Diagnosing Childhood-onset Inborn Errors of Metabolism by Next Generation Sequencing

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ABSTRACT

Background: Inborn errors of metabolism (IEMs) underlie a substantial proportion of paediatric disease burden but their genetic diagnosis can be challenging using the traditional approaches.

Methods: We designed and validated a Next Generation Sequencing (NGS) panel of 226 IEM genes, created six overlapping phenotype-based sub-panels and tested 102 individuals, who presented clinically with suspected childhood-onset IEMs.

Results: In 51/102 individuals, NGS fully or partially established the molecular cause or identified other actionable diagnoses. Causal mutations were identified significantly more frequently when the biochemical phenotype suggested a specific IEM or a group of IEMs ($p<0{\cdot}0001$), demonstrating the pivotal role of prior biochemical testing in guiding NGS analysis. The NGS panel helped to avoid further invasive, hazardous, lengthy or expensive investigations in 69% individuals ($p<0{\cdot}0001$). Additional functional testing due to novel or unexpected findings had to be undertaken in only 3% of subjects, demonstrating that use of NGS does not significantly increase the burden of subsequent follow-up testing. Even where a molecular diagnosis could not be achieved, NGS-based approach assisted in the management and counselling by reducing the likelihood of a high-penetrant genetic cause.

Conclusions: NGS has significant clinical utility for the diagnosis of IEMs. Biochemical testing and NGS analysis play complementary roles in the diagnosis of IEMs. Incorporating NGS into the diagnostic algorithm of IEMs can improve the accuracy of diagnosis.

KEY WORDS

Inborn errors of metabolism; metabolic disorders; next generation sequencing

MAIN TEXT

Inborn errors of metabolism (IEMs) result in a range of childhood- or adult-onset phenotypes and are caused by the disruption of biochemical pathways. IEMs occur in 1 in 800 to 2500 births [1 2]. Metabolic investigations are an integral part of their diagnostic process [3 4] and are essential for enabling management that may prevent disability, limit disease or be curative [5-9]. IEM investigations can be broadly divided in two overlapping categories – (i) screening tests (newborn screening or phenotype-directed) to detect abnormal biochemical markers (e.g. acylcarnitine profile, organic acid profile in urine, or amino acid profiles in plasma/urine) or (ii) specific assays to detect precise deficiencies (e.g. specific pathognomonic biomarkers, cellular complementation or enzyme activity assays) [10]. However, some IEMs lack reliable biochemical markers or if present, the relevant investigations can be invasive, prohibitively expensive, time consuming or offered only on an *ad hoc* basis by research laboratories. Even for IEMs with accessible biochemical markers, genetic testing is often needed to improve the accuracy of diagnosis, determine prognosis, for cascade carrier testing and prenatal or pre-implantation diagnosis. In the era of personalised medicine, genetic testing will facilitate emerging genotype-specific therapies [11].

Next Generation Sequencing (NGS) has changed the diagnostic paradigm of rare genetic disorders [12-17]. Diagnostic yield is a key parameter of the clinical utility of NGS [18]. Systematic evaluation of diagnostic yields of NGS across different disease groups will help in the rational integration of NGS into clinical practice. IEMs are potentially excellent candidates for an NGS-based approach due to their extreme genetic heterogeneity and complexities of the traditional diagnostic approach.

However, the role of NGS in the diagnostic algorithm of IEMs and its broader clinical impact remain to be established. Here we report 102 individuals who underwent NGS for suspected childhood-onset IEM in a clinical diagnostic setting.

Methods and Results

Panel design and sequencing

A list of over 500 known IEM genes was prepared in April 2014 [19] (www.omim.org). From this list we excluded mitochondrial disorders and congenital disorders of glycosylation because their genetic heterogeneity remains substantially unresolved (Supplementary Table S1). This makes whole exome (WES) or whole genome sequencing (WGS) more appropriate for these two groups. Finally, we selected 226 IEM genes with predominantly neurodevelopmental phenotypes that are genetically heterogeneous, or are diagnosed through specialist investigations which are invasive, expensive or have poor availability. We created overlapping sub-panels based on biochemical and clinical phenotypes to optimise the bioinformatic analysis and minimise secondary findings. The sub-panels were – (i) amino acid and neurotransmitter defects; (ii) organic acidaemias and vitamins-related disorders; (iii) disorders of fatty acid oxidation or ketone metabolism and hyperammonaemia; (iv) carbohydrate metabolism defects; (v) lysosomal disorders and neuronal ceroid lipofuscinoses and (vi) peroxisomal disorders. A full list of the selected genes and the sub-panels is provided in Table S2.

