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ORIGINAL ARTICLE

Supplementary testing after negative or inconclusive exome sequencing results

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ABSTRACT

Background: Accurate diagnosis benefits patients and their families by directing clinical management; predicting recurrence risks; providing prognosis; and preventing the invasive, time-consuming, and costly diagnostic odyssey. The present study aimed at evaluating the usefulness and clinical utility of supplementary testing (deletion/duplication, targeted genome methylation analysis, and whole mitochondrial genome testing) after inconclusive or negative exome results and the outcome of the supplementary testing.

Methods: A total of 3,505 clinical exome sequencing results were evaluated, and cases with supplementary testing were analyzed for the accuracy and validity of the supplementary testing.

Results: The present study cohort comprised 26 cases where supplementary testing was ordered (12 inconclusive results and 14 negative results). Out of the 12 inconclusive results, only one case was positive for supplementary testing (1/12) and none of the negative cases (0/14).

Conclusion: For most cases, supplementary testing to negative exome sequencing failed to identify any possible explanation of the disorder, concluding that supplementary testing has limited clinical utility.

Keywords: Exome sequencing, deletion duplication, methylation, mitochondrial genome, Saudi Arabia.

Introduction

Accurate diagnosis benefits patients and their families by directing clinical management; predicting recurrence risks; providing prognosis; and preventing the invasive, time-consuming, and costly diagnostic odyssey. Apart from its tangible benefits, confirming a clinical diagnosis is therapeutic for the patient and the family. Establishing diagnosis in patients with complex disorders involves a stepwise approach from history taking to physical examination, with further complementary tests such as radiography and metabolite analysis, and genetic testing. However, many patients who undergo extensive genetic testing still need to be diagnosed. Exome sequencing (ES) became one of the leading diagnostic tools for genetic diseases, with a hit rate ranging from 25% to 58% (1,2). Also, ES provides further advantages, faster results, and a cost-efficient testing strategy (3,4). ES is a powerful tool to end a diagnostic odyssey. However, limitations of the current practice of ES include limited detection of copy number variation (CNV) changes based on the used bioinformatics pipeline, limited detection of variation in the mitochondrial genome and inability to detect methylation changes. Performing CNV analysis increases the diagnostic yield of ES cases by 4.2% (1). And while methylation testing is a powerful tool in cancer diagnosis with a 95% accuracy in predicting cancerous versus normal tissue (5) according to a recent study tested the utility of a new diagnostic network for methylation testing in mendelian disorders, a hit rate of 27.6% (57/207) was achieved (6). For the mitochondrial genome (mtDNA), a study published in 2018 showed that the mitochondrial genome test hit rate was 20% (23/117) (7). Furthermore, in a previous study, we showed that genome sequencing (GS) has limited clinical utility compared to the re-analysis of ES raw data (1). In this study, we aim to evaluate the usefulness and clinical utility of further testing beyond or after

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inconclusive or negative exome results and the outcome of supplementary testing, including deletion/duplication, targeted methylation testing and mitochondrial genome analysis post-negative or inconclusive ES results.

Subjects and Methods

A total of 3505 ES cases were evaluated during from 2018 to 2020. Testing was done either in-house or at other CAP-accredited laboratories. Analysis was done for patients at King Abdulaziz Medical City, Ministry of National Guard - Health Affairs, Riyadh. All clinical reports were investigated, and all cases with supplementary testing were further evaluated for accuracy and validity of the testing after the ES results (initial exome result, date of testing, clinical chart note documenting the requested test, indication of the further testing) (Figure 1). Only cases with high suspicion of specific phenotypes and negative or inconclusive exome results are considered in this analysis. Inconclusive results mean that a possible explanation of the primary indication of testing might be detected but would still need to fully explain the phenotype from a molecular point of view. For example, one pathogenic or likely pathogenic variant in a gene is related to the disorder clinically.

