Original Article

Exome Sequencing in Children

Undiagnosed Developmental Delay and Neurological Illness

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Summary

Background: In developed countries, global developmental disorders are encountered in approximately 1% of all children. The causes are manifold, and no exogenous cause can be identified in about half of the affected children. The parallel investigation of the coding sequences of all genes of the affected individual (whole exome sequencing, WES) has developed into a successful diagnostic method for identifying the cause of the problem. It is not yet clear, however, when WES should best be used in routine clinical practice in order to exploit the potential of this method to the fullest.

Methods: In an interdisciplinary study, we carried out standardized clinical phenotyping and a systematic genetic analysis (WES of the index patient and his or her parents, so-called trio WES) in 50 children with developmental disturbances of unclear etiology and with nonspecific neurological manifestations.

Results: In 21 children (42% of the collective), we were able to identify the cause of the disorder by demonstrating a mutation in a gene known to be associated with disease. Three of these children subsequently underwent specific treatment. In 22 other children (44%), we detected possibly etiological changes in candidate genes not currently known to be associated with human disease.

Conclusion: Our detection rate of at least 42% is high in comparison with the results obtained in other studies from Germany and other countries to date and implies that WES can be used to good effect as a differential diagnostic tool in pediatric neurology. WES should be carried out in both the index patient and his or her parents (trio-WES) and accompanied by close interdisciplinary collaboration of human geneticists and pediatricians, by comprehensive and targeted phenotyping (also after the diagnosis is established), and by the meticulous evaluation of all gene variants.

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Divergence from the expected psychomotor development (global developmental delay) is observed in around 1% of children in the industrialized countries (1, 2). Global developmental delay thus accounts for a considerable proportion of all cases of neuropediatric illness, although there are no reliable statistical data on the prevalence of neurological diseases in childhood. The symptoms of global developmental disorders are often unspecific, so that in many cases no precise diagnosis is possible. Establishment of the diagnosis is, however, a necessary precondition for initiating any disease-specific treatment that may be available, drawing up an individualized support and prevention program, assessment of the developmental prognosis, and accurate estimation of the risk of similar disorders in the patient's siblings or other family members.

There are multiple different factors that may be responsible for developmental delay, and in around half of the children affected no causative exogenous factor is identified (3). Probably most of these cases are of genetic origin (3–9). Genetic techniques therefore play a crucial role in identifying the cause and pinpointing the diagnosis in this group of patients. In the past, molecular genetic tests were extremely laborious, usually proceeding "gene by gene." However, the recently introduced "next-generation" sequencing (NGS) enables analysis of large numbers of genes (right up to the whole human genome) in a short amount of time at reasonable cost (10–12) *(Table 1)*.

The ethical aspects of such all-embracing genetic analysis techniques have been and continue to be widely discussed (13–16). Particularly intensively debated topics are the explanation of and consent to genetic analysis, storage of and access to genetic data, and how to deal with incidental findings. The latter are genetic variants that happen to be identified in the course of NGS-based analyses. They are not related to the index patient's illness, but mean there is an increased likelihood of a second, independent disease in the patient (and possibly his/her relatives). Country-specific and international guidelines and regulations vary widely in their recommendations on how to deal with incidental observations, and the debate is probably far from over. There is unanimity, however, on the need actively to include patients and guardians in the discussion of these questions.

Despite this, NGS methods have become established in routine genetic diagnosis. The technique

TABLE 1

Comparison of sequencing techniques

	Next-generation sequencing (NGS)			Single-gene sequencing
	Whole-genome sequencing (WGS)	Whole-exome sequencing (WES)	Panel diagnostics	Sanger sequencing
Portion of genetic material analyzed	Entire genome	All known exons, individual regulatory sequences	Exons of selected genes	Exons of a single selected gene
Scope of analysis	~3 billion base pairs = 3000 Mb	~1% of the genome + regulatory sequences + miRNA = ~50 Mb	Depending on the genes/ exons selected, several Kb to a few Mb	Depending on the gene/exon selected, a few Kb
Number of variants identified	> 3 000 000	15 000–40 000	Few or none	Few or none
Detection	Point mutations, indels, numerical and structural chromosome variants	Point mutations, indels	Variants in the selected genes	Variants in the selected gene/ exon
	Incidental findings	Incidental findings	No incidental findings	No incidental findings
	New disease genes	New disease genes	No new disease genes	No new disease genes

Kb, Kilobase (1 Kb = 1000 base pairs); Mb, megabase (1 Mb = 1 000 000 base pairs); miRNA, microRNA; indels, insertions/deletions

generally used in Germany is "panel diagnostics," in which a defined number of genes are investigated depending on the disease of interest. In highly heterogeneous illnesses (e.g., unspecific childhood developmental disorders) with hundreds of associated genes it does not make sense to limit the amount of genes to be analyzed. Patients affected by such diseases should be offered analysis of their entire genetic coding material (i.e., all genomic regions that are translated into proteins = all exons of the circa 20 000 human genes), known as whole-exome sequencing (WES).

Numerous studies from various countries have shown that the causes of developmental disorders and neurological illnesses in childhood can often be uncovered by means of WES (8, 17–25). The success rate of WES has been reported as 25 to 68% and is particularly high when:

- The study group is highly selected (17, 22)
- The exome sequencing is extended to parents (trio exome sequencing) and/or other family members (family exome sequencing) (17, 26)
- The analysis of the exome data embraces all known genes, not only those associated with a human illness at the given point in time (27, 28).

