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Posted Date: 12 June 2024

doi: 10.20944/preprints202406.0760.v1

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Article

Rare-ID: The First Neonate Diagnostic Approach Using Genome Sequencing for Complex Pediatric Cases in a Greek Cohort

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Abstract: Newborn screening using biochemical tests is a widespread practice. However, the recent availability of genetic sequencing has enabled the rapid screening of many monogenic disorders. The purpose of our study was to assess the outcomes of whole exome sequencing (WES) and whole genome sequencing (WGS) as the primary newborn screening test. This cohort study enrolled 26 symptomatic neonates and infants exhibiting a spectrum of neurological symptoms and issues. Genome sequencing (GS) identified relevant diagnostic variants in 9 patients (point mutations, CNVs and one case of aneuploidy), suggesting that using GS as the primary newborn screening test in a general newborn population enhances traditional screening's detection capabilities. This evidence-based finding may support considering GS as a critical method for first-tier screening.

Keywords: newborn screening; neonates; WES; WGS; genome screening; genome sequencing

1. Introduction

Newborn screening (NS) strives to detect treatable disorders before symptoms appear, enabling early medical intervention. NS involves muscle tone and hearing tests, heart and respiratory rates. However, the cornerstone of neonatal screening involves collecting a blood sample to detect various metabolic, hematologic, endocrine, and hereditary disorders. Ideally, this sample is obtained within 24-48 hours of birth[1]. Blood is typically drawn from the infant's heel and placed on a filter paper card for processing (Guthrie's card)[2,3].

NS originated in the 1960s with the introduction of screening for phenylketonuria (PKU), which became widely implemented by the 1970s. In 1968, long before the era of modern genetic testing, Wilson and Jungner published criteria to determine the conditions for which carrier testing should be done[4]. It is remarkable that, more than 50 years later, the criteria are still relevant and useful for considering the design of expanded carrier screening (ECS) panels. As Sanger sequencing became available in the 1970s, the molecular cause of more diseases was revealed[5]. In 1989, the discovery of deleterious mutations in the *CFTR* gene as the underlying cause of cystic fibrosis (CF), opened the door to guidelines recommending that carrier screening for CF be widely offered in 1997[6]. The early 2000s marked a significant expansion of NS programs, propelled by the introduction of tandem mass spectrometry, enabling rapid and comprehensive screening for a broader range of diseases[7]. In 2001, the American College of Medical Genetics and Genomics (ACMG) together with the American Gynecological Association issued guidelines for screening for CF[8], and in 2004 for screening for fragile X[9]. During this timeframe, evidence emerged that spinal muscular atrophy (SMA) was prevalent in many ethnicities. In response, the ACMG in 2008 published guidelines for nationwide carrier screening for SMA[10]. In 2009, the first "expanded screening" platform was commercialized that allowed the option of simultaneous testing of multiple genes. In 2012, The National Human Genome Research Institute and the Eunice Kennedy Shriver National Institute of Child Health and Human Development launched a \$25 million grant program aimed at investigating the potential applications of genome sequencing (GS) NS. This initiative sought to promote studies addressing the complexities and potentials of integrating WES or WGS into newborn healthcare[3]. In 2016 thirteen ACMG guidelines for expanded screening were issued, and since 2017 we are discussing carrier testing in the era of genomic medicine[11].

The emergence of next-generation sequencing (NGS) has transformed the diagnostic approach to rare disorders. Before the advent of NGS, diagnosing these disorders often relied on sequential targeted genetic testing, which yielded a diagnosis in about half of the cases[12,13]. For the remaining undiagnosed patients, the journey to diagnosis was prolonged, with limited options beyond traditional Sanger sequencing [14,15]. Unlike the Sanger method, whole exome sequencing (WES) and whole genome sequencing (WGS) enable the simultaneous sequencing of millions of short fragments covering the coding and noncoding regions, respectively. This technological advancement has led to remarkable breakthroughs, with 25–50% of previously undiagnosed patients receiving a diagnosis and the identification of new disease genes [16–21]. The genomic data obtained through NGS not only facilitates precise diagnosis but also enables personalized medical management. Continuous technological advancements are anticipated to broaden the scope of NS, offering enhanced detection capabilities over time.

