Novel Mutations in the Asparagine Synthetase Gene (ASNS) Associated With Microcephaly

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Microcephaly is a devastating condition defined by a small head and small brain compared to the age- and sex-matched population. Mutations in a number of different genes causative for microcephaly have been identified, e.g., MCPH1, WDR62, and ASPM. Recently, mutations in the gene encoding the enzyme asparagine synthetase (ASNS) were associated to microcephaly and so far 24 different mutations in ASNS causing microcephaly have been described. In a family with two affected girls, we identified novel compound heterozygous variants in ASNS (c.1165G > C, p.E389Q and c.601delA, p.M201Wfs*28). The first mutation (E389Q) is a missense mutation resulting in the replacement of a glutamate residue evolutionary conserved from Escherichia coli to Homo sapiens by glutamine. Protein modeling based on the known crystal structure of ASNS of E. coli predicted a destabilization of the protein by E389Q. The second mutation (p.M201Wfs*28) results in a premature stop codon after amino acid 227, thereby truncating more than half of the protein. The novel variants expand the growing list of microcephaly causing mutations in ASNS.

Keywords: microcephaly, asparagine synthetase, mutation, compound heterozygous, exome sequencing, genetic variants

BACKGROUND

Microcephaly is a devastating condition defined by a small head compared to the age- and sex-matched population, often defined as a head circumference more than three standard deviations below the age- and sex-matched means. In most cases, microcephaly ("small head") is associated with microencephaly ("small brain"); Gilmore and Walsh, 2013). Children with microcephaly often have impaired cognitive development, facial distortions, hyperactivity, seizures, and other brain and neurological impairments. While some patients only show rather mild disabilities, other patients are severely affected and require lifelong intensive care. Both, genetic as well as acquired causes have been described for congenital microcephaly and several genes have been identified causing autosomal recessive primary microcephaly (Faheem et al., 2015). These genes mostly affect...
mitosis of neural progenitors, resulting in reduced numbers of neurons. Furthermore, mutations in other genes have been associated with microcephaly, which affect cellular processes other than neuronal development (Morris-Rosendahl and Kaindl, 2015).

Originally in 2013, three causative mutations in the gene coding for asparagine synthetase (ASNS) have been identified in patients suffering from microcephaly in four families (Ruzzo et al., 2013; ASNS deficiency, ASNSD; OMIM #615574). Since then several mutations were reported in patients with microcephaly in scientific publications (Ruzzo et al., 2013; Alfadhel et al., 2015; Ben-Salem et al., 2015; Palmer et al., 2015; Gataullina et al., 2016; Seidahmed et al., 2016; Gupta et al., 2017; Sacharow et al., 2017; Sun et al., 2017; Yamamoto et al., 2017; Abhyankar et al., 2018; Galada et al., 2018; for an overview see Gupta et al., 2017; Lomelino et al., 2017 and Table 1) and three others have been reported in poster form or in the internet (Table 1) from almost all around the world.

Asparagine synthetase is a metabolic enzyme (EC 6.3.5.4) catalyzing the reaction L-aspartate + L-glutamine + ATP + H₂O → L-asparagine + L-glutamate + AMP + PPI (Horowitz and Meister, 1972). ASNS is expressed almost ubiquitously with higher expression levels in brain but very low levels in liver (Ruzzo et al., 2013; Lomelino et al., 2017). Consequently, it has been proposed that insufficient supply of asparagine within the brain underlies brain malformation and malfunction in ASNSD (Ruzzo et al., 2013). As ASNS metabolically connects the four amino acids L-aspartate, L-asparagine, L-glutamate, and L-glutamine also a dysregulation of the balance of these amino acids in the brain might contribute to the pathophysiology of ASNSD (Lomelino et al., 2017; Sacharow et al., 2017). ASNS has been intensively studied in cancer research as tumors capable to synthesize asparagine de novo via ASNS may display resistance to the treatment by asparaginase (Horowitz et al., 1968; Aslanian et al., 2001; Richards and Kilberg, 2006), and ASNS expression in human solid tumors was correlated with survival prognosis (Zhang et al., 2013; Panosyan et al., 2016). Furthermore, glutamine-dependent asparagine synthesis via ASNS is indispensable for endothelial cell growth which is important for angiogenesis supporting invasive tumor growth and metastasis (Folkman, 2002; Huang et al., 2017). Consistently, in fibroblasts derived from patients with ASNSD proliferation was markedly reduced under conditions of asparagine deprivation (Palmer et al., 2015; Sacharow et al., 2017). However, while the association of ASNS mutations and microcephaly is now well-established, the pathophysiology of these mutations is still unclear.