It has not yet been adequately investigated how exome sequencing can be effectively integrated into concrete clinical routine (5).

Many questions remain unanswered. These include what patients should be offered WES, to what extent it should be extended to the patient's relatives, and in what clinical and analytical context it should take place.

To address these questions, we carried out a single-center pilot study of 50 children with undiagnosed developmental delay and neurological illness presumed to be of genetic origin. Thorough clinical examination, biochemical analyses, electroencephalography, and diagnostic imaging were accompanied by trio WES.

The aim of this study was to establish a practical procedure for phenotyping and genotyping that would achieve a high rate of diagnosis for unspecific neurological illnesses of childhood and could be accommodated into the clinical routine.

Method

Study design

The study was a joint project of the Department of Pediatrics and the Institute of Human Genetics at the University Medical Center Hamburg-Eppendorf, Germany. Over a period of 17 months, a total of 50 consecutive children with undiagnosed neurological illnesses underwent a standardized assessment program in the context of a complex neuropediatric diagnostic work-up (German surgical procedure code [OPS] 1–942) *(Figure 1, eMethods)*. The inclusion criteria were as follows:

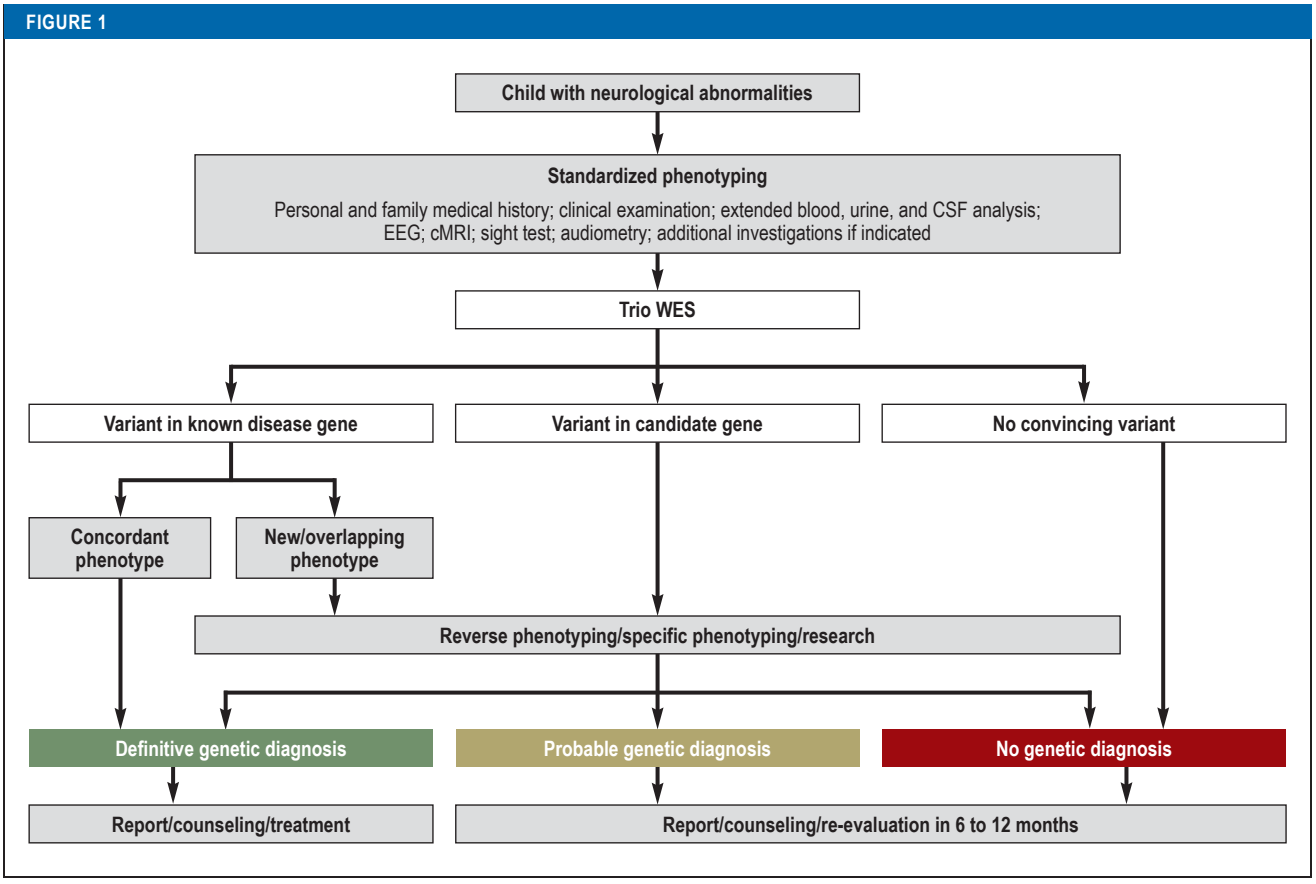
- Neurological symptoms (e.g., global developmental delay, ataxia, seizures)
- Suspected genetic etiology (i.e., no sign of serious perinatal complications, infection, injury, other exogenic factors)
- No specific provisional diagnosis
- Signed consent from the parents, following appropriate explanation, for exhaustive investigations including trio WES

The study was approved by the ethics committee of the Hamburg Medical Association (project number PV3802).