However, only one variant is detected, and the disorder is an autosomal recessive disorder. A negative result means ES failed to identify any variants that would explain the phenotype. The supplementary testing included in this study is one of three, either deletion/duplication analysis for a specific gene, which means a change in gene dosage that can not always be detected with next-generation sequencing testing. Deletion/duplication analysis includes looking at a missing or duplicated part of that gene in one or both strands. This is usually triggered by inconclusive exome results or a high suspicion of the specific diagnosis. The second form of supplementary testing is targeted methylation analysis which explores the epigenetic modification that causes changes in genetic regulation secondary to the addition of methyl group to the DNA, which can cause changes to DNA expression without altering the DNA sequence (8), with clinical presentation suspecting disorder related to methylation defect. The third form of supplementary testing is whole mitochondrial genome testing by analyzing mitochondrial DNA, which is of maternal origin. The mitochondria are known as the powerhouse of the cell. Changes in energy production can cause disease. This is indicated by either family history, clinical phenotype or abnormal basic laboratory testing like high lactate (9). All supplementary testing must be done at a clinical laboratory and clinical grade analysis. All research results are excluded from this study. This study was approved by the Institutional Research Board of King Abdullah International Medical Research Center, #RC19/315/R.

Results

Out of the 3,505 ES cases, there were 26 cases where supplementary testing was requested due to either inconclusive or negative exome results with high suspicion of a specific phenotype.

Deletion/duplication analysis

Out of the 26 samples we reviewed, 16 cases with either inconclusive or negative exome results in 10 cases with inconclusive ES results identified where a heterozygous variant in a gene with an autosomal recessive mode of inheritance has been detected and could explain the phenotype (Table 1: cases 1-10). However, ES failed to identify the second pathogenic variant, and supplementary testing was requested. Only 1/16 (6.25%) cases were positive for deletion/duplication analysis. Case number 5 (Table 1: case 5) presented with abnormal renal morphology with enlarged kidney and neonatal hypoglycemia with abnormal newborn screening suggestive of medium-chain acyl-CoA dehydrogenase deficiency (MCAD) (OMIM #201450), ES identifies one heterozygous, pathogenic variant in *ACADM* gene, NM_001286043.1:c.715C>T, p.(Arg239Cys), deletion/duplication analysis of *ACADM* (Figure 2) identified heterozygous deletion encompasses exon 8 of *ACADM* gene and establishes the diagnosis of MCAD. However, in 15/16 cases, the supplementary analysis results of deletion-duplication analysis were negative, for example, case number 1 (Table 1: case 1), which was tested for deletion-duplication after the inconclusive ES result presented with hypothyroidism, elevated liver enzymes and diabetes mellitus, ES showed heterozygous pathogenic variant in *ATP8B1* and associated with benign recurrent intrahepatic cholestasis (OMIM #243300), due to these results and the clinical presentation of elevated liver enzymes, the physician ordered deletion -duplication testing to exclude heterozygous CNV changes that ES did not detect. However, results are negative for deletion or duplication of the *ATP8B1* gene. A complete list of all cases is present in Table 1.

Targeted methylation analysis

Targeted methylation testing was requested in 6 cases (Table 1: cases 17-22) after initial ES results, 5 cases had negative initial ES results, and 1 was inconclusive when the initial ES testing was done. For the five negative ES cases, Silver-Russell syndrome was suspected in four cases (Table 1: Cases 18, 20-22). The four cases presented with multiple phenotypes related to Silver-Russell syndrome, including intrauterine growth retardation, small for gestational age, premature birth, short stature, abnormal facial shape, abnormal skull morphology, abnormal heart morphology, vertebral segmentation defect, failure to thrive and global developmental delay, but all four cases had negative results. The last case (Table 1: case 19) presented with delayed speech and language development, hyperactivity, intellectual disability and muscular hypotonia and was tested for Angelman syndrome but was also negative. Furthermore, an additional ES case was inconclusive for an unrelated homozygous variant in the *C1QA* gene (Table 1: case 17). However, based on the patient's phenotype, the clinician did not pursue further testing on that gene and instead tested for Russel Silver methylation, but unfortunately, the result was negative.

