-020-0919-x) contains supplementary material, which is available to authorized users.

ACKNOWLEDGEMENTS

We thank the families for their participation in this study. The authors extend their appreciation to the Deputyship for Research and Innovation, Ministry of Education in Saudi Arabia for funding this research work (project number 821).

DISCLOSURE

The authors declare no conflicts of interest.

REFERENCES

1. Alfadhel M, El-Hattab AW. Asparagine synthetase deficiency. In: Adam MP, Ardinger HH, Pagon RA, et al., editors. GeneReviews. Seattle, WA: University of Washington; 1993.
2. Lomelino CL, Andring JT, McKenna R, Kilberg MS. Asparagine synthetase: function, structure, and role in disease. J Biol Chem. 2017; 292:19952–19958.
3. Zhang YP, Lambert MA, Cairney AE, Wills D, Ray PN, Andrulis IL. Molecular structure of the human asparagine synthetase gene. Genomics. 1989;4:259–265.
4. Ruzzo EK, Capo-Chichi JM, Ben-Zeev B, et al. Deficiency of asparagine synthetase causes congenital microcephaly and a progressive form of encephalopathy. Neuron. 2013;80:429–441.
5. Gutierrez JA, Pan YX, Koroniak L, Hiratake J, Kilberg MS, Richards NG. An inhibitor of human asparagine synthetase suppresses proliferation of an L-asparaginase-resistant leukemia cell line. Chem Biol. 2006;13: 1339–1347.
6. Palmer EE, Hayner J, Sachdev R, et al. Asparagine synthetase deficiency causes reduced proliferation of cells under conditions of limited asparagine. Mol Genet Metab. 2015;116:178–186.
7. Sacharow SJ, Dudenhausen EE, Lomelino CL, et al. Characterization of a novel variant in siblings with asparagine synthetase deficiency. Mol Genet Metab. 2018;123:317–325.
8. Ben-Salem S, Gleeson JG, Al-Shamsi AM, et al. Asparagine synthetase deficiency detected by whole exome sequencing causes congenital microcephaly, epileptic encephalopathy and psychomotor delay. Metab Brain Dis. 2015;30:687–694.
9. Seidahmed MZ, Salih MA, Abdulbasit OB, et al. Hyperekplexia, microcephaly and simplified gyral pattern caused by novel ASNS mutations, case report. BMC Neurol. 2016;16:105.
10. Sprute R, Ardicli D, Oguz KK, et al. Clinical outcomes of two patients with a novel pathogenic variant in ASNS: response to asparagine supplementation and review of the literature. Hum Genome Var. 2019;6:24.
11. Alfadhel M, Alrifai MT, Trujillano D, et al. Asparagine synthetase deficiency: new inborn errors of metabolism. JIMD Rep. 2015;22:11–16.
12. Alrifai MT, Alfadhel M. Worsening of seizures after asparagine supplementation in a child with asparagine synthetase deficiency. Pediatr Neurol. 2016;58:98–100.
13. Shaheen R, Maddirevula S, Ewida N, et al. Genomic and phenotypic delineation of congenital microcephaly. Genet Med. 2019;21:545–552.
14. Monies D, Abouelhoda M, AlSayed M, et al. The landscape of genetic diseases in Saudi Arabia based on the first 1000 diagnostic panels and exomes. Hum Genet. 2017;136:921–939.
15. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17:405–424.
16. Almontashiri NA, Chen HH, Mailloux RJ, et al. SPG7 variant escapes phosphorylation-regulated processing by AFG3L2, elevates mitochondrial ROS, and is associated with multiple clinical phenotypes. Cell Rep. 2014;7:834–847.
17. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res. 2003;31: 3381–3385.
18. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 1997;25:4876–4882.
19. Bhidayasiri R, Truong DD. Startle syndromes. Handb Clin Neurol. 2011; 100:421–430.
20. Capo-Chichi JM, Boissel S, Brustein E, et al. Disruption of CLPB is associated with congenital microcephaly, severe encephalopathy and 3-methylglutaconic aciduria. J Med Genet. 2015;52:303–311.
21. Sun J, McGillivray AJ, Pinner J, et al. Diaphragmatic eventration in sisters with asparagine synthetase deficiency: a novel homozygous ASNS mutation and expanded phenotype. JIMD Rep. 2017;34:1–9.
22. de Koning TJ, Klomp LW, van Oppen AC, et al. Prenatal and early postnatal treatment in 3-phosphoglycerate-dehydrogenase deficiency. Lancet. 2004;364:2221–2222.
23. Haberle J, Shahbeck N, Ibrahim K, et al. Glutamine supplementation in a child with inherited GS deficiency improves the clinical status and partially corrects the peripheral and central amino acid imbalance. Orphanet J Rare Dis. 2012;7:48.
24. Yamamoto T, Endo W, Ohnishi H, et al. The first report of Japanese patients with asparagine synthetase deficiency. Brain Dev. 2017;39: 236–242.