Phenotype documentation

The comprehensive assessment program always included detailed questioning about the medical history of the patient and their family, extensive clinical

FIGURE 1



Study protocol including evaluation algorithm for genetic variants
cMRI, Cranial magnetic resonance imaging; CSF, cerebrospinal fluid; EEG, electroencephalography; trio WES, whole-exome sequencing of index patient and parents

evaluation by a neuropediatrician and a clinical geneticist, wide-ranging investigation of blood, urine, and cerebrospinal fluid parameters, diagnostic imaging (1.5-T or 3-T cranial magnetic resonance imaging (cMRI), and electroencephalography (EEG). Depending on the clinical findings, other tests were added in individual patients, for example further diagnostic imaging procedures (e.g., MR spectroscopy), audiometry, echocardiography, or ophthalmological examination. We documented the reported medical history and the clinical data systematically using the Phenomizer software (29), which is based on Human Phenotype Ontology (HPO) (30). The degree of cognitive and/or physical impairment was classified according to Zhang et al. (31) *(eTable 1)*.

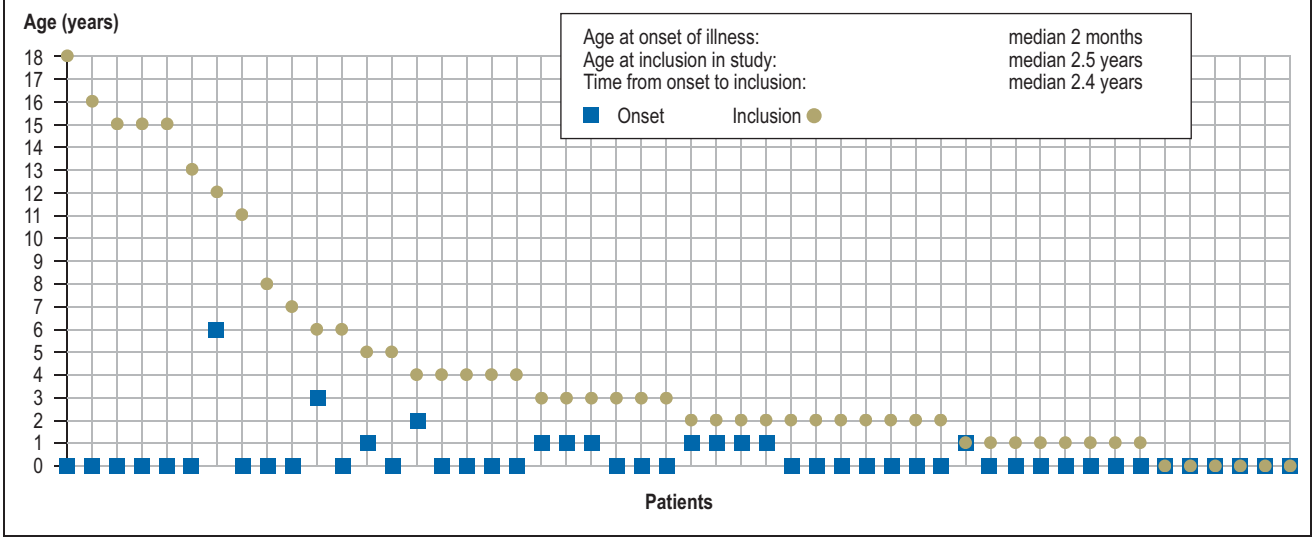
Genotype documentation

The genetic data were obtained from WES of EDTA blood from each index patient and their biological parents (trio WES). The WES procedure is described in the *eMethods*. Closer attention was paid to genetic variants which were very rarely identified in the population (minor alleles frequency [MAF]; occurrence of the rarer allele in the population <0.01%) and which, according to several different bioinformatic prediction algorithms, impact negatively on gene function (functionally relevant variants). The variants filtered out in this way were mostly nonsynonymous point mutations (variants that changed the amino acid sequence in the coded protein), losses/gains of one or several base pairs (indels) or losses/gains of submicroscopic chromosome regions (microdeletions/microduplications; copy number variations [CNVs]). These affected a known disease gene or a candidate gene. More detailed information on the WES procedure and the classification of variants and genes can be found in the *eMethods*.

Interdisciplinary interpretation of findings and reverse phenotyping

In each individual case, the members of a multidisciplinary team consisting at least of pediatricians and human geneticists discussed the findings and assessed the potential relevance of the genetic variants identified in the context of the medical history and the patient's symptoms. Whenever necessary, other experts (e.g., neuroradiologists) were added to the panel. In many cases the team ordered additional specific clinical investigations (reverse phenotyping) to enable more detailed assessment of a genetic variant. The results of reverse phenotyping were then interpreted by the assembled team.

FIGURE 2



Age at onset of illness (square) and age at inclusion in study (circle) for each of the 50 patients

Statistical analysis

Descriptive statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS, version 23).

Results

Study participants

The median age of the 50 unrelated children (19 girls [38%], 31 boys [62%]) at study inclusion was 2.5 years (range 4 days to 18 years; *Figure 2*). Thirty-three (66%) of them had already undergone genetic testing (chromosome analysis, array-based comparative genomic hybridization [array CGH]), and/or single-gene sequencing. In no case had the results been abnormal (for details see the *eMethods*).

Results of phenotyping

The first symptoms were observed at a median age of 2 months (0 days to 6 years; *Figure 2*). Sixteen patients (32%) had delayed development of motor functions and/or speech, six (12%) showed abdominal symptoms (e.g., omphalocele, esophageal stenosis/atresia, feeding difficulties), and five (10%) evinced ophthalmological abnormalities (e.g., congenital cataract, nystagmus, strabismus). A median 2.4 years (4 days to 18 years; *Figure 2*) elapsed between occurrence of the first symptoms and inclusion in the study.