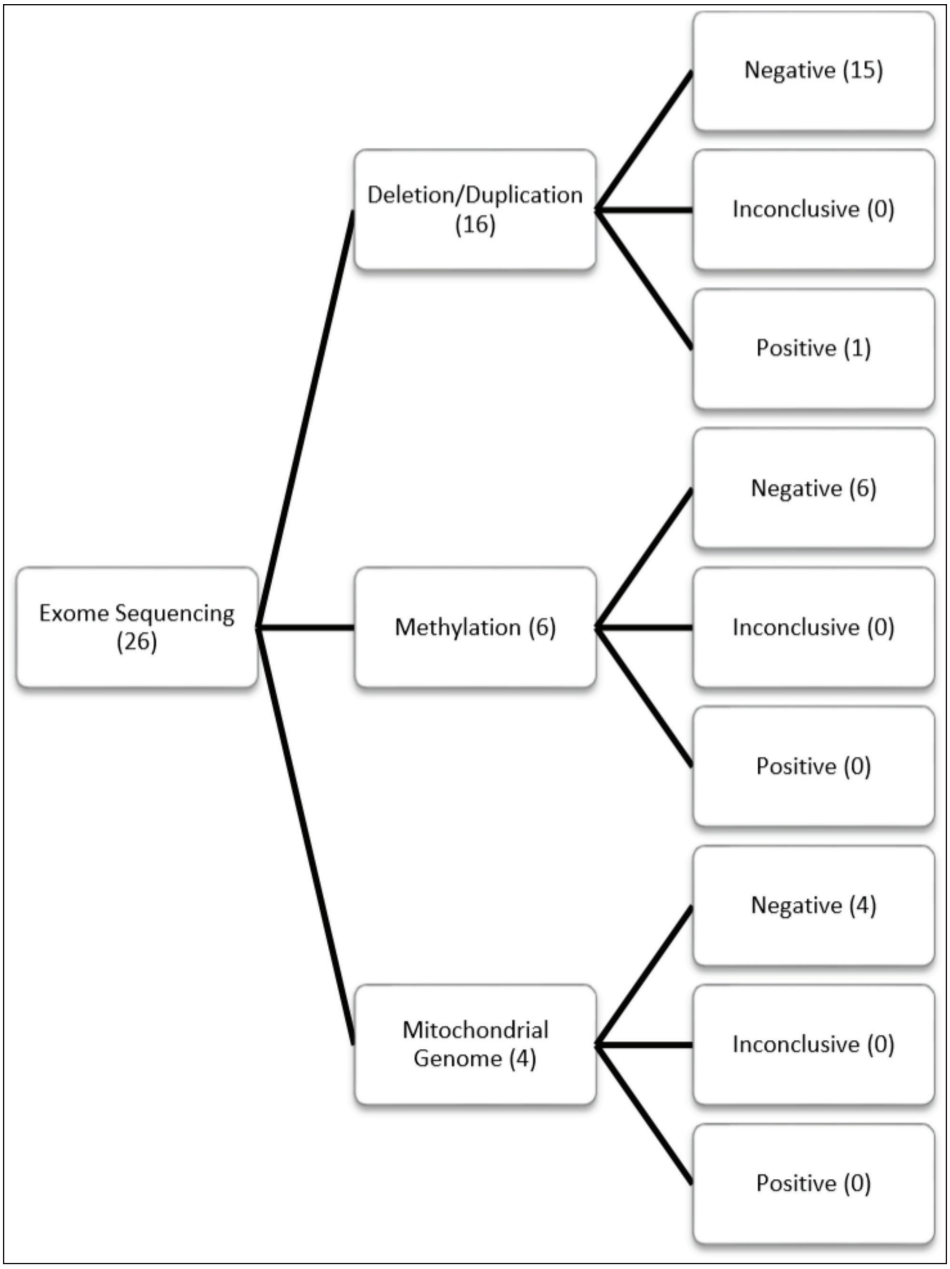

Figure 1. *Number of patients that underwent supplementary testing after ES flow diagram.*