We designed a targeted enrichment to sequence all the exons (+/-50 base pairs), of the canonical transcripts of the selected 226 genes. Additionally, we included all the published pathogenic intronic variants for the selected genes (information taken

from http://www.hgmd.cf.ac.uk). Using manufacturers' protocols, DNA samples from peripheral blood were enriched by an Agilent SureSelect Custom Design target-enrichment kit (Agilent, Santa Clara, CA, USA) and sequenced with the Illumina HiSeq 2500 (Illumina, Inc., San Diego, CA, USA). Subsequent sequence alignment, variant calling and annotation and filtering were performed as reported previously[20]. If a heterozygous variant in a clinically relevant recessive-disorder-linked gene was identified, then ExomeDepth program [21] was used to identify call copy number variants (CNVs) in the relevant gene.

Sensitivity of the assay was determined by comparing calls from four cell lines, with published genotypes, derived from The International HapMap Project and the 1000 Genomes Project (NA19194, HG01970, NA19005, NA18907; Corriell Institute, 403 Haddon Avenue Camden, New Jersey, 08103, USA). In total, 1216 known single nucleotide variants, within the region of interest, were cross-referenced with the locally generated NGS data. This analysis did not identify any false-positive or false-negative calls. We undertook NGS runs on four individuals with previously known mutations in at least one of the 226 selected genes and confirmed the ability of the assay to identify the expected mutations.

A repeat independent enrichment and sequencing run on one of the four cell lines was undertaken and results compared to test assay-repeatability, which demonstrated 100% match with original run along with comparable coverage for the region of interest. In summary, we designed and validated a robust targeted NGS analysis assay for a comprehensive range of IEMs, that has a minimum 50X coverage for >97% bases and a specificity of 100% and sensitivity of 100% within exons +/-5

bases. No known areas in the 226 selected genes consistently failed to achieve this coverage.

Clinical data collection, reporting and statistical analysis

This work is a retrospective evaluation of a clinical diagnostic service and ethical approval was not required. Samples along with completed clinical proformas (Supplementary Information) were accepted from clinicians for analysis of either a single sub-panel or any combination of sub-panels. Clinical and biochemical profiling of most patients was undertaken prior to the referral for NGS analysis by IEM specialists or geneticists from tertiary centres (Table 1, Table S3). NGS panel analysis was performed for 102 individuals with suspected IEMs of childhood-onset.

A multi-disciplinary team (MDT) comprising of clinical scientists, clinical geneticists, IEM specialists and genetic counsellors was constituted for clinical correlation of results. Findings were reported under three categories (i) **primary** - likely clinically significant pathogenic variants; (ii) **secondary** - deleterious but likely incidental carrier findings; and (iii) **variants of unknown clinical significance** (VUS) – variants with insufficient population frequency data to evaluate their deleteriousness. All primary and secondary point mutations were confirmed with bi-directional Sanger sequencing and CNVs were confirmed by dosage assays. Where relevant and possible, parental segregation studies were performed.

Primary pathogenic variants were reported in 51/102 individuals (Table 1). In 51/102 no primary pathogenic variants were detected (Table S3). In 18/102 (8 with and 10 without primary pathogenic findings) secondary pathogenic carrier variants were

reported (Table S4). In total, 89 pathogenic variants were identified across 58/226 genes in 61 individuals. Of these, 27/89 (30%) were novel and the remainder have been previously reported in the literature as pathogenic variants.

For overall analysis of distribution of diagnostic yields and carrier frequencies in this study, the data were grouped according to the complexity of the presenting phenotype and number of sub-panels requested. χ^2 tests were performed using IBM SPSS Statistics v24. Results of these analyses are summarised in Figure 1.