We here report two novel mutations in ASNS in a German family with two girls suffering from microcephaly, which are inherited in a compound heterozygous manner in the family.

**COMPLIANCE WITH ETHICAL STANDARDS**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed written consent was obtained from all individual participants or their legal representatives (parents) included in the study. The study was approved by the ethical committee of the University of Halle.

**CASE PRESENTATION**

The two affected sisters were born to healthy non-consanguineous German parents with unremarkable family history. Patient 1 was a full-term born female baby with a weight of 3,320 g (50th percentile) and a height of 51 cm (10th percentile). The head circumference at birth measured 29.5 cm, corresponding to a circumference below the 3rd percentile in female neonates. Of note, a microcephaly was already observed in routine ultrasonography during pregnancy. Until the age of 2 years, the head circumference was determined in short time intervals demonstrating an increase in size that parallels the standard growth curves with respective to the gradient angle in the first 6 months, but flattens markedly out at 1 and 2 years of age. At 2 years of age, the head circumference measured 37.5 cm (<3rd percentile), reflecting a progressive severe microcephaly, which was confirmed by MRI imaging (Figure 1A). Clinical symptoms in patient 1 became apparent immediately after birth with tonic-clonic seizures, relapsing vomiting, and feeding difficulties. In addition, a muscle hypertonus and a tetraspastic movement dysfunction was noted. During further infantile development, the girl did not achieve any major developmental milestones and never attained the ability to sit, stand, or walk. Throughout life, no voluntary movements of limbs or any parts of the body have been observed. A severe mental retardation was diagnosed from infantile development on with severe deficits in perception and an inability to establish any form of targeted contact with other individuals. In line with the majority of patients with ASNSD, a cortical blindness was diagnosed. Though, the hearing ability is not significantly impaired and acoustic stimulation is used as therapeutic measures of treatment. In addition, a cutis verticis gyrate was diagnosed around the age of 10 years.