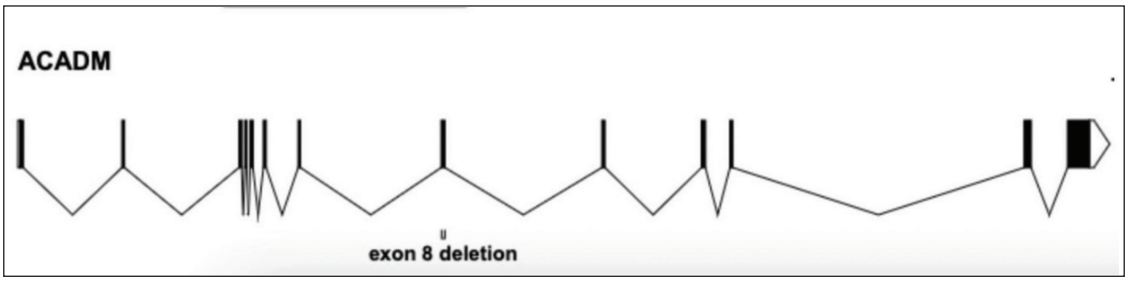

Figure 2. *ACADM: exon 8 deletion.*

Mitochondrial genome

There were 4 cases in total tested for mitochondrial genome after the ES results (Table 1: case 23-26). Three of these cases (Table 1: case 24-26) were ES-negative and were followed by mitochondrial testing. However, all 3 had negative results. Phenotypically all three cases were heterogeneous and had no specific phenotype. For instance, one case (Table 1: case 24) presented with lactic acidosis, and another presented with hypoglycemia (Table 1: case 25). Moreover, a case (Table 1: case 23) was ES inconclusive for the *GRIN2A* gene. However,

Table 1. *26 cases with initial test and supplementary testing.*

Case number	HPO	Initial test	Initial result	Initial result	Supplementary test	Supplementary test result
1	Hypothyroidism (HP:0000821), Elevated liver enzymes (HP:0002910), Diabetes mellitus (HP:0000819)	ES	Inconclusive	Heterozygous, pathogenic (PS4, PM2, PP2, PP3, PP4, PP5) variant in ATP8B1 gene NM_005603:c.1594G>A, p.(Ala532Thr), (AR)	ATP8B1 Deletion-Duplication	Negative
2	Direct hyperbilirubnemia (HP:0002904), Rickets (HP:0002748)	ES	Inconclusive	Heterozygous, VUS (PM2, PP2, PP3) variant in ABCB11 gene NM_003742.2:c.2092C>T, p.(Arg698Cys), (AR)	ABCB11 Deletion-Duplication	Negative
3	Apnea (HP:0002104), Hypotonia (HP:0001290), gastro-esophageal reflux (HP:0002020), vesicoureteral reflux (HP:0000076)	ES	Inconclusive	Heterozygous, pathogenic (PS4, PM2, PP1, PP3, PP4, PP5) variant in POMT1 gene NM_007171.3:c.1241+4_1241+7del, (AR)	POMT1 Deletion-Duplication	Negative
4	Bone marrow failure (HP:0005528)	ES	Inconclusive	Heterozygous, VUS (PM2,PP2) variant in FANCA gene NM_000135.2:c.3697G>A, p.(Ala1233Thr), (AR) Heterozygous, VUS variant in FANCG gene NM_004629.1:c.1156C>G, p.(Pro386Ala), (AR) Heterozygous, VUS (PM2,BP4) variant in PALB2 gene NM_024675.3:c.1102A>T, p.(Asn368Tyr), (AR,AD)	FANCA, FANCG, PALB2 Deletion-Duplication	Negative
5	Abnormal renal morphology (HP:0012210), Abnormal renal physiology (HP:0012211), Enlarged kidney (HP:0000105), Hyperinsulinemic hypoglycemia (HP:0000825), Hypoglycemia (HP:0001943), Lethargy (HP:0001254), Multicystic kidney dysplasia (HP:0000003), Neonatal hypoglycemia (HP:0001998)	ES	Inconclusive	Heterozygous, pathogenic (PP5, PM2, PM5, PP2, PP3) variant in ACADM gene NM_001286043.1:c.715C>T,p.(Arg239Cys), (AR)	ACADM gene Deletion-Duplication Heterozygous deletion encompassing exon 8 of the ACADM gene	Positive
6	Facial dysmorphism (HP:0001999), global developmental delay (HP:0001263), agenesis of corpus callosum (HP:0001274) , seizure disorder (HP:0001250), tetralogy of Fallot (HP:0001525), congested lung with frequent lung collapse (HP:0002107)	ES	Inconclusive	Heterozygous, likely pathogenic variant in SMG9 gene NM_019108.3:c.701+4A> G, (AR)	SMG9 gene Deletion-Duplication	Negative
7	Abnormality of blood and blood-forming tissues (HP:0001871), Behavioral abnormality (HP:0000708), Bone marrow hypocellularity (HP:0005528), Depressivity (HP:0000716), Fever (HP:0001945), Leukopenia (HP:0001882), Specific learning disability (HP:0001328)	ES	Inconclusive	Heterozygous, VUS (PM2, PP3) variant in NBN gene NM_002485.4:c.737G>A, p.(Gly246Asp), (AR)	NBN gene Deletion-Duplication	Negative