Discussion

The relative merits and limitations of WGS, WES and custom targeted-design have been discussed in the literature [12 14-17 20 22-24]. We chose bespoke targeted enrichment to guarantee a uniformly high coverage of the regions of interest. Our approach allowed for the inclusion of known intronic mutations that would escape detection via exome sequencing. Notably, we identified a deep-intronic *PTS* c.84-323A>T mutation in subject #30 (Table 1) with tetrahydrobiopterin-deficient hyperphenylalaninemia (OMIM 261640).

Our cohort of 102 individuals includes five adults, three individuals with known onset of disease in childhood and two parents (subjects #11 and #12) of a sibship of three deceased children with suspected GM1 gangliosidosis (OMIM 230500). In 42/51 individuals the reported primary pathogenic variant was fully consistent with their biochemical and clinical features (Table 1). In a further 7/51 individuals, heterozygous pathogenic variants were detected in relevant recessive disorder genes (subjects #3, 6, 8, 15, 27, 42, 50). These may be incidental carrier findings or

partial diagnoses where a second variant escaped detection. In 2/51 individuals the genetic diagnosis did not fully explain the presenting phenotype (Tyrosinaemia type I, OMIM 276700 in subject #47 and peroxisomal acyl-CoA oxidase deficiency, OMIM 264470 in subject #51). Incidental additional diagnoses with clear management implications were made in two individuals with other confirmed or possible diagnoses (Fabry disease, OMIM 301500 in subject #11 and galactokinase deficiency, OMIM 230200 in subject #51). Overall, the panel provided clinically useful and actionable information in half (51/102) of the individuals tested.

Identification of causal mutations had direct clinical impact on a number of families and individuals (Table 1). We diagnosed an ultra-rare condition in subject #41 (HMG-CoA synthase-2 deficiency, OMIM 605911) that could not have been identified just on clinical or biochemical features. Diagnosis of glycogen storage disorder 0 (OMIM 240600) in subject #43 led to a change in management strategy from frequent bolus feeds to regular overnight feeds, necessitating gastrostomy insertion. Accurate genetic diagnosis enabled targeted cascade genetic testing for a number of relatives and subsequently led to diagnostic confirmation in three siblings. Five prenatal tests were offered following NGS results and one fetus was confirmed to be affected. Although we have not undertaken a formal cost-benefit analysis, the cost of NGS was comparable with the traditional diagnostic approach in mutation positive cases (Table 1) (Table S7).

When clinical or biochemical features suggested a specific single gene defect or a particular sub-group of IEMs, NGS analysis led to a confirmed diagnosis in 39/66

(59%) individuals, whereas when an IEM was suspected but clinical and biochemical features were nonspecific, genetic diagnoses were made in only 3/36 by this approach (8%) ($p<0{\cdot}0001$). This underlines the role of biochemical investigations and phenotyping in directing NGS data analysis.

The likelihood of confirming a genetic diagnosis in individuals with a suspected specific single gene defect was high (24/34, 71%). Admittedly, a panel-based approach in such cases appears counterintuitive. However, targeted clinical sequencing is not available in the UK for 16/34 suspected single gene defects.. improves access to genetic testing for IEMs.

Certain biochemical markers, such as very long chain fatty acids (VLCFA, indicative of peroxisomal disorders), methylmalonic aciduria (MMA, indicative of methylmalonic acidaemia or cobalamin metabolism defects) or specific enzyme deficiencies, led to better diagnostic yields than markers such as isolated hypoglycaemia or hyperammonaemia (Figure 1B). Our results suggest that for certain groups of disorders, NGS can be reliable, cost-effective, quicker and less invasive than follow-up biochemical testing. For example, confirmatory biochemical diagnosis of specific peroxisomal disorders, MMA or cobalamin metabolism defects usually requires functional testing in cultured fibroblasts, necessitating a skin biopsy followed by lengthy highly specialist complementation or enzyme studies [25 26].

The diagnostic yield of NGS for hypoglycaemia and hyperammonaemia was comparatively lower than for VLCFA or MMA (Figure 1B), but consistent with the known positive predictive value of these markers [27 28]. For hypoglycaemia, the

NGS diagnostic yield (27%) is comparable to that of diagnostic fasting (22%) [27]. Fasting requires hospital admission and is potentially hazardous, though it may still have a role in optimising management [29]. For hyperammonaemia, the NGS yield (45%) is comparable to that of standard confirmatory tests (54%) [28]. For both hypoglycaemia and hyperammonaemia, the yield improved in the presence of an additional biochemical marker (Figure 1B).