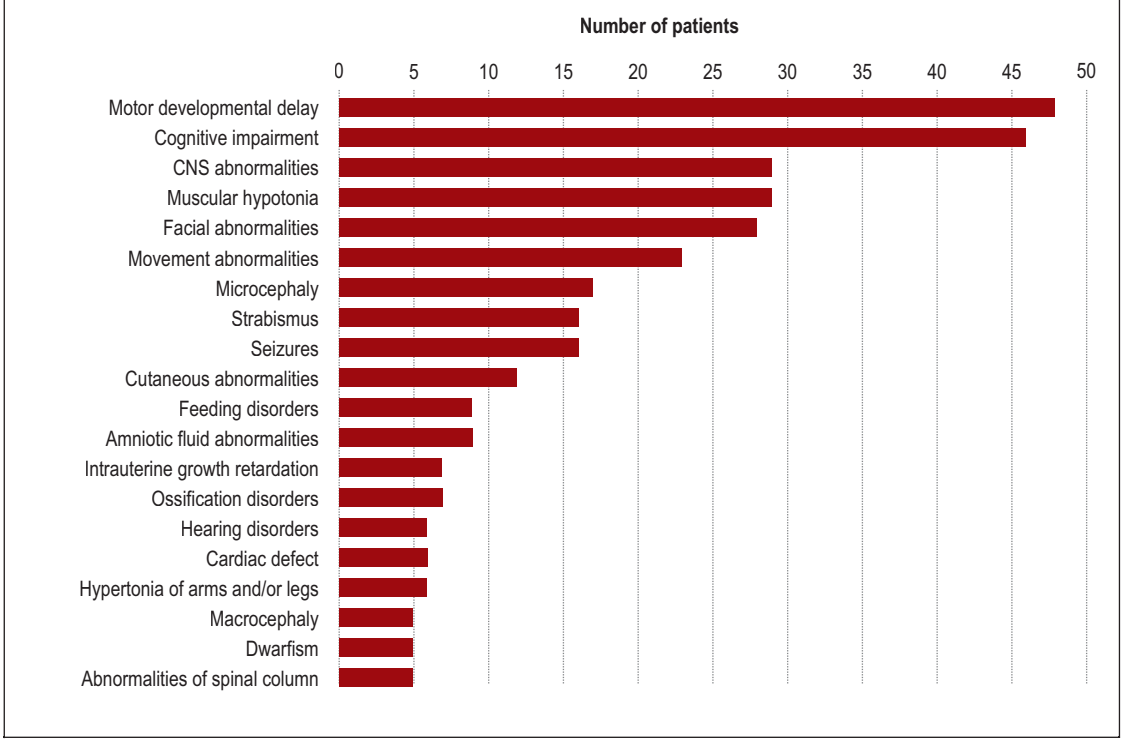
In line with the inclusion criteria, all 50 patients had neurological symptoms at the time of entry into the study. These comprised global developmental delay (88%), cognitive impairment without motor symptoms (4%), or motor symptoms without cognitive impairment (8%). As classified according to Zhang et al. (31), the developmental disorder was mild in 10% of patients, moderate in 32%, severe in 56%, and profound in one patient. Psychomotor development was categorized in 68% of patients as "gradually progressive," in 6% as "stagnating," and in 26% as "regressive." At the time of clinical examination, body weight, body length, and head circumference were abnormal (<-2 standard deviations [SD] or $>+2$ SD according to Kromeyer-Hauschild et al. [32]) in 13 (26%), 12 (24%), and 21 (42%) of patients, respectively. Thirty-six patients (72%) showed abnormalities of the head and neck (e.g., facial dysmorphism), 34 (68%) had abnormalities of the musculature (e.g., muscular hypotonia), and 28 (56%) exhibited ocular abnormalities (e.g., strabismus) *(Figure 3)*. Further details of the phenotyping findings are documented in the *eMethods* and in *eTable 2*.

Results of genotyping

In 21 patients (42%) we found a pathogenic variant (mutation) in a disease gene known at the time of analysis or a known disease-related microdeletion *(Table 2, eTable 3)*. Twelve (57.1%) of these 21 mutations were *de novo* events, i.e., they had arisen in the index patient. In nine index patients (42.9%) the mutations were biallelic (on both alleles of the gene affected), so that the inheritance was autosomal recessive. Strikingly, during the course of our study variants in three genes *(CHAMP1, SSR4,* and *SON)* initially classified as candidate genes were convincingly shown by newly published data to be mutations in new disease genes.

In an additional 22 patients (44%) we identified a variant in candidate genes that probably caused illness. The disease association of these candidate genes has been or is currently being investigated in international cooperation projects. Since the conclusion of our work, two of these 22 candidate genes

FIGURE 3



Clinical characterization of the study group according to the HPO system (HPO, Human Phenotype Ontology)

have been confirmed as disease genes and the research to date supports a disease association for some of the others.In only seven patients there (14%) were no abnormal genetic findings.