Continued

Case number	HPO	Initial test	Initial result	Initial result	Supplementary test	Supplementary test result
8	Dysmorphic features (HP:0001999), hydrocephalus (HP:0000238), large VSD (HP:0001629), multicystic dysplastic left kidney (HP:0000003), ectopic echogenic right kidney (HP:0000086), repaired inguinal hernia (HP:0000023), right periventricular white matter punctate lesions.	ES	Inconclusive	Heterozygous, pathogenic (PS4, PM2, PM5, PP2, PP3) variant in FKRP gene NM_001039885.2:c.898G>A, p.(Val300Met), (AR)	FKRP gene Deletion-Duplication	Negative
9	Abnormality of the cerebral white matter, cafe-au-lait spot (HP:0000957), delayed fine motor development (HP:0010862), delayed gross motor development (HP:0002194), delayed speech and language development (HP:0000750), global developmental delay (HP:0001263), hyporeflexia (HP:0001265), infantile-onset (HP:0003593), motor delay (HP:0001270), muscular hypotonia (HP:0001252), pointed chin (HP:0000307), protruding ear (HP:0000411), triangular face (HP:0000325).	ES	Inconclusive	Heterozygous, pathogenic (PP5, PM1, PM2, PM5, PP2, PP3) variant in SMPD1 gene NM_000543.4:c.1267C>T, p.(His423Tyr), (AR)	SMPD1 gene Deletion-Duplication	Negative
10	Abnormal facial shape (HP:0001999), Brachydactyly (HP:0001156), Central hypotonia (HP:0001252), Depressed nasal bridge (HP:0005280), Generalized hypotonia (HP:0001290), Global developmental delay (HP:0001263), Hypertelorism (HP:0000316), Left ventricular outflow tract obstruction (HP:0032092), Left-to-right shunt (HP:0012382), Low-set ears (HP:0000369), Mitral regurgitation (HP:0001653), Patent ductus arteriosus (HP:0001643), Patent foramen ovale (HP:0001655), Sandal gap (HP:0001852), Single transverse palmar crease (HP:0000954), Systolic heart murmur (HP:0031664), Tricuspid regurgitation (HP:0005180), Umbilical hernia (HP:0001537), Upslanted palpebral fissure (HP:0000582)	ES	Inconclusive	Heterozygous, pathogenic (PVS1, PP5, PM2, BP4) variant in ACADVL gene NM_001270447.1:c.134C>A, p.(Ser45*), (AR)	ACADVL gene Deletion-Duplication	Negative

Continued

Case number	HPO	Initial test	Initial result	Initial result	Supplementary test	Supplementary test result
11	Adrenal Insufficiency (HP:0000846), Recurrent hypoglycemia (HP:0001988)	ES	Negative	Negative	TBX19 gene Deletion-Duplication	Negative
12	Atrial septal defect (HP:0001631), Hydronephrosis (HP:0000126), congenital bilateral hip dislocation (HP:0008780), supraventricular tachycardia (HP:0004755)	ES	Negative	Negative	KCNQ1 gene Deletion-Duplication	Negative
13	11 pairs of ribs (HP:0000878), Abnormal facial shape (HP:0001999), Abnormality of the dentition (HP:0000164), Abnormality of the nasal bridge (HP:0000422), High, narrow palate (HP:0002705), Kyphosis (HP:0002808), Large fontanelles (HP:0000239), Low-set ears (HP:0000369), Metatarsus adductus (HP:0001840), Microretrognathia (HP:0000308), Muscular hypotonia (HP:0001252), Poor head control (HP:0002421), Retrognathia (HP:0000278), Talipes equinovarus (HP:0001762), Webbed neck (HP:0000465), Wide intermamillary distance (HP:0006610)	ES	Negative	Negative	SMN1 gene Deletion-Duplication	Negative
14	Abnormal enzyme/coenzyme activity (HP:0012379), Bacteremia (HP:0031864), Cesarean section, Failure to thrive (HP:0001508), Fever (HP:0001945), Food intolerance (HP:0012537), Gastroparesis (HP:0002578), Laryngomalacia (HP:0001601), Left ventricular hypertrophy (HP:0001712), Muscular hypotonia (HP:0001252), Muscular hypotonia of the trunk (HP:0008936), Patent ductus arteriosus (HP:0001643), Patent foramen ovale (HP:0001655), Polyhydramnios (HP:0001561), Small for gestational age (HP:0001518), Stridor (HP:0010307), Vocal cord paralysis (HP:0001605), Vomiting (HP:0002013).	ES	Negative	Negative	SMN1 gene Deletion-Duplication	Negative