Due to relapsing incidents of aspiration, a fundoplication and the insertion of a percutaneous endoscopic gastrostoma (PEG) were undertaken at the age of 4 years. She received an intrathecal pain-pump implantation at the age of 9 years in order to provide a constant supply with the muscle-relaxant drug baclofen. At the age of 16 years the girl demonstrated a profound psychomotor retardation with an apathetic behavior and a severe deficit in perception and no signs of improvement in comparison to the initial clinical picture during infancy. The patient showed a height of 135 cm (<3rd percentile), a weight of 49 kg (10th percentile), and a head circumference of 45 cm (<3rd percentile) at that age. She suffers from hebdomadal tonic-clonic epileptic seizures and frequent painful spasms of the upper extremities together with events of opisthotonus.
| No. | Position in coding sequence NM_133436.3 | Position in protein sequence NP_597680.2 | Published by (+ comments) | dbSNP entry for the base position in coding sequence NM_133436.3 [ClinVar/clinical significance] |
|-----|--------------------------------------|--------------------------------------|--------------------------|-----------------------------------------------|
| 1   | c.17C > A | p.A6E | Ruzzo et al. (2013) Neuron 80, 429–441 | rs398122975 C > A (p.A6E) [pathogenic/likely pathogenic] and C > T (p.A6V) |
| 2   | c.146G > A | p.R49Q | Sacharow et al. (2017) Molecular Genetics and Metabolism 123, 317–325 | rs769236847 G > A (p.R49Q) [likely pathogenic] |
| 3   | c.198_202delATATC | p.K68Nfs*10 | Reed (2016) Poster at Annual Clinical Genetics Meeting | |
| 4   | c.224A > G | p.N75S | Galada et al. (2018) Congenital Anomalies (Kyoto) | rs747624770 C > A (p.R49Q) [likely pathogenic] |
| 5   | c.434T > C | p.L145S | Yamamoto et al. (2017) Brain & Development 39, 236–242 | |
| 6   | c.601delA | p.M201Wfs*28 | This publication | |
| 7   | c.728T > C | p.V243A | Abhyankar et al. (2018) Clinical Case Reports 6, 200–205 | rs148111963 T > C (p.V243A) [likely pathogenic] |
| 8   | c.740T > G | p.L247W | Yamamoto et al. (2017) Brain & Development 39, 236–242 | |
| 9   | c.866G > C | p.G289A | Palmer et al. (2015) Molecular Genetics and Metabolism 116, 178–186 | rs369633015 G > A (p.G289D) |
| 10  | c.1010C > T | p.T337I | Palmer et al. (2015) Molecular Genetics and Metabolism 116, 178–186 | |
| 11  | c.1019G > A | p.R340H | Sun et al. (2017) JIMD Reports 34, 1–9 | |
| 12  | c.1084T > G | p.F362V | Ruzzo et al. (2013) Neuron 80, 429–441 | rs398122973 T > G (p.F362V) [pathogenic] |
| 13  | c.1097G > A | p.G366E | Abhyankar et al. (2018) Clinical Case Reports 6, 200–205 | |
| 14  | c.1138G > T | p.A380S | Gupta et al. (2017) Metabolic Brain Disease 32, 1899–1900 | rs758183057 G > C (p.A380P) |
| 15  | c.1165G > C | p.E389Q | This publication | rs948326794 G > A (p.E389*); Variant reported as c.970C > T/p.R324*; c.1219C > T/p.R407*; as reference sequences are mixed (NM_001178076.1; NM_133436.3) |
| 16  | c.1193A > G | p.Y398C | Alfadheli et al. (2015) JIMD Reports 22, 11–16 | rs773348232 —T (p.Y398Lfs*18) [likely pathogenic] |
| 17  | c.1193A > G | p.Y398C | Alfadheli et al. (2015) JIMD Reports 22, 11–16 | rs773348232 —T (p.Y398Lfs*18) [likely pathogenic] |
| 18  | c.1211G > A | p.R404H | Galada et al. (2018) Congenital Anomalies (Kyoto) | rs774808316 G > A (p.R404H) |
| 19  | c.1219C > T | p.R407* | Seidahmed et al. (2016) BMC Neurology 16, 105 | rs1140424 C > T (p.R407*) |
| 20# | c.1279-1281[TCC] | p.S427P | http://thejoyofjules.blogspot.de/2016/12/genetics-results-asparagine-synthetase.html | 3 SNPs, one for every position in the codon: c.1279 rs105751841 T > C (p.S427P) [likely the mutation] [likely pathogenic] |

(Continued)
that last for a few minutes. The electro-encephalogram (EEG) demonstrates a severe, diffuse brain dysfunction in addition to the characteristic pattern of tonic-clonic epileptic seizures. At present, the medication comprises sulthiame, tizanidine, phenobarbital, and valproic acid. In addition, the girl receives constant physiotherapy and attends a social organization for blind, handicapped children during daytime.

The second patient is the sibling of patient 1 and was born 3 years after her sister (for a comparison of both patients see also Figure 1B). Patient 2 has a healthy twin brother. Both children were born in the 38th week of gestation via cesarean section after uncontrollable labor. The newborn girl presented with a birth weight of 2,450 g (<3rd percentile), a height of 45 cm (<3rd percentile), and a head circumference of 30 cm (<3rd percentile). Microcephaly had been diagnosed already during pregnancy via ultrasonography. Comparable to her sister, the relative increase in head circumference of patient 2 paralleled the standard curve during the first 6 months of age, but then flattened out with a head circumference of 37 cm (<3rd percentile) at the age of 2 years, reflecting a severe, progressive microcephaly. Overall, the clinical symptoms of patients 1 and 2 are nearly identical. In detail, patient 2 developed the first neurological symptoms at the age of 4 weeks with epileptic seizures, muscle hypertonus, and a progressive tetraspastic motor dysfunction. Like her sister, she suffers from a cortical blindness. A cutis verticis gyrate was also diagnosed during childhood. Due to gastroesophageal reflux and feeding problems the girl received a fundoplication and the insertion of a PEG at the age of 1 year. Again, no developmental milestones with regard to motor development were achieved, and the patient never developed the ability to carry out voluntary movements or axial control. She showed a severe psychomotor retardation and pronounced deficits in perception from early infancy on, which did not significantly improve during further development and parallels the clinical picture of her older sister. The girl is 124 cm tall (<3rd percentile), has a body weight of 43 kg (25th percentile), and a head circumference of 43 cm (<3rd percentile). The EEG demonstrates a severe diffuse brain dysfunction together with an overall slowing and flattening of the amplitudes. The girl suffers from relapsing tonic-clonic seizures in addition to a severe tetraspasticity. The medication comprises baclofen, sulthiame, gabapentin, and levetiracetam next to physical therapy. Like her sister, she is attending a social organization for blind, handicapped children. At time of submission, patient 1 was 19 years old and her sister, patient 2, 16 years of age. Both patients show a stable, overall unaltered health situation regarding the specific disease syndrome as well as with respect to the general health condition within the last years. Both patients live in their family at home.