Consequences for clinical care

In 17 (81%) of the 21 patients with a mutation in a known disease gene, diagnosis resulted in recommendations to change/modify the clinical management. In 14 children (66.7%) the recommendations affected the prevention and monitoring program (e.g., the institution of new follow-up investigations or discontinuation of existing investigations), and in 13 patients (62%) the individual support measures (e.g., use of sign language or a talker, planning of future education) were involved. Moreover, three patients (3/21 = 14%; 3/50 = 6%) were offered specific treatment: In an 11-month-old girl with developmental regression, severe muscular hypotonia, and eye movement disorders, treatment with L-Dopa was initiated immediately after the detection of compound heterozygous mutations in the tyrosine hydroxylase gene (TH-Segawa syndrome, OMIM #605407). This led to swift regression of the neurological symptoms and to catch-up development over the course of time. In a 4-year-old boy with global developmental delay, dystonia, and gait abnormality, treatment with trihexyphenidyl was started after identification of a *de novo* mutation in *SGCE* (dystonia type 11, OMIM #159900). Follow-up showed decreased severity of the dystonic movement disorder and improved gait. Detection of a homozygous mutation in *ARSA* (metachromatic leukodystrophy, OMIM #250100) in a 3-year-old boy with peripheral neuropathy and leukodystrophy led to his inclusion in a treatment study *(eTable 4* and *eTable 5)*.

Systematic documentation and comparative statistical evaluation of the implementation of the treatment recommendations and their success were not among the aims of our study and were in any case not compatible with the selected study design.

Discussion

Our standardized search for the cause of undiagnosed developmental delay and neurological illnesses in a pediatric collective revealed mutations in known disease genes compatible with the phenotype in 21 (42%) of 50 patients. This involved wide-ranging clinical, biochemical, and instrument-based investigations together with trio WES. Our rate of identification of the cause of developmental disorders in a general pediatric cohort is towards the high end of the spectrum of results from international research: comparable studies from, among other countries, Israel, the UK, and the USA report diagnosis rates of 25 to 49% (8, 20, 22, 24). Our findings emphasize that pediatric patients with unspecific neurological symptoms can often be helped by means of the powerful genetic diagnostic technique of exome sequencing.

TABLE 2

Findings of whole-exome sequencing (WES)

	Number of patients
Findings	**50 (100%)**
– Mutation in a known disease gene	21 (42.0%)
– Variant in a candidate gene	22 (44.0%)
– No convincing variant	7 (14.0%)
Characterization of the mutations in known disease genes	**21 (100%)**
– Inheritance	
– *De novo*	12 (57.1%)
– Autosomal recessive	9 (42.9%)
– X-chromosomal	0 (0%)
– Mutation type	
– Missense	11 (52.4%)
– Nonsense	3 (14.3%)
– Frameshift	4 (19.0%)
– CNV	3 (14.3%)

CNV, Copy number variations

Individual out-of-hospital exome sequencing is covered by German health insurance under EBM fee schedule code number 11514 (remuneration currently circa €3300). However, the necessary individual approval by the health insurance provider is rarely forthcoming. In the hospital setting, genetic diagnosis, including WES, can be carried out under OPS code 1-942.

The mutations affected 20 different disease genes in the 21 confidently diagnosed patients, reflecting the extreme heterogeneity of childhood developmental disorders and neuropediatric disorders and underlining the necessity of systematic genetic analyses (WES) in this group. Our experience leads us to recommend trio WES, because confident and effective identification of *de novo* (newly arising) mutations is facilitated by comparing the genetic variants in the index patient with those found in the patient's parents. In fact, *de novo* mutations comprise a high proportion of the genetic causes of disease: in our study 57% of the mutations arose *de novo*, and in the DDD study the figure was as high as 65% (9).

Analysis of the genetic data was not limited to known disease genes, and indeed a high number of probably disease-relevant variants were found in various candidate genes. Even before the conclusion of the study, three genes we had originally categorized as candidate genes *(CHAMP1, SSR4, SON)* were reclassified as "new disease genes" (33, 34). After completion of the study there were 22 index patients (44%) with a probably disease-relevant variant in a candidate gene. We are convinced, on the basis of the trio WES data, bioinformatic predictions, published research, and data from cooperating study groups, that in the course of time most of these candidate genes will be confirmed as disease relevant and reclassified as new disease genes. As of November 2018, two of them *(DHX30, ANO3)* had already been reclassified, increasing our rate of diagnosis to 46% (23 of the 50 index patients).

Conclusion

This pilot study has confirmed the high diagnostic potential of exome sequencing in a heterogeneous cohort of neuropediatric patients whose unspecific symptoms gave no clues to the diagnosis. The high rate of successful diagnosis was achieved by means of a interdisciplinary clinical–genetic approach, with:

- Detailed phenotyping, followed, whenever necessary, by specific investigations (reverse phenotyping)
- Exome sequencing of the index patient and their parents (trio WES)
- Comprehensive evaluation of the entire data obtained by exome sequencing
- Interdisciplinary interpretation of the clinical and genetic data
- Close cooperation and data sharing with physicians and study groups in Germany and other countries

Under these conditions exome sequencing is a highly powerful genetic investigation technique that can be used in patients with disease of probable genetic origin in whom no specific diagnosis is suspected. In pinpointing the diagnosis, it opens the door to the specific treatments that are increasingly becoming available for genetic diseases.

Limitations

One limitation of our study is the relatively small number of patients. However, data from independent international studies seem to show that the central results can be reproduced in a larger cohort of patients. It must be borne in mind that the detection rates relate to the data available at the time the study was carried out.

Acknowledgments

We are grateful to the patients who took part in this study and their families. We also thank all the clinicians who contributed data as well as the molecular geneticists, bioinformaticians, and laboratory technicians who lent us their assistance.