There were 23 incidences of carrier findings across 21 genes in 18/102 (17%) individuals (Table S4). By dividing the assay into sub-panels we minimised incidental carrier findings. VUS were detected in 85/102 (83%) of individuals, with a median of 3 VUS detected per individual (range 0-24) (Table S5). These results reflect the importance of pre-test counselling and consent for NGS-based tests.

Scientific analysis of variants to determine the pathogenicity is challenging and compounded by the large number of variants identified in multi-gene NGS analyses. We identified 89 pathogenic mutations across 58 genes, thus expanding the mutational spectrum for these genes. If there was insufficient prior evidence to confirm the significance of the detected genetic variant we undertook reverse phenotyping using clinical, biochemical or in silico approaches (e.g. subject #41 with HMG-CoA synthase-2 deficiency, OMIM 605911) [30]. Interestingly, even though 27/89 pathogenic variants were novel, the burden of additional functional testing due to NGS results was minimal and had to be considered in only 3% of subjects (Table 1). In most cases, review of previously performed biochemical tests was sufficient for correlation of unexpected NGS findings. In comparison, the NGS approach helped to avoid invasive, hazardous, lengthy or expensive investigations in

69% (Tables 1 and S2). This demonstrates the robustness of available variant and mutation databases and in silico tools for this group of disorders and underscores the clinical utility of this approach.

A proportion of the 51/102 individuals (Table S3) in whom we failed to achieve a genetic diagnosis may have mutations in genes that are not part of our panel. In addition, certain classes of mutations may have escaped detection due to technical limitations of the assay. Additionally, certain biochemical phenotypes such as recurrent hypoglycaemia or hyperammonaemia may be multi-factorial in origin in some patients, making it challenging to achieve precise genetic diagnoses.

Notably, using NGS is clinically useful even in cases where a molecular diagnosis is not achieved because a 'negative' NGS result can substantially reduce the likelihood of a high-penetrant single gene disorder and thus informing the management of the patient and counselling of the families. Furthermore, NGS was the only practical approach in several cases or helped to avoid inconvenient or invasive investigations. Even in these 'negative' cases, the cost of NGS was comparable to the investigations that would otherwise have been performed (Table S6). These individuals could form a focussed cohort for future WES or WGS research studies.

Although the potential for NGS in a few selected groups of IEMs has been previously reported, the use of NGS for multiple groups of IEMs has not been studied extensively [15 31-39] (summarised in Figure S1). The results presented here establish the utility of NGS for diagnosis of multiple groups of IEMs in clinical practice. Figure 2 illustrates how the use of NGS may be incorporated into the

diagnostic algorithm of IEMs. We have demonstrated that biochemical tests are pivotal for the diagnosis of IEMs as they help focus genetic testing and facilitate NGS analysis. In addition, we have demonstrated that NGS can help to avoid invasive, expensive or hazardous biochemical confirmatory studies in specific situations, can improve access to genetic testing and may be a comparable approach in cost terms to alternative investigations. Overall, incorporating NGS into the diagnostic algorithm of IEMs can improve the accuracy of diagnosis. Further work is required to formally assess the cost-effectiveness of NGS and explore the optimal approach to the timing of NGS in the diagnosis of IEMs.

The future of genomic techniques in the diagnosis of IEMs is likely to involve WES and WGS, the latter conferring particular advantages including the ability to provide equal coverage across the genome, detect copy number variants, indels, intronic and regulatory variants. However, the pivotal role of biochemical testing as we demonstrate here and the potential ability of genomic testing to reduce the need for additional studies will remain relevant to WES and WGS.

What is already known on this topic (3 statements, max 25 words per statement)

Next generation sequencing has changed the diagnostic paradigm for a number of rare genetic disorders.

Genetic testing in inborn errors of metabolism is important for accuracy of diagnosis, to provide information on prognosis, for cascade carrier testing and for prenatal or pre-implantation diagnosis.

Though research based NGS testing for IEM has been developed, the place of NGS in a clinical diagnostic setting has not been established.

What this study adds (3 statements, max 25 words per statement)

NGS based testing can improve the accuracy of diagnosis of IEM and potentially avoid the need for invasive or expensive functional testing.