**LABORATORY INVESTIGATIONS**

**Reference Sequence**

As some discrepancies and inconsistencies exist about in the denomination of mutations in ASNS partly due to the use of different reference sequences (see results and Table 1), all positions in the ASNS gene are annotated to RefSeq NM_133436.3, Ensembl ENST00000394309.3, GRCh37 in this publication. Amino acid positions were assigned using NCBI NP_597680.2 and UniProtKB P082431 as reference. Whole exome sequence data were mapped and aligned to Human Genome Build GRCh37/hg19.

### TABLE 1 | Continued

| No. | Position in coding sequence NM_133436.3 | Position in protein sequence NP_597680.2 | Published by (→ comments) | dbSNP entry for the base position in coding sequence NM_133436.3 [ClinVar:clinical significance] |
|-----|--------------------------------------|--------------------------------------|----------------------------|------------------------------------------------------------------------------------------------|
| 21  | c.1439C > T                           | p.S480F                              | Gataulina et al. (2016) Neuropediatrics 47, 399–403 | rs75403007 C > T (p.S480F) [likely pathogenic] |
| 22  | c.1466T > A                           | p.V489D                              | Yamamoto et al. (2017) Brain & Development 39, 236–242 | |
| 23# | c.1555-1557[CGT]                      | p.R519H                              | http://thejoyofjules.blogspot.de/2016/12/genetics-results-asparagine-synthetase.html | 2 SNPs, first and second position of the codon: c.1555 COSMIC database C0091093405 c.1555 C > T (p.R519C) |
|     |                                      |                                      |                           | c.1556 rs56870377 G > A (p.R519H) (likely the mutation) [likely pathogenic] |
| 24  | c.1623_1624delGA                      | p.W541Cfs*5                          | Yamamoto et al. (2017) Brain & Development 39, 236–242 | rs1624 rs755055587 A > T (p.I542F) |
| 25  | c.1648C > T                           | p.R550C                              | Gataulina et al. (2016) Neuropediatrics 47, 399–403 | rs3981292974 C > T (p.R550C) [likely pathogenic] |
|     |                                      |                                      |                           | |
| 26  | c.1649G > A                           | p.R550H                              | Galada et al. (2018) Congenital Anomalies (Kyoto) | rs552452349 G > A (p.R550H) |

Variants are given in the order of their occurrence in the coding sequence. *Amino acid change reported in an online blog without annotation of the coding sequence position and change. NCBI/ClinVar – aggregates information about genomic variation and its relationship to human health [Landrum et al., 2016].
Whole Exome Sequencing

A total of 50 ng of genomic DNA were used for paired-end libraries synthesis with the Nextera DNA Library Prep kit (Illumina) according to the instructions of the manufacturer. A pool of up to eight libraries was used for exomes enrichment and indexing with the Nextera Rapid Capture Expanded Exomes kit (Illumina). Cluster generation was performed with the Illumina cBot at a concentration of 10 nM using an Illumina cBot. Paired-end reads of 100 bp were sequenced with an Illumina HighScan-SQ sequencer at the sequencing core facility of the Faculty of Medicine (University Leipzig) using version 3 chemistry and flowcell according to the instructions of the manufacturer. After base calling with Real-Time Analysis software 1.13 (Illumina) demultiplexing of raw reads, adapter trimming, and quality filtering was done accordingly (Stokowy et al., 2014). Resulting read pairs were mapped to the human genome (hg19) using the BWA aligner (Li and Durbin, 2010). Mapped reads were further processed for variant calling according to the Best Practices workflow (DePristo et al., 2011; Van der Auwera et al., 2013) suggested by the Broad Institute using GATK 3.4 tools (McKenna et al., 2010). Finally, genomic variants were annotated and filtered using wANNOVAR1, a web based tool for the functional annotation of genetic variants (Yang and Wang, 2015). Variant filtering was applied for rare Mendelian disease using the

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1http://wannovar.wglab.org
default parameter settings. Briefly, non-synonymous coding and splicing variants were considered candidates if their minor allele frequency (MAF) in the 1000 Genomes Project and gnomAD exome databases was ≤ 1% for a recessive mode of inheritance or ≤ 0.01% for a dominant inheritance pattern. In addition, the same MAF thresholds were applied to exclude variants that occurred in an in-house exome database of unaffected control individuals.