The purpose of our study was the detection of genes and genomic regions responsible for severe genetic disorders in neonates using the WES and WGS method.

2. Materials and Methods

We encountered a cohort of 26 neonates and infants under the age of 6 months, admitted to the neonatal intensive care unit (NICU), presenting with a variety of medical concerns, including hypotonia, hypotonia, respiratory problems, low birth weight, insufficient weight gain, feeding difficulties, structural brain abnormalities, dysmorphic facial features, microcephaly, neonatal convulsions, head tremors, encephalitis due to infection, metabolic disorders, skeletal abnormalities, congenital heart disease, pulmonary function disorders, gastrointestinal and ophthalmological disorders (Table 1).

Initially, WES was conducted for the first 17 neonates to unravel the genetic underpinnings of their conditions. Consequently, we adapted our protocol to leverage the comprehensive coverage provided by WGS, a decision substantiated by the notable increase in diagnostic yield observed in our initial findings. Our analytical approach was multifaceted, targeting a spectrum of genetic aberrations including point mutations, copy number variations (CNVs), mitochondrial DNA (mtDNA) variations, and nucleotide repeat expansions (WES: point mutations and indels, WGS: point mutations, indels, CNVs, mtDNA).

Table 1. Neonates and infants of the study, their phenotype and age of onset and referral.

A/a	WES/WG S	Phenotype	Age of symptoms	Age of referral
1	WES	Hypotonia, respiratory distress	birth	22d
2	WES	Hypotonia, brain abnormality (enlarged cisterna magna or cerebellomedullary cistern), short neck, livedo reticularis	20d	1.5m
3	WES	Microcephaly, hypodontia, non-febrile seizures, dysmorphic facial features, absent speech, lack of mobility, epileptic encephalopathy	10d	1m
4	WES	Metabolic acidosis, hypotonia, vomiting, diarrhea, endocrine disorders, disorders of intermediary metabolism	2d	1m
5	WES	Head/neck tremor	5.5 m	6m
6	WES	Neonatal convulsions, molybdenum cofactor deficiency, epileptic encephalopathy	birth	6d
7	WES	Trunk hypotonia, ventriculomegaly, limb hypertonia, head lag, MRI abnormalities (lateral ventricles, corpus callosum)	birth	22d
8	WES	Infantile spasms, epilepsy, leukoencephalopathy, MRI abnormalities, sensorineural hearing loss, feeding difficulties, EEG abnormalities	4d	40d
9	WES	COVID-19 encephalitis	5d	5d
10	WES	Lissencephaly	N/A	3.5m
11	WES	Hypoplasia/skeletal abnormalities of lower limbs, varus forefoot, cryptorchidism	birth	2d
12	WES	Near-miss sudden infant death syndrome, acute encephalopathy, convulsions, hypoglycemia, MRI abnormalities (cytotoxic edema, cerebral hemispheres, cerebellum)	5m	6m
13	WES	Low birth weight, convulsions, limb hypertonia, head lag, dysmorphic facial features, MRI abnormalities (basal ganglia, white matter), poor weight gain, small for gestational age (SGA), respiratory failure	birth	7m
14	WES	Extremely low birth weight, small for date infant, intrauterine growth restriction, dysmorphic features, saddle-nose deformity	birth	1m
15	WES	Neonatal convulsions	2d	20d
16	WES	Apnea, bradycardia with accompanying cyanosis	1m	2m
17	WES	Infantile spasms	3d	24d
18	WGS	Hypotonia, muscle weakness, micrognathia, congenital lower limb abnormalities, respiratory failure, feeding difficulties, jaundice, clubfoot, overstretching knee,	birth	10d