Compliance with ethical standards

We obtained signed consent from each study participant or, if applicable, their parents. All procedures in studies with human participants complied with the ethical standards of the institution and country concerned and adhered to the tenets of the Helsinki Declaration of 1964 and its subsequent revisions or to comparable ethical standards.

Conflict of interest statement

Prof. Meitinger is the director of a molecular genetics laboratory authorized to issue private invoices at Rechts der Isar Hospital, TUM, Munich.

Key messages

- The achieved diagnosis rate of at least 42% underlines the efficiency of exome sequencing as a method for detection of the genetic causes of developmental delay and neurological illness in childhood.
- Exploitation of the potential of exome analysis to the full involves comprehensive phenotyping of the patient, extension of exome analysis to the patient's parents, and detailed analysis of the genetic data.
- Exome analysis can, in the absence of a concrete provisional diagnosis, be used as the first step of the genetic diagnostic work-up in neuropediatric patients. In no way does it replace explicit phenotyping of the child affected.
- Timely use of exome analysis enables early diagnosis and thus avoidance or termination of a diagnostic odyssey for the patient and their family, and also hastens the use of any specific treatment that may be available in the individual case.
- The complex nature of this detailed genetic analysis, including the ethical aspects, must be explicitly discussed in advance with the patients or their guardians.

Dr. Hempel and Prof. Kubisch also work at the Martin Zeitz Center for Rare Diseases in Hamburg. This center is a project partner of the health care project TRANSLATE NAMSE.

Prof. Muntau is a project partner of the health care project TRANSLATE NAMSE.

The remaining authors declare that no conflict of interest exists.

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► **Supplementary material**
For eReferences please refer to:
www.aerzteblatt-international.de/ref1219

eMethods, eTables:
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Supplementary material to:

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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).

eTABLE 1

Classification of mental retardation (MR) according to Zhang et al. (e5)

Level	Degree of disability(IQ)	Adults	Children
0	None	No impairment	Development appropriate for age
1	Borderline (<70)	Attendance of regular schools for several years; needs little support	Milestones reached at normal ages; slight developmental delay apparent in first years at school
2	Very mild (<65)	Attendance of regular schools for a few years; needs a lot of support; simple skills in reading, writing, and numeracy	Milestones reached at normal ages for the first few years; slight developmental delay apparent from age of 2 to 3 years
3	Mild (<50)	Good language comprehension, even long sentences are understood; limited skills in reading, writing, and numeracy	Achievement of milestones delayed by a few months; apparent from the age of 1 to 2 years
4	Moderate (<35)	Good language comprehension; speaks short sentences, makes a lot of gestures	Achievement of milestones delayed by several months; apparent from the 2nd year of life
5	Severe (<20)	Understands simple common sentences; speaks two-to three word sentences, makes a lot of gestures; can walk	Achievement of milestones delayed by several months to a year; apparent from the 1st year of life
6	Very severe (<10)	Understands single words; speaks single words or not at all; can walk unsteadily/with assistance	Developmental delay by several years; becomes apparent in first 6 months of life
7	Profound	No or only slight reaction; can sit and stand with assistance, walks rarely or not at all	No unassisted sitting at age of 5 years

IQ, Intelligence quotient

eTABLE 2

Findings of instrument-based diagnostic methods

Method	Number of patients with abnormalities
EEG	20 of 47 (42.6%)
cMRI	29 of 50 (58.0%)
Audiometry	8 of 16 (50.0%)
Sight test	23 of 37 (62.2%)

cMRI, Cranial magnetic resonance imaging; EEG, electroencephalography

eTABLE 3

Patients with mutations in known disease genes or CNVs known to cause disease

Patient	Consanguinity of parents	Other family members with similar symptoms	Phenotype (cardinal symptoms)	OMIM disease gene	Variant	Mutation nucleotide	Mutation protein	Inheritance	Associated disease	OMIM disease number
a) Mutations in known disease genes with concordant phenotype										
1	No	No	Severe global developmental delay, regression, dysmorphism	*del 15q11q13*	CNV	chr.15: 23,684,690–28,544,611x1		*De novo*	Angelman syndrome	# 105830
2	No	No	Severe global developmental delay, congenital cataract, microcephaly	*COL4A1*	Missense	c.1973C>A	p.Gly658Val	*De novo*	BSVD	# 607595
3	No	No	Mild global developmental delay, dystonia, gait disorder	*SGCE*	Nonsense	c.771_772delAT	p.Cys258Stop	*De novo*	DYT11	# 159900
4	No	No	Moderate global developmental delay, short stature, facial dysmorphism	*ANKRD11*	Nonsense	c.4886G>C	p.Ser1629Stop	*De novo*	KBG syndrome	# 148050
5	No	No	Severe global developmental delay, short stature, dysmorphism	*KAT6A*	Nonsense	c.1096G>A	p.Arg366Stop	*De novo*	MRD32	# 616268
6	No	No	Severe global developmental delay, spastic tetraplegic cerebral palsy, microcephaly	*RNASEH2B*	Missense	c.529G>A; c.529G>A	p.Ala177Thr; p.Ala177Thr	Autosomal recessive	AGS2	# 610181
7	Yes	No	Severe global developmental delay, regression, movement disorder, myelinization disorder	*RNASEH2C*	Missense	c.205G>A; c.205G>A	p.Arg69Trp; p.Arg69Trp	Autosomal recessive	AGS3	# 610329
8	No	No	Moderate global developmental delay, ataxia, stereotypes	*MYT1L*	Nonsense	c.223G>A	p.Arg75Stop	*De novo*	MRD39	# 616521
9	No	Yes	Severe global developmental delay, leukodystrophy, cerebellar hypoplasia	*TREX1*	Missense	c.290G>A; c.290G>A	p.Arg97His; p.Arg97His	Autosomal recessive	AGS1	# 225750
10	No	Yes	Mild global developmental delay, regression, hypotonia	*TH*	Missense	c.1273C>T; c.1096G>T	p.Glu425Lys; p.Leu366Met	Autosomal recessive	Segawa syndrome	# 605407
11	No	No	Severe global developmental delay, microcephaly, autism, stereotypes	*MBD5*	CNV	chr.2: 149,130,689–149,227,038		*De novo*	MRD1	# 156200
12	No	No	Mild global developmental delay, neurodegenerative disorder	*FA2H*	Missense	c.1T>G	p.Met1Leu	Autosomal/ recessive	SPG35	# 612319
13	Yes	No	Moderate global developmental delay, regression, neuropathy, pyloric stenosis	*ARSA*	Missense	c.679G>C	p.Arg227Gly	Autosomal/ recessive	MLD	# 250100