Biochemical testing and NGS are complementary in the diagnosis of IEM.

NGS testing in patients with suspected IEM can significantly reduce the need for other invasive and expensive investigations.

TABLE 1: Primary pathogenic variants from IEM NGS panel analysis for individuals with childhood-onset disease.

Results are grouped by sub-panels - (AMN, FAOD, KET), (LSD, NCL), (OA, VIT), (AA, NT), (CHO), (PER), followed by combinations.

Key – AA Disorders of amino acid metabolism including phenylketonuria, and cerebral organic acid disorders; AMN Disorders associated with hyperammonaemia; CHO Disorders of carbohydrate metabolism; FAOD Fatty acid oxidation defects including multiple acyl-CoA dehydrogenase deficiency; KET Disorders of ketogenesis or ketolysis; LSD Lysosomal disorders: includes lysosomal storage disorders, transport defects and protease defects; NCL Neuronal ceroid lipofuscinoses; NT Disorders of neurotransmission (includes pterins, tyrosinaemia); OA Organic acidaemias, including disorders of branched chain amino acid catabolism, 3-methylglutaconic acidurias; PER Peroxisomal biogenesis disorders and disorders of single peroxisomal enzymes; VIT Folate and cobalamin defects, also riboflavin transport defects, and biotin-responsive disorders.
Het heterozygous, hom homozygous, hemi hemizygous comp het compound heterozygous.
^ Denotes invasive or hazardous or lengthy or expensive investigations that were avoided by using the NGS approach.

*Three *SUMF1* variants were identified. Of the two *cis* variants (c.776A>T p.(Asn25911e), c.797C>T p.(Pro266Leu)), (c.776A>T p.(Asn25911e) is considered to be the more pathogenic.[40] **Previously reported synonymous change resulting in a splicing defect.[41]

Subject #	Approximate age at investigation	Key biochemical and clinical features	Result	Zygosity	Interpretation and clinical impact	Investigations avoided by NGS	Investigations added by NGS
Single sub-panel tested: (AMN, FAOD, KET)							
1	<1 year	Neonatal encephalopathy, severe hyperammonaemia, low arginine. Suspected argininosuccinic aciduria.	*ASL* c.749T>A [p.(Met250Lys)]	hom	Diagnosis of argininosuccinic aciduria confirmed	Single gene mutation analysis or ^enzyme analysis in red blood cells followed by single gene analysis	None
2	<1 year	Encephalopathy, hypertonia. Hyperammonaemia with elevated citrulline. Suspected citrullinaemia.	*ASS1* c.970+5G>A, c.892delG [p.(?), (Glu298ArgfsTer18)]	comp het	Diagnosis of citrullinaemia confirmed	Single gene mutation analysis Cascade testing of relatives undertaken	None
3	1-11 years	Motor delay, muscle weakness during illness, memory and concentration deficits. Elevated medium chain acylcarnitines. Suspected Multiple acyl-CoA dehydrogenase deficiency (MADD).	*ETFDH* c.5ldupT [p.(Ala18CysfsTer5)]	het	Possible diagnosis of MADD (no second variant found)	^Sequential mutation analysis of multiple genes	None
4	1-11 years	Severe progressive myopathy with myoglobinuria and hepatic steatosis, recurrent	*ETFDH* c.413T>G [p.(Leu138Arg)], c.1333T>C	comp het	Diagnosis of MADD confirmed	^Sequential mutation analysis of multiple	None

