eMETHODS

Method

Study design

The 50 index patients were admitted consecutively to the Department of Pediatrics, University Medical Center Hamburg-Eppendorf for complex neuropediatric diagnostic work-up (German surgical procedure code OPS 1–942). All patients underwent the following standardized program of investigations:

- At least detailed questioning regarding the medical history of the index patient and family members
- Extended clinical assessment
- Wide-ranging examinations of blood, urine, and cerebrospinal fluid parameters
- Cranial magnetic resonance imaging (cMRI) at 1.5 T or 3 T
- Electroencephalography (EEG)
- Trio WES

The diagnostic procedures, including trio WES, were billed as "complex neuropediatric diagnostic work-up with extended genetic work-up" (OPS 1–942.2) or "complex neuropediatric diagnostic work-up with neurometabolic laboratory testing and/or infectiological/autoimmune inflammatory laboratory testing with extended genetic work-up" (OPS 1–942.3).

Documentation of genotype—WES technique

The exome sequencing was performed at the Institute of Human Genetics, Helmholtz Center Munich. All known coding DNA fragments from a patient and their biological parents were enriched with the SureSelect Human All Exon 50Mb V5 Kit (Agilent, Santa Clara, CA, USA) and sequenced using the HiSeq2500 system (Illumina, San Diego, CA, USA). The reads were assigned to the reference genome "human genome assembly hg19" (UCSC Genome Browser) with the aid of the Burrows-Wheeler Aligner (BWA, v.0.5.87.5). The detection of genetic variants (deviations from the norm) was achieved by means of SAMtools (v0.1.18), PINDEL (v 0.2.4t), and ExomeDepth (v1.0.0). In this way 95 to 99% of the exome sequences were covered at least 20-fold.

Documentation of genotype—classification of variants and genes

The genetic data generated by exome sequencing were analyzed at the Institute of Human Genetics, University Medical Center Hamburg-Eppendorf.

The disease relevance of a given identified variant was evaluated with the aid of a number of variables: MAF, assessment by bioinformatic prediction programs, comparison with databases (e.g., Database of Exome Aggregation Consortium, ExAC [e1]; Online Mendelian Inheritance of Man, OMIM [e2]), and current knowledge of the coded protein and its function.

A variant in a gene was classified as causing disease (i.e., as a mutation) if:

- It affected a known disease gene
- The very same variant had previously been described as causing disease, or the variant was comparable in type with known disease-causing variants in the same gene
- There was overlap in phenotype between our patient and the published patients with causative mutations in the same gene

We defined known disease genes as those whose association with disease had been demonstrated by published clinical–genetic and/or functional data.

A variant in a gene was classified as probably causing disease if:

- It affected a candidate gene (see below)
- More than one prediction program (Polyphen 2, SIFT and CADD) classified it as pathogenic
- In the case of a *de novo* mutation, it was not listed in the ExAC database

A gene was classified as a candidate gene if:

- Based on the trio WES results it was the only one of the patient's genes in which a rare variant was found, or based on the trio WES results and bioinformatic prediction algorithms it was classified as probably causing disease
- Previously published data (e.g., functional in-vivo or in-vitro studies) pointed to disease association in humans or there was, via Genematcher (3), contact to other study groups who had also detected variants in the same gene in patients with overlapping symptoms

Phenotyping results

Sociodemographic data

For 49 patients (98%) data were available on the consanguinity of the parents. The parents' ethnicity was documented for 45 patients (90%). The parents came from 12 different countries, including Germany (23 [46%]), Turkey (5 [10%]), Iran, Afghanistan, and Kosovo (each 3 [6%]). Nine patients (18%) had consanguineous parents. The consanguineous couples were from Germany (1 [2%]), Turkey, Iran, Afghanistan (each 2 [4%]), Egypt, and Pakistan (each 1 [2%]).

Perinatal abnormalities

Abnormal features of gestation or delivery had been reported for 18 patients (36%): an abnormal amount of amniotic fluid in nine cases (18%), vaginal bleeding in three (6%), intrauterine growth retardation (IUGR) in seven (14%), and premature delivery in five (10%). Other complications (e.g., placental insufficiency, gestational diabetes) were documented in individual cases. Nine patients showed abnormal measurements at birth: One patient was small for gestational age (SGA) (birth weight, length, and head circumference <−2 standard deviations [SD] according to Voigt et al. [e4]), and isolated microcephaly was reported for two patients. In five patients (10%) birth weight and/or body length were <−2

SD, and in one patient birth weight and head circumference were >+ 2 SD.

Imaging, functional analysis, and metabolic analysis

The EEG results were abnormal in 20 (43%) of 47 patients, for example "pathological waking EEG with generalized susceptibility to seizures and activation by hyperventilation and photostimulation" or "pathological waking EEG with inconstant bilateral temporal foci and left temporal susceptibility to seizures." The findings on cMRI were abnormal in 29 (58%) of 50 patients, for instance "midline deformity with hypoplasia of the corpus callosum and absent septum pellucidum. Prominent cerebellar tonsils, but currently no Chiari I. Residual hemorrhages" or "suspicion of small right frontal subdural hematoma with no appreciable space-occupying effect. Increasing diffuse leptomeningeal and subarachnoid contrast-enhancing substrate over time, primarily compatible with a progressive angiomatous process with leptomeningeal accentuation." Ophthalmological examination revealed abnormalities in 23 (62%) of 37 children, e.g., "left: optic nerve hypoplasia," "right/left: mild tortuosity of veins," or "right/left: hyperopia, astigmatism *(eTable 2)*. Blood gas analysis (BGA), performed in 45 index patients, revealed elevated lactate concentrations in 13 cases (29%) and striking base excess in nine patients (20%) (decreased levels in two children, elevated levels in seven). Of the 49 children who underwent cerebrospinal fluid analysis, one (2%) showed an elevated glucose concentration, 12 (25%) had abnormal lactate levels (low in two children, high in 10), and three children (6%) displayed elevated concentrations of protein. The majority of patients underwent extended metabolic screening, including analysis of amino acids in plasma and cerebrospinal fluid together with determination of acylcarnitines in dried blood spots and measurement of organic acids in urine. In no patient was there a constellation of biochemical findings pointing to a specific disease.

Genetic analysis had been carried out previously in 33 children (66%). Four had undergone chromosome analysis; five, array-CGH analysis; 10, chromosome analysis plus array-CGH analysis; five, individual or panel genetic analysis; and nine, individual or panel genetic analysis plus chromosome analysis or array-CGH analysis. None of these earlier analyses had revealed any abnormalities (e.g., chromosome analysis; normal male karyotype 46,XY; array-CGH analysis: no abnormal findings; *ARX* genetic analysis: no abnormal findings).