b) Mutations in known disease genes with extension of phenotype										
14	No	No	Severe global developmental delay, severe encephalopathy, dystonia	*GLE1*	Missense	c.1706G>A; c.1750C>T	p.Arg569His; p.Arg584Trp	Autosomal/ recessive	LCCS1, LAAHD	# 253310, # 611890
15	Yes	Yes	Severe global developmental delay, tapeto-retinal degeneration, coordination disorder	*RPIA*	Missense	c.627G>C; c.627G>C	p.Trp209Cys; p.Trp209Cys	Autosomal/ recessive	RPIA deficiency	# 608611
16	No	No	Moderate delay in motor development, connective tissue weakness	*IGHMBP2*	Missense	c.745G>A; c.61C>T	p.Asp249Asn; p.Arg21Cys	Autosomal/ recessive	DSMA1, CMT2S	# 604320, # 616155
17	No	No	Severe global developmental delay, hypo-tonia, hearing and vision deficiencies	*CHD2*	Missense	c.1854A>T	p.Glu618Asp	*De novo*	EEOC	# 615369
c) Mutations initially categorized as variants in candidate genes but reclassified as mutations in new disease genes in the course of the study										
18	No	No	Severe global developmental delay, micro-cephaly, muscular hypotonia	*CHAMP1*	Frameshift	c.1865_1866delAC	p.Asp622 Glufs*7	*De novo*	MRD40	# 616579
19	No	No	Moderate global developmental delay, short stature, deafness	*del Xq28 (SSR4, PLXNB3, SRPK3, IDH3G)*	CNV	chr.X: 153,034,617–153,060,208		*De novo*	CDG1Y	# 300934
20	Yes	No	Severe global developmental delay, cardiac abnormalities, muscular hypotonia, facial dysmorphism	*SON*	Frameshift	c.268delC	p.Ser90 Valfs*59	*De novo*	ZTTK syndrome	# 617140
21	No	No	Severe global developmental delay, cardiac abnormalities, epilepsy, renal cyst	*SON*	Frameshift	c.4055delC	p.Pro1352 Glnfs*14	*De novo*	ZTTK syndrome	# 617140

AGS2, Aicardi–Goutières syndrome 2; AGS3, Aicardi–Goutières syndrome 3; BSVD, brain small vessel disease with or without ocular anomalies; CDG1Y, congenital disorder of glycosylation type 1y; CMT2S, Charcot–Marie–Tooth disease, axonal, type 2S; CNV, copy number variations; DSMA1, spinal muscular atrophy, distal, autosomal recessive 1; DYT11, dystonia 11; EEOC, epileptic encephalopathy childhood onset; KBG syndrome, designation composed of the initials of the surnames of the first three patients described; LAAHD, lethal arthrogryposis with anterior horn cell disease; LCCS1, lethal congenital contracture syndrome 1; MLD, metachromatic leukodystrophy; MRD1, mental retardation, autosomal dominant 1; MRD32, mental retardation, autosomal dominant 32; MRD39, mental retardation, autosomal dominant 39; MRD40, mental retardation, autosomal dominant 40; OMIM, Online Mendelian Inheritance in Man; SPG35, spastic paraplegia, autosomal recessive 35; ZTTK syndrome, Zhu–Tokita–Takenouchi–Kim syndrome