MRI abnormalities				
19	WGS	Feeding difficulties, trunk hypotonia, weight loss	9d	17d
20	WGS	Tachypnea, prolonged expiration breathing, wheezing	2d	55d
21	WGS	Infantile spasms	3d	38d
22	WGS	Focal seizures, hypotonia, epileptic encephalopathy, MRI abnormalities (basal ganglia), EEG abnormalities	16d	23d
23	WGS	Premature birth, neurodevelopmental delay, Duane type 3, mild eye contact and communication	5m	5m
24	WGS	Micrognathia, hypotonia upper limb, hypertonia lower limb, Failure to thrive, renal agenesis	5m	5m
25	WGS	Jaundice, thrombocytopenia, anemia, hepatosplenomegaly, intraventricular hemorrhage grade 2-3	birth	28d
26	WGS	Hypoxic ischemic encephalopathy, MRI abnormalities, EEG abnormalities, seizures, hypertonia, absence of breastfeeding reflexes (without congenital anomalies)	birth	56d

Massive parallel sequencing of the genome was performed using PCR-Free library preparation. In brief, genomic DNA samples extracted from peripheral blood underwent processing at the BGI facility. Subsequently, sequencing was conducted utilizing the BGI DNB sequencer (DNBseq) with paired-end sequencing reads of 150 base pairs. The average median sequence coverage was >35x. Raw data analysis involved a combined approach utilizing two algorithms: the Genome Analysis Toolkit (GATK)[22], known for its effectiveness in germline variant detection [23,24], and, and the DeepVariant (DeepV) method, which employs deep learning techniques for variant detection[25,26]. Research by AlDubayan et al. demonstrated that the integration of these algorithms produces highly accurate results, with sensitivity nearing 99.9%[27]. Additionally, several in silico tools were employed to predict variant pathogenicity (SIFT, LRT, MutationTaster, FATHMM, PROVEAN, MetaSVM, MetaLR, MetaRNN, M-CAP, PrimateAI, DEOGEN2, BayesDel addAF, BayesDel noAF, LIST-S2, FATHMM MKL Coding, FATHMM XF Coding).

Regarding WGS samples, variant identification of the CNVs, including insertions or deletions of one or more exons or large genomic regions (Del/Dups), was obtained from WGS analysis data using an inhouse bioinformatic analysis pipeline.

The evaluation of the annotated variants was conducted on proprietary platform (NsClinical - Neoscreen Genomics App). In order to validate genome sequencing results, we designed primers based on the paired end reads and performed Sanger sequenced.

3. Results

In our study, the diagnostic yield of genetic testing using WES or WGS in symptomatic neonates and infants was found to be 34.6%, mostly in infants presenting with congenital anomalies, hypotonia, and epilepsy, according to ACMG criteria, providing crucial insights that guided their clinical management and treatment strategies. GS detected diagnostic variants in 9 cases of the study (34.6%). These variants included point mutations (78%), CNVs (11%) and one case of aneuploidy (11%). Details on 9 individuals, disease causing variants found and disorders are presented in Table 2. The inheritance pattern included autosomal recessive-AR (44%), autosomal dominant-AD (n=56%). We didn't detect any x-linked disorder, which is of course due to randomly sampling. In 6 cases (patient numbers 1,2,3,8,17,24) variants found were compared to parental samples in order to determine if they were *de novo* or inherited from the biological parents. In cases 17 and 24 parental

sequencing showed that *KCNQ2* and *EHMT1* variants were not inherited (*de novo* variants). On the contrary, parents of patients in cases 1, 2, 3 and 8 found to be both heterozygous for the respective variant.

Table 2. Overview of Patient Information and Genetic Findings Neonates and infants of the study, disease causing variants found, inheritance patterns and the corresponding diagnosed disorders.