Sanger Sequencing
To validate the mutations in the ASNS coding sequence found by Whole Exome Sequencing (WES), all ASNS exons were sequenced in all family members and three independent healthy control samples using Sanger sequencing on an ABI PRISM 3130 × 1 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, United States).

Protein Modeling
The ASNS protein was modeled using the I-Tasser online server (Zhang Lab², University of Michigan; Zhang, 2009; Roy et al., 2012; Yang and Zhang, 2015). ASNS-wild-type, -M201Wfs*28 and -E389Q FASTA sequences were loaded and modeled using the crystal structure from *E. coli* as template (PDB 1CT9; DOI: 10.2210/pdb1ct9/pdb). The best fitting model for each query (positive C-score, model 1 for each) was downloaded and visualized using PyMOL software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC³).

Identification of Mutations in ASNS
Using WES, the coding sequences of the genome of the two affected girls, their unaffected brother as well as their healthy parents were analyzed with wANNOVAR⁴ (Yang and Wang, 2015). We assumed two models for the analysis: (1) a rare recessive Mendelian disease model and (2) a de novo rare dominant Mendelian disease model. Pedigree structure excluded an autosomal dominant model of inheritance as both parents are unaffected. Further, X-chromosomal dominant inheritance was excluded for the same reason, and X-chromosomal recessive inheritance was excluded because of the unaffected father. All genetic variants consistent with a de novo dominant disease model (variants not detected in parents or brother) could be excluded because only one of the affected sisters carried the variant. The rare recessive Mendelian disease model in wANNOVAR assumes that in case of an autosomal inherited disease at least two deleterious alleles (compound heterozygous or homozygous) need to be present in a gene. A gene was considered as a candidate if mutations were detected in both affected girls. Of those, all genes except for ASNS could be excluded, because either (a) identical variants were detected in the unaffected brother; (b) the mother was homozygous for the variants; (c) the haplotype was derived from the mother; or (d) the variant was found ≥ 2× in the controls, or have been found homozygous in a high number in the gnomAD database. Within ASNS, which has previously been associated with congenital microcephaly (Ruzzo et al., 2013; Gupta et al., 2017), two new mutations were identified: c.1165G > C, p.E389Q and c.601delA, p.M201Wfs*28 (Table 2; all positions in the ASNS gene are annotated to Refseq NM_133436.3, Ensembl ENST00000394309.3, GRCh37). Segregation in the family revealed compound heterozygous genotypes in both patients and confirmed the autosomal-recessive mode of inheritance. The unaffected brother carried only one of the variants (Figure 1C).

The WES data were validated by Sanger Sequencing. The mutations c.601delA and c.1165G > C co-segregated with the disease in the family and were not found in population matched controls (Figure 1C and Table 2). In addition, four further known polymorphisms were detected that did not co-segregate with the disease. Two are located in the 5′ UTR or the intron respectively (rs58521276 [Exon 1], rs796621224 [Intron 1-2]), one in exon 5 (rs1049674), and one in exon 10 (rs1049677). The newly identified mutations were not found in dbSNP, Exome Sequencing Project (ESP), Exome Aggregation Consortium (ExAC), Human Gene Mutation Database (HGMD; public version), and 1000 Genomes databases, suggesting that these variants are very rare in the population. The novel genetic variants in ASNS described in this work have been submitted to the ClinVar database (c.601delA: accession number: SCV000778369; c.1165G > C: accession number: SCV000778370).