Continued

Case number	HPO	Initial test	Initial result	Initial result	Supplementary test	Supplementary test result
15	Abnormality of lateral ventricle (HP:0030047), Abnormality of the choroid plexus (HP:0007376), Abnormality of the musculature (HP:0003011), Absent speech (HP:0001344), Delayed ability to walk (HP:0031936), Delayed gross motor development (HP:0002194), Feeding difficulties (HP:0011968), Full cheeks (HP:0000293), Generalized hypotonia (HP:0001290), Global developmental delay (HP:0001263), Growth delay (HP:0001510), Intracranial hemorrhage (HP:0002170), Moderate global developmental delay (HP:0011343), Muscle weakness (HP:0001324), Neonatal hypotonia (HP:0001319), Polyhydramnios (HP:0001561), Premature birth (HP:0001622), Proptosis (HP:0000520), Skeletal muscle atrophy (HP:0003202), Small for gestational age (HP:0001518), Triangular mouth (HP:0000207), Turricephaly (HP:0000262), Wide nasal bridge (HP:0000431)	ES	Negative	Negative	SMN1 gene Deletion-Duplication	Negative
16	Ataxia (HP:0001251), Brachycephaly (HP:0000248), Broad-based gait (HP:0002136), Delayed fine motor development (HP:0010862), Delayed gross motor development (HP:0002194), Delayed speech and language development (HP:0000750), Dermoid cyst (HP:0025247), Frequent falls (HP:0002359), Gait ataxia (HP:0002066), Generalized-onset seizure (HP:0002197), Global developmental delay (HP:0001263), Hirsutism (HP:0001007), Hyperalaninemia (HP:0003348), Lactic acidosis (HP:0003128), Long eyelashes (HP:0000527), Mongolian blue spot (HP:0011369), Motor delay (HP:0001270), Neonatal onset (HP:0003623),	ES	Negative	Negative	SYNE1 gene Deletion-Duplication	Negative

Continued

Case number	HPO	Initial test	Initial result	Initial result	Supplementary test	Supplementary test result
	Neonatal respiratory distress (HP:0002643), Periorbital dermoid cyst (HP:0030668), Premature birth (HP:0001622), Seizures (HP:0001250), Small for gestational age (HP:0001518), Synophrys (HP:0000664), Tremor (HP:0001337), Triangular face (HP:0000325)					
17	Deeply set eye (HP:0000490), Frontal bossing (HP:0002007), Hypodontia (HP:0000668), Micrognathia (HP:0000347), Pointed chin (HP:0000307), Prominent supraorbital ridges (HP:0000336), Short nose (HP:0003196), Systemic lupus erythematosus (HP:0002725), Thin upper lip vermilion (HP:0000219), Triangular face (HP:0000325)	ES	Inconclusive	Homozygous, VUS (PM1,PM2,PP5) variant in C1QA gene NM_001347465.1:c.470G>A p.(Gly 157Asp), (AR)	Russell Silver Methylation	Negative
18	Abnormal heart morphology (HP:0001627), abnormality of the genitourinary system (HP:0000119), atrial septal defect (HP:0001631), hydronephrosis (HP:0000126), medullary nephrocalcinosis (HP:0012408), nephrocalcinosis (HP:0000121), premature birth (HP:0001622), scoliosis (HP:0002650), small for gestational age (HP:0001518), ventricular septal defect (HP:0001629), vertebral fusion (HP:0002948), vertebral segmentation defect (HP:0003422)	ES	Negative	Negative	Russell Silver Methylation	Negative
19	Attention deficit hyperactivity disorder (HP:0007018), Autism (HP:0000717), Autistic behavior (HP:0000729), Delayed speech and language development (HP:0000750), Hyperactivity (HP:0000752), Intellectual disability (HP:0001249), mild Joint hypermobility (HP:0001382), Muscular hypotonia (HP:0001252), Pulmonic stenosis (HP:0001642), Systolic heart murmur (HP:0031664), Violent behavior (HP:0008760)	ES	Negative	Negative	Angelman Methylation	Negative