A/ a	Patient no	WES/W GS	Phenotype	Gene/Inherit ance	Disease causing variant	Disorder
1	1	WES	Hypotonia, respiratory distress	EXOSC3 AR, hom	c.92G>C, p.Gly31Ala	Pontocerebellar hypoplasia, type 1B
2	2	WES	Hypotonia, brain abnormality	EXOSC3 AR, hom	c.92G>C, p.Gly31Ala	Pontocerebellar hypoplasia, type 1B
3	3	WES	Microcephaly, dysmorphic facial features, epileptic encephalopathy	MOCS1 AR, hom	c.1508_1509del, p.Glu503Alafs*103	Molybdenum cofactor deficiency A
4	8	WES	Infantile spasms, hypotonia	ECHS1 AR, hom	c.476A>G, p.Gln159Arg	Mitochondrial short-chain enoyl-CoA hydratase 1 deficiency
5	9	WES	COCID-induced encephalitis infection	RANBP2 AD, het	c.1966A>G, p.Ile656Val	Encephalopathy, acute, infection-induced, 3, susceptibility to
6	17	WES	Infantile spasms	KCNQ2 AD, het <i>de novo</i>	c.1229dupC, p.Pro411Alafs*7	Developmental and epileptic encephalopathy 7 / Myokymia / Seizures, benign neonatal, 1
7	21	WGS	Infantile spasms	CNV 938kb, het (COL9A3, SLC17A9, CHRNA4, KCNQ2, EEF1A2, RTEL1)	del(20)(q13.33) chr20:62751315_63689 630del	Epilepsy, phsychomotor retardation, mild dysmorphic facial features, skeletal anomalies
8	23	WGS	Premature birth, neurodevelopmental delay, Duane syndrome type 3	Aneuploidy	Trisomy 9 mosaic	IUGR, mental retardation, developmental delay, dysmorphic features, low-set ears, microphthalmia, congenital heart disease, urogenital abnormalities, skeletal abnormalities, cetral nervus system

					defects (hydrocephaly, Dandy-Walker)
9	24	WGS	Micrognathia, hypotonia upper limb, hypertonia lower limb, failure to thrive	<i>EHMT1</i> AD, het <i>de novo</i>	c.3614C>A, p.Pro1205His Kleefstra syndrome 1

Case 1 refers to a 12-day-old male infant, born at 36 weeks by caesarean section. This neonate showed generalized hypotonia from birth and due to extremely reduced respiratory effort had to be intubated. According to the family history, the first two children died in infancy with a similar phenotype with congenital hypotonia. The first child was hospitalized in the NICU and died 12 months old, while the second child was also hospitalized and died after 2.5 months. Parents referred two other deaths during neonatal period and infancy of unexplained etiology in the family. Parents come from the same village, so consanguinity between them could not be excluded. Based on the entire clinical and family history, a genetic disease of AR inheritance was suspected.

WES revealed a pathogenic variant in *EXOSC3* gene in homozygosity, causing Pontocerebellar hypoplasia type 1b (OMIM#614678). It is a severe autosomal recessive neurologic disorder characterized by underdevelopment of the pons and cerebellum in the brain, along with severe motor deficits resulting from spinal cord motor neuron loss, hypotonia, respiratory and nutritional issues[28]. There is no targeted but only symptomatic treatment, the infant died in infancy. However, the diagnosis could help the couple for a subsequent pregnancy by giving the choice of prenatal or pre-implantation diagnosis.

Case 8 involved a 40-day-old male infant who experienced an episode of spasms in 4th day of life. The couple's history includes two miscarriages, and they have a healthy 3.5-year-old girl. WGS detected a pathogenic variant in the *ECHS1* gene in homozygosity, causing mitochondrial short-chain enoyl-CoA hydratase 1 deficiency. It is an autosomal recessive metabolic disease characterized by psychomotor and developmental delay, hypotonia, encephalopathy (Leigh-like), epilepsy [29]. Moreover, this enzyme found to be involved in the valine catabolism pathway. Diagnosis is important because exclusion of valine from patient's diet changes the outcome of the disease. Also, it could help the couple for a subsequent pregnancy by giving the choice of prenatal or preimplantation diagnosis.