Prediction of Functional Consequences and Protein Modeling
The two novel mutations were inherited by the patient’s family in a compound heterozygous manner (Figure 1C). Mutation c.1165G > C [p.E389Q] exchanges glutamate by glutamine at position 389 of the protein (Figure 1C), which was predicted as a most likely damaging mutation by all prediction tools used (Table 2). Mutation c.601delA [p.M201Wfs*28] results in a frame shift and induces a new stop codon after amino acid 227 thereby deleting more than half of the protein (total wild-type protein size 561 aa). While both new variants are not recorded yet in the public databases (see above), a variant different from the c.1165G > C mutation is described in Ensembl and dbSNP at the same ASNS coding sequence position (rs948326794 c.1165G > T, MAF < 0.01) leading to a premature stop codon. This variant was submitted by TOPMED (Goncalo Abecasis, Center for Statistical Genetics, Biostatistics Department, Ann Arbor, MI, United States). No further information on this variant is currently available in the literature and – to our knowledge – no cases of microcephaly carrying this variant have been reported so far.

Modeling of the 3D structure of ASNS was performed to localize the position of the mutated amino acids and the impact of the mutation on the protein structure. As no structure of the human ASNS has been reported so far, the model was based on the structure of *E. coli* ASNS (Figure 1D; accession number 1CT9 in the RCSB PDB database) as described previously (Gupta et al., 2017; Lomelino et al., 2017; Sacharov et al., 2017; Yamamoto et al., 2017). The ASNS enzyme consists of two protein domains, the glutamine amidotransferase domain (aa 2 to 191;
TABLE 2 | Sequencing result in cases and controls.

| Location | Exon 1 | Intron 1–2 | Exon 5 | Exon 10 |
|----------|--------|-----------|--------|--------|
| Variant  | rs85521276 | rs796621224 | Del A (*) | G > C (*) |
| rs-number | 5′ UTR | c.601delA | p.M201Wfs*28 | p.E389Q |
| Position in cDNA | Inton | c.629T > A | p.V210E | p.L403L |
| Position in protein | Missense | Missense | Synonymous codon | |
| Functional consequence | UTR variant | Intron variant | Frameshift variant | |
| Index patient | A/A | A/A | Del A/A | T/T |
| Sister | A/Ins A | A/Ins A | A/A | T/T |
| Brother | A/Ins A | A/Ins A | A/A | T/T |
| Father | A/Ins A | A/Ins A | A/A | T/T |
| Mother | A/Ins A | A/Ins A | A/A | T/T |
| Control 1 | A/Ins A | A/Ins A | A/A | T/T |
| Control 2 | A/A | A/A | Del A/A | T/T |
| Control 3 | A/Ins A | A/Ins A | A/A | T/T |
| Prediction-Tools | Polyphen | n.d. | n.d. | 0.09 |
| | SIFT | n.d. | n.d. | 0.4 |
| | SNP&GO | n.d. | n.d. | 0.347 |
| | MutPred | n.d. | n.d. | 0.22 |

(*) Mutations found in the family. UTR, untranslated region; Ins, insertion; Del, deletion; n.d., not determined/no prediction possible. Polyphen (http://genetics.bwh.harvard.edu/pph2/): 0–0.452 benign; 0.453–0.955 possibly damaging; 0.956–1 probably damaging. SIFT (http://sift.jcvi.org/): value ≤0.05 damaging, value ≥0.05 benign. SNP&GO (http://snps-and-go.biocomp.unibo.it/snps-and-go/): value ≥0.5 most likely damaging. MutPred (http://mutpred.mutdb.org/): value = probability of a damaging mutation. Reference Sequence NM_133436.3.

Within ASNS, these mutations are scattered throughout the coding sequence of the glutaminase and the ASNS domains, but also affecting amino acids between the two domains and the very C-terminal amino acids in the protein (Figures 2A,B). All mutated amino acids are highly conserved among different species from zebrafish to humans (Supplementary Figure 1). Strikingly, while in zebrafish E389 is replaced by aspartate (Supplementary Figure 1), the glutamate at this position is also present in Drosophila melanogaster (NP_993132.1), Saccharomyces cerevisiae (NP_011640.1), and even E. coli (WP_000337071.1; data not shown).

The clinical data of many of the patients with ASNSD reported so far has recently been summarized (Gupta et al., 2017). An obvious heterogeneity between all these patients is the different lethality and life span (Figure 2C). This analysis has to be considered with caution as other factors besides the direct consequence of the ASNS mutations can affect the life span in addition to the ASNS mutation. Furthermore, several patients were still alive at the time of publication so their life span is unknown. Nevertheless, some mutations appear to be associated with shorter life span, i.e., higher severity of the disease (e.g., R550C; Figure 2C).