Continued

Case number	HPO	Initial test	Initial result	Initial result	Supplementary test	Supplementary test result
20	Abnormal facial shape (HP:0001999), Abnormal skull morphology (HP:0000929), Clubbing (HP:0001217), Delayed ability to sit (HP:0025336), Delayed ability to walk (HP:0031936), Delayed fine motor development (HP:0010862), Delayed speech and language development (HP:0000750), Dry skin (HP:0000958), Ectopic kidney (HP:0000086), Growth hormone deficiency (HP:0000824), Motor delay (HP:0001270), Reduced bone mineral density (HP:0004349), Reduced subcutaneous adipose tissue (HP:0003758), Severe failure to thrive (HP:0001525), Short nail (HP:0001799), Short stature (HP:0004322)	ES	Negative	Negative	Russell Silver Methylation	Negative
21	Abnormal liver morphology (HP:0410042), Abnormal pulmonary valve morphology (HP:0001641), Abnormal respiratory system morphology (HP:0012252), Abnormal skull morphology (HP:0000929), Abnormality of the gallbladder (HP:0005264), Abnormality of the ribs (HP:0000772), Cesarean section, Global developmental delay (HP:0001263), Hyperechogenic kidneys (HP:0004719), Inguinal hernia (HP:0000023), Intrauterine growth retardation (HP:0001511), Muscular hypotonia (HP:0001252), Nasogastric tube feeding (HP:0040288), Nephrolithiasis (HP:0000787), Oral-pharyngeal dysphagia (HP:0200136), Patent ductus arteriosus (HP:0001643), Poor suck (HP:0002033), Premature birth (HP:0001622), Recurrent lower respiratory tract infections (HP:0002783), Renal hypoplasia/aplasia (HP:0008678), Respiratory distress (HP:0002098), Severe failure to thrive (HP:0001525), Small for gestational age (HP:0001518)	ES	Negative	Negative	Russell Silver Methylation	Negative

Continued

Case number	HPO	Initial test	Initial result	Initial result	Supplementary test	Supplementary test result
22	Blue nevus (HP:0100814), Coxa valga (HP:0002673), Cyanosis (HP:0000961), Delayed speech and language development (HP:0000750), Diarrhea (HP:0002014), Dry skin (HP:0000958), Dyspnea (HP:0002094),(HP:0100559), Premature birth (HP:0001622), Respiratory failure requiring assisted ventilation (HP:0004887), Snoring (HP:0025267), Lower limb asymmetry (HP:0100559), Eczema (HP:0000964) Elevated alkaline phosphatase (HP:0003155), Elevated serum aspartate aminotransferase (HP:0031956), Fetal distress (HP:0025116), Flushing (HP:0031284), Global developmental delay (HP:0001263), Lactic acidosis (HP:0003128), Low anterior hairline (HP:0000294)	ES	Negative	Negative	Russell Silver Methylation	Negative
23	Abnormality of higher mental function (HP:0011446), elevated urinary 3-hydroxybutyric acid (HP:0040155), hepatic steatosis (HP:0001397), hypoglycemia (HP:0001943), increased circulating free fatty acid level (HP:0030781), recurrent hypoglycemia (HP:0001988), seizure (HP:0001250), tonic seizure (HP:0032792)	ES	Inconclusive	Heterozygous, VUS (PM2, PP2, PP3) variant in GRIN2A gene NM_000833.3:c.905C>T p.(Ala302Val), (AD)	Mitochondrial Genome	Negative
24	Ataxia (HP:0001251), Brachycephaly (HP:0000248), Broad-based gait (HP:0002136), Delayed fine motor development (HP:0010862), Delayed gross motor development (HP:0002194), Delayed speech and language development (HP:0000750), Dermoid cyst (HP:0025247), Frequent falls (HP:0002359), Gait ataxia (HP:0002066), Generalized-onset seizure (HP:0002197), Global developmental delay (HP:0001263), Hirsutism (HP:0001007), Hyperalaninemia (HP:0003348), Lactic acidosis (HP:0003128), Long eyelashes (HP:0000527), Mongolian blue spot (HP:0011369), Motor delay (HP:0001270), Neonatal onset (HP:0003623),	ES	Negative	Negative	Mitochondrial Genome	Negative