It's worth noting that a case of Trisomy 9 with evidence of mosaicism (T9M) has also been reported (case 23). Trisomy 9 is an extremely rare chromosomal abnormality, frequently appearing in mosaic form. It has been associated with high neonatal mortality, while surviving individuals often experience severe intellectual disabilities [30,31]. Structural variants played a minor role, with case 21 being heterozygous for a CNV of 938 kb (deletion) that disrupted *COL9A3*, *SLC17A9*, *CHRNA4*, *KCNQ2*, *EEF1A2*, *RTEL1*, contributing to her phenotype (Table 2).

Thorough genetic counseling was provided to all infants' families, and potential treatment implications were discussed with the referring physician. In 33% of cases, the diagnosis aided in treatment selection for treatable conditions, while in all cases, it enhanced the overall quality of family life by preventing consanguinity, avoiding diagnostic challenges, and providing valuable information regarding reproductive options.

4. Discussion

NS is a population-wide initiative aimed at alleviating the impact of diseases that substantially affect neonates. We stand at the threshold of a new era in neonatal screening, where genomics is anticipated to emerge as a pivotal component alongside metabolomics. The key advantage of WES and WGS lies in their ability to examine a wide array of diseases for early intervention without limitations. The widespread integration of WES and WGS into clinical practice has enhanced the diagnosis of rare Mendelian disorders. By identifying pre-symptomatic children, WES and WGS

enable the exploration of therapeutic interventions aimed at averting or mitigating disabilities. Moreover, they offer valuable data for advancing the development of new pharmaceutical treatments through research initiatives. While WES is not commonly applied in newborns, its application in cases of critically ill infants has demonstrated a considerable diagnostic yield for genetic disorders, thus impacting their management and prognosis[32,33].

Numerous studies indicate that the diagnostic yield of WES typically ranges from 25% to 35%, with an average of 28% based on aggregated data[34,35], while WGS consistently demonstrates diagnostic rates ranging from 40% to 60%, with an average of 49% [18,20,36–49]. This signifies an almost two fold increase in diagnostic efficacy when choosing WGS over WES, as WGS enables the identification of single-nucleotide variants, small insertions/deletions, CNVs, balanced and complex structural variations, multiple repeat expansions, and mitochondrial variants[21,40,50–52]. This broader scope enables the examination of intronic, intergenic, and regulatory regions implicated in diseases often overlooked by other testing methods. In our study, if we had conducted WGS in all cases, we might have achieved an even greater diagnostic yield.

Despite their initial higher cost, the decreasing expenses of sequencing and the capacity of WES, and even more WGS, to supplant multiple testing methods like Chromosomal Microarray Analysis (CMA) and mtDNA sequencing, leading to reduced overall healthcare expenditures by replacing sequential genetic tests with a single comprehensive assay, not only resulting in quicker diagnoses and improved patient outcomes, but also reducing the financial strain on their families[53–56]. However, further research is necessary to evaluate the cost-effectiveness of this strategy.

Genetic NS holds significant promise, but it also raises several important questions and concerns, for instance over the privacy of a child's genetic information and how it could be used in the future. One of the key areas of debate is which diseases should be included in the screening program. Should it strictly focus on those conditions that have a known treatment, or should it also include diseases where early detection could offer benefits such as access to social care and support, or regular monitoring for possible complications? Additionally, there may be disparities in access to genetic screening and treatment based on socioeconomic status or geographic location. Another area of concern is how to handle findings related to adult-onset diseases or those with incomplete penetrance or incidental findings. Should there be included in the conclusion of the screening report? This raises ethical dilemmas, as some individuals may carry rare potentially pathogenic variants that could be overlooked in a healthy adult but raise concerns when found in an asymptomatic newborn[57]. There is also a risk of false alarms in such cases, which could lead to unnecessary anxiety, confusion, and additional tests for the family. Therefore, careful consideration and clear guidelines are needed to navigate these complex issues and ensure that genetic newborn screening is both effective and ethical[58][59].