DISCUSSION

Mutations in ASNS have recently been identified as the cause of microcephaly and severe defects in brain development in human patients (Ruzzo et al., 2013). In a family with two girls with microcephaly, we have identified two novel mutations. These mutations have not been reported in the
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While the mutations in ASNS have been unequivocally identified as the cause of the disease condition, the pathophysiology remains enigmatic. Two aspects need to be considered in this context: (1) What is the effect of the mutation on the ASNS protein? (2) Why does a (assumed) loss in ASNS activity result in the phenotype observed in the patients?

Firstly, it has been suggested that mutations in ASNS lead to reduced expression (Ruzzo et al., 2013), and/or to a reduced stability of the protein (Yamamoto et al., 2017). The E389Q mutation is located in the second helix of a helix-loop-helix motif and the A380S mutation in the first helix of this motif.
destabilizes the ASNS protein (Gupta et al., 2017). Consistently, prediction tools suggest a destabilization of the ASNS-E389Q protein. E389 is conserved throughout evolution from E. coli to humans, with the exception of Danio rerio where it is replaced by aspartate, indicating that the negative charge of the amino acid at this position is important for ASNS function. E389 is localized rather on the surface of the ASNS protein near the binding pocket of ATP and aspartate (Gupta et al., 2017); however, whether and how E389 contributes to the binding of these molecules remains to be addressed by further investigations. Furthermore, mutations introducing a premature stop codon, like the c.601delA [p.M201Wfs*28] mutation reported here, often result in expression of non-functional truncated proteins, which may be cleared by lysosomal degradation immediately after translation. Alternatively, nonsense mediated decay of mutant mRNA might result in fast degradation of the respective mRNAs (Miller and Pearce, 2014; Ottens and Gehring, 2016). However, while no measurements of the specific activity of the different ASNS mutants have been reported so far, it is very likely that – by whatever mechanism – the observed ASNS mutations finally result in a reduced or absent enzymatic activity of ASNS. This is consistent with the impaired growth of fibroblasts in the absence of Asn as reported for the G289A/T337I compound heterozygous mutation (Palmer et al., 2015).

Secondly, how a reduced activity of ASNS result in microcephaly? ASNS synthesizes Asn and Glu from Asp and Gln; and it has been suggested that loss of ASNS activity might result in a decrease in Asn availability in the brain (Ruzza et al., 2013). However, Asn is generally considered as a non-essential amino acid and can be provided to the developing fetus in sufficient amounts to enable normal development of peripheral organs. While no data is available on the permeability of Asn through the blood–brain barrier in the developing human fetus, it has therefore been suggested that lack of ASNS activity disturbs an intricate metabolic balance of amino acids including Asn, Asp, Glu, and Gln resulting in disturbed brain development and function (Sacharow et al., 2017). Interestingly, other deficiencies of enzymes involved in the synthesis of non-essential amino acids including serine, glutamine, and proline have been reported, which all show a severe neurological and/or neurodevelopmental phenotype (de Koning, 2017). With the exception of skin deficiencies reported for several cases, these neurological symptoms appear to be a rather isolated phenotype also in these conditions similar to ASNSD (de Koning, 2017). Therefore, as “it is puzzling how a ubiquitously expressed enzyme defect can affect the central nervous system in such a specific way” (de Koning, 2017), the pathomechanisms of these mutations of enzymes of amino acid biosynthesis are still enigmatic and deserve further investigations.

In conclusion, we here present two novel mutations in ASNS identified in a family with two children with microcephaly. These add to the recently rapid growing list of reported mutations in ASNS in children with this condition. However, while identification of mutations will help to diagnose patients, it will still be a long way to unravel the underlying pathophysiology or even to develop treatment for the patients.

AUTHOR CONTRIBUTIONS

DS, AS, PGH, UW, SK, JB, and KK: acquisition of data. DS, AS, RSt, JK, UW, SK, JTH, RSc, KK, KH, PK, and JH: analysis and interpretation of data. PK and JH: conception of the project. KH, PK, and JH: supervised the project. DS, RSt, PK, and JH with input of all coauthors: wrote the paper. All authors approved the final version of the MS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2018.00245/full#supplementary-material

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