eTABLE 4

Recommended management of treatment in the 21 patients with a genetic diagnosis

Patient	Phenotype (cardinal symptoms)	OMIM disease gene	Associated disease	Individually recommended measures
1	Severe global developmental delay, regression, dysmorphism	*del 15q11q13*	Angelman syndrome	Preventive (EEG monitoring initiated), symptomatic (speech therapy modified)
2	Severe global developmental delay, congenital cataract, microcephaly	*COL4A1*	BSVD	Preventive (ophthalmological monitoring modified, nephrological monitoring initiated)
3	Mild global developmental delay, dystonia, gait disorder	*SGCE*	DYT11	Curative (trihexyphenidyl), symptomatic (physiotherapy modified)
4	Moderate global developmental delay, short stature, facial dysmorphism	*ANKRD11*	KBG syndrome	None
5	Severe global developmental delay, short stature, dysmorphism	*KAT6A*	MRD32	Preventive (EEG, cardiological, ophthalmological, and orthopedic monitoring initiated), symptomatic (ergotherapy and speech therapy modified)
6	Severe global developmental delay, spastic tetraplegic cerebral palsy, microcephaly	*RNASEH2B*	AGS2	Preventive (cardiological monitoring discontinued, orthopedic and ophthalmological monitoring initiated), symptomatic (physiotherapy modified)
7	Severe global developmental delay, regression, movement disorder, myelinization disorder	*RNASEH2C*	AGS3	Preventive (neurological and ophthalmological monitoring initiated), symptomatic (physiotherapy adjusted)
8	Moderate global developmental delay, ataxia, stereotypes	*MYT1L*	MRD39	None
9	Severe global developmental delay, leukodystrophy, cerebellar hypotrophy	*TREX1*	AGS1	None
10	Mild global developmental delay, regression, hypotonia	*TH*	Segawa syndrome	Curative (L-Dopa), preventive (EEG monitoring initiated), symptomatic (physiotherapy adjusted, ergotherapy initiated)
11	Severe global developmental delay, microcephaly, autism, stereotypes	*MBD5*	MRD1	Preventive (EEG monitoring initiated)
12	Mild global developmental delay, neurodegenerative disorder	*FA2H*	SPG35	Preventive (EEG-, urological, ophthalmological, and orthopedic monitoring initiated), symptomatic (physiotherapy modified, baclofen/Botox discussed)
13	Moderate global developmental delay, regression, neuropathy, pyloric stenosis	*ARSA*	MLD	Curative (inclusion in an MLD study), preventive (referral for specialist consultation)
14	Severe global developmental delay, severe encephalopathy, dystonia	*GLE1*	LCCS1, LAAHD	None
15	Severe global developmental delay, tapetoretinal degeneration, coordination disorder	*RPIA*	RPIA deficiency	Preventive (EEG monitoring initiated), symptomatic (physiotherapy modified)
16	Moderate delay in motor development, connective tissue weakness	*IGHMBP2*	DSMA1, CMT2S	Symptomatic (physiotherapy modified)
17	Severe global developmental delay, hypotonia, hearing and vision deficiencies	*CHD2*	EEOC	Preventive (EEG monitoring initiated)
18	Severe global developmental delay, microcephaly, muscular hypotonia	*CHAMP1*	MRD40	Symptomatic (speech therapy modified)
19	Moderate global developmental delay, short stature, deafness	*del Xq28 (SSR4, PLXNB3, SRPK3, IDH3G)*	CDG1Y	Preventive (coagulation and EEG monitoring, gastroenterological referral initiated), symptomatic (physiotherapy modified)
20	Severe global developmental delay, cardiac abnormalities, muscular hypotonia, facial dysmorphism	*SON*	ZTTK syndrome	Preventive (nephrological and dental monitoring, hearing test initiated; avoidance of radiography), symptomatic (speech therapy modified)
21	Severe global developmental delay, cardiac abnormalities, epilepsy, renal cyst	*SON*	ZTTK syndrome	Preventive (nephrological and dental monitoring, hearing test initiated; avoidance of radiography), symptomatic (ergotherapy adjusted, speech therapy initiated)

AGS2, Aicardi–Goutières syndrome 2; AGS3, Aicardi–Goutières syndrome 3; BSVD, brain small vessel disease with or without ocular anomalies; CDG1Y, congenital disorder of glycosylation type 1y; CMT2S, Charcot–Marie–Tooth disease, axonal, type 2S; DSMA1, spinal muscular atrophy, distal, autosomal recessive 1; DYT11, dystonia 11; EEOC, epileptic encephalopathy childhood onset; KBG syndrome, designation composed of the initials of the surnames of the first three patients described; LAAHD, lethal arthrogryposis with anterior horn cell disease; LCCS1, lethal congenital contracture syndrome 1; MLD, metachromatic leukodystrophy; MRD1, mental retardation, autosomal dominant 1; MRD32, mental retardation, autosomal dominant 32; MRD39, mental retardation, autosomal dominant 39; MRD40, mental retardation, autosomal dominant 40; OMIM, Online Mendelian Inheritance in Man; SPG35, spastic paraplegia, autosomal recessive 35; ZTTK syndrome, Zhu–Tokita–Takenouchi–Kim syndrome

eTABLE 5

Overview of recommendations for management of treatment in the 21 patients with a genetic diagnosis

	Number of patients
Change in management	**17 (81.0%)**
Preventive measures:	14 (66.7%)
− Initiation of specific preventive measures recommended (e.g., monitoring [EEG, cardiological, ophthalmological, orthopedic], avoidance of radiography)	14 (66.7%)
− Initiation of specific preventive measures recommended (ophthalmological monitoring)	1 (4.8%)
− Discontinuation of specific preventive measures recommended (cardiological monitoring)	1 (4.8%)
Symptomatic measures:	13 (61.9%)
− Initiation of specific symptomatic measures recommended (ergotherapy, speech therapy)	2 (9.5%)
− Modification of specific symptomatic measures recommended (speech therapy, ergotherapy, physiotherapy)	13 (61.9%)
Curative measures:	3 (14.3%)
− Initiation of specific curative measures recommended (trihexyphenidyl, L-Dopa, inclusion in MLD study)	3 (14.3%)
No change in management	**4 (19.0%)**

EEG, Electroencephalography; L-Dopa, L-3,4-dihydroxyphenylalanine; MLD, metachromatic leukodystrophy