Overall, this cohort underscores the critical importance of prompt and precise genetic diagnoses in ensuring optimal healthcare delivery. Our findings indicate that WES/WGS should be considered as the main diagnostic tool for genetic disorders in newborns and infants.

Author Contributions: Conceptualization: Y. L. Loukas; Methodology: Y. L. Loukas, K. Anagnostopoulou, G.Thodi, A. Dinopoulos; Software: Y. L. Loukas, C. Gavalas; Validation: Y. L. Loukas, K. Anagnostopoulou, G.Thodi; Formal analysis: Y. L. Loukas, K. Anagnostopoulou, G.Thodi; Investigation: Y. L. Loukas, K. Anagnostopoulou, G.Thodi, A. Dinopoulos; Resources: Y. L. Loukas, K. Anagnostopoulou, G.Thodi, A. Dinopoulos, M. Spanou, R. Pons, K. Tziouvas, G. Vartzelis, E. Skouteli, E. Loukatou, A. Charitou, K. Douros, S. Sihanidou, M. Giorgi, A. Stephanede, M. Angeli, M. Nikolaidou, E. Kokkinou, I. Kouri, V. Koute; Data curation: Y. L. Loukas, K. Anagnostopoulou, G.Thodi, A. Dinopoulos, M. Spanou, R. Pons, K. Tziouvas, G. Vartzelis, E. Skouteli, E. Loukatou, A. Charitou, K. Douros, S. Sihanidou, M. Giorgi, A. Stephanede, M. Angeli, M. Nikolaidou, E. Kokkinou, I. Kouri, V. Koute; Writing-Original Draft Preparation: M. Alvanou; Writing-Review and Editing: Y. L. Loukas, K. Anagnostopoulou, G.Thodi, A. Dinopoulos, M. Spanou, R. Pons, K. Tziouvas, G. Vartzelis, E. Skouteli, E. Loukatou, A. Charitou, K. Douros, S. Sihanidou, M. Giorgi, A. Stephanede, M. Angeli, M. Nikolaidou, E. Kokkinou, I. Kouri, V. Koute; Visualization: Y. L. Loukas; Supervision: Y. L. Loukas, A. Dinopoulos; Project Administration: Y. L. Loukas, A. Dinopoulos; Funding Acquisition: Y. L. Loukas.

Funding: The Rare-ID project is fully funded by the Single Action State Aid regarding of Research, Technological Development & Innovation “RESEARCH – CREATE – INNOVATE”.

Informed Consent Statement: Informed consent was obtained from the parents of all subjects involved in the study.

Acknowledgments: The Rare-ID project is fully funded by the Single Action State Aid regarding of Research, Technological Development & Innovation “RESEARCH – CREATE – INNOVATE”, Partnership Agreement (PA) 2014-2020 with the contribution of significant resources originating from the European Structural and Investment Funds (ESIF) of the European Union.

Conflicts of interest: The authors declare no conflict of interest.

Abbreviations

ACMG	<i>American College of Medical Genetics and Genomics</i>
CF	<i>cystic fibrosis</i>
CMA	<i>chromosomal microarray analysis</i>
CNVs	<i>copy number variations</i>
ECS	<i>expanded carrier screening</i>
GS	<i>genome sequencing</i>
mtDNA	<i>mitochondrial DNA</i>
NGS	<i>next-generation sequencing</i>
NICU	<i>neonatal intensive care unit</i>
NS	<i>newborn screening</i>
PKU	<i>phenylketonuria</i>
SMA	<i>spinal muscular atrophy</i>
VUS	<i>variant of uncertain significance</i>
WES	<i>whole exome sequencing</i>
WGS	<i>whole genome sequencing</i>

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