Continued

Case number	HPO	Initial test	Initial result	Initial result	Supplementary test	Supplementary test result
	Neonatal respiratory distress (HP:0002643), Periorbital dermoid cyst (HP:0030668), Premature birth (HP:0001622), Seizures (HP:0001250), Small for gestational age (HP:0001518), Synophrys (HP:0000664), Tremor (HP:0001337), Triangular face (HP:0000325)					
25	Hypoglycemia (HP:0001943), Seizures (HP:0001250), Gastroesophageal reflux (HP:0002020), Vomiting (HP:0002013), Choking episodes (HP:0030842), Premature birth (24 weeks) (HP:0001622), Pulmonary hemorrhage (HP:0040223), Sepsis (HP:0100806), Grade II preterm intraventricular hemorrhage (HP:0030750), Patent ductus arteriosus (HP:0001643), Small for gestational age (HP:0001518), Arterial thrombosis (HP:0004420), Gastroparesis (HP:0002578), Enterocolitis (HP:0004387), Hypopigmentation of the skin (HP:0001010), Hyperpigmentation of the skin (HP:0000953), Lichenoid skin lesion (HP:0031452), Nevus (HP:0003764), Hydronephrosis (HP:0000126), Abnormality of the lower limb (HP:0002814), Coarse facial features (HP:0000280), Depressed nasal bridge (HP:0005280), Frontal bossing (HP:0002007), Hypertelorism (HP:0000316).	ES	Negative	Negative	Mitochondrial genome	Negative
26	Gait disturbance (HP:0001288), Hip dysplasia (HP:0001385), Seizure (HP:0001250), Spastic diplegia (HP:0001264), Spasticity (HP:0001257)	ES	Negative	Negative	Mitochondrial Genome	Negative

AD: Autosomal dominant; AR: Autosomal recessive; VUS: Variant of uncertain significance; ES: Exome sequencing.

this gene could not explain the phenotype, leading the clinician to exclude the gene and pursue mitochondrial genome testing instead, but the result was negative.

Inconclusive versus negative ES results

The hit rate of positive results after inconclusive ES results is 1/12 (8.3%) for supplementary testing to ES. However, if the ES results are negative, supplementary testing fails to identify any further possible explanation of the disorder.

Discussion

Establishing a clinical diagnosis is one of the main goals in health care - advances in molecular testing aid for better diagnosis. For example, the diagnostic yield for ES is clearly in the lead with a 25% to 58% hit rate (2), supplementary testing after inconclusive or negative exome like deletion/duplication testing, targeted methylation analysis or mitochondrial genome testing has low or no hit rate (10). Previous studies estimated the hit rate of methylation analysis in mendelian disorder to be around 27% (6). However, we could not establish the diagnosis of any disorder related to methylation defect despite the suspected phenotype of disorders related to methylation defect and the possible clinical phenotype presentation in the included cohort.

One major consideration of this study is the targeted population. The majority of marriages in the included cohort are consanguineous marriages (~75%), previously we showed that around 84% of detected disorders in our population results from homozygous variants in autosomal recessive disorders (11), which might explain the lower hit rate of any supplementary testing for disorders that are unlikely related to consanguinity like heterozygous deletion, or disorders in the mitochondrial genome which is maternally inherited or defects in methylation or imprinting that are unlikely linked to the autosomal recessive mode of inheritance.

Even though supplementary testing is essential, how crucial and beneficial it is when both results and testing cost are considered. Previously we showed that ES is the most cost-effective diagnostic testing even compared to GS (1). Furthermore, we showed that solo ES compared to extended family testing, also has limited clinical advantages (12). In this study, we showed supplementary testing triggered either by initial exome results or suspected phenotype with negative or inconclusive results also has no major advantages on top of ES and hence until the price of GS is equal to or lower than ES, solo ES would consider as the most cost-effective testing approach.

Conclusion

ES is considered the best approach in terms of diagnostic yield and cost-effectiveness. Supplementary testing (deletion/duplication testing, targeted methylation analysis or whole mitochondrial genome testing) beyond negative or inconclusive ES has lower diagnostic yield with limited clinical advantages. It is recommended only when another biomarker establishes the diagnosis and as a confirmatory tool for the molecular defects of disorders diagnosed by other methods.

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Declaration of conflicting interests
The authors of this article have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Ethical approval
Ethical approval was granted by the Institutional Research Board of King Abdullah International Medical Research Center with approval number RC19/315/R.

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