		hypoglycaemia. Elevated acylcarnitines, urine hexanoylglycine and lactate. Suspected Multiple acyl-CoA dehydrogenase deficiency (MADD).	[p.(Trp445Arg)]			genes Cascade testing of relatives undertaken	
5	<1 year	Hypoglycaemia, biochemical tests suggestive of CPT II deficiency. Hypertrophic cardiomyopathy, abnormal renal cortex and IUGR.	*CPT2* c.1838G>A [p.(Gly613Glu)]	hom	Diagnosis of CPT II deficiency confirmed. Prenatal testing undertaken, fetus affected	Single gene mutation analysis and/or ^enzyme activity in muscle tissue or fibroblasts	None
6	1-11 years	Recurrent hypoglycaemia, minimal ketonuria, previously abnormal C14:1 carnitine. Possible very long chain Acyl-CoA dehydrogenase (VLCAD) deficiency	*ACADVL* c.1591C>T sub-[p.(Arg531Trp)]	het	Carrier finding or possible diagnosis of VLCAD deficiency (but no second variant found)	Single gene mutation analysis	None
7	1-11 years	Haematological abnormalities, persistently raised lysine in urine and plasma. Suspected lysinuric protein intolerance	*SLC7A7* c.625+1G>A	hom	Diagnosis of Lysinuric protein intolerance achieved	Single gene mutation analysis	None
8	1-11 years	Reye-like illness. Hypoglycaemia, hyperammonaemia, elevated lactate and ALT. Suspected FAOD.	*ACADVL* c.631 G>A [p.(Gly211Ser)]	het	Carrier finding or possible diagnosis of VLCAD deficiency (but no second variant found)	^Sequential mutation analysis of multiple genes	None
9	1-11 years	Profound learning disability, inflammatory bowel disease, biochemical diagnosis of citrullinaemia	*ASS1* c.1168G>A [p.(Gly390Arg)]	hom	Diagnosis of citrullinaemia confirmed	Single gene mutation analysis	None
10	1-11 years	Recurrent myositis with raised creatine kinase. Acylcarnitine profile suggests FAOD, possible CPT II deficiency.	*CPT2* c.338C>T [p.(Ser113Leu)]	hom	Diagnosis of CPT II deficiency confirmed	Single gene mutation analysis	None
Single sub-panel tested: (LSD, NCL)							
11	Adult (18+ years)	Parent of three decreased children with suspected GM1 gangliosidosis	*GLB1* c.75+2dupT *GLA* c.1153A>G [p.(Thr385Ala)]	het hemi	Diagnosis of GM1 gangliosidosis in deceased children confirmed Incidental diagnosis of Fabry disease	Single gene mutation analysis None	None Enzyme assay in leucocytes (for confirmation of Fabry disease)
12	Adult (18+ years)	Parent of three decreased children with suspected GM1 gangliosidosis	*GLB1* c.75+2dupT	het	Diagnosis of GM1 gangliosidosis in deceased children confirmed	Single gene mutation analysis	None
13	1-11 years	Global developmental delay. Elevated urine GAGs and reduced activity of multiple lysosomal sulfatases. Suspected multiple sulfatase	*SUMF1* c.776A>T [p.(Asn259Ile)], c.797C>T [p.(Pro266Leu)], c.836C>T,	comp het	Diagnosis of multiple sulfatase deficiency achieved.	Single gene mutation analysis	None

		deficiency.	[p.(Ala279Val)]*				
14	<1 year	Suspected galactosialidosis	*CTSA* c.1284deIG [p.(Trp428CysfsTer18)]	hom	Diagnosis of galactosialidosis confirmed	Skin biopsy, fibroblast culture, enzyme activity in fibroblasts and single gene mutation analysis	None
15	<1 year	Biochemical diagnosis of suspected mucolipidosis type III. One *GNPTAB* mutation already known.	*GNPTAB* c.3503_3504deITC [p.(Leu1168GlnfsTer5)]	het	Possible diagnosis of mucolipidosis type III alpha/beta (no second variant found)	None, one mutation already known	None
16	1-11 years	Lower limb spasticity, deteriorating vision, loss of speech. Reduced arylsulfatase A activity. Suspected metachromatic leukodystrophy	*ARSA* c.465+1 G>A	hom	Diagnosis of metachromatic leukodystrophy confirmed. Prenatal testing undertaken – fetus unaffected	Single gene mutation analysis, urinary sulfatides	None
17	12-17 years	Biochemical (enzyme) diagnosis of mucolipidosis type III, no mutations found in GNPTAB	*GNPTG* c.196C>T [p.(Arg66Ter)]	hom	Diagnosis of Mucolipidosis III gamma achieved Genetic testing of sibling undertaken and found to be unaffected.	^Sequential mutation analysis of multiple genes Cascade testing of relatives undertaken	None
18	1-11 years	Developmental delay, behavioural disturbance. Elevated urine heparan sulfate with deficient HGSNAT activity in leukocytes, biochemical diagnosis of MPS IIIC.	*HGSNAT* c.947G>A [p.(Trp3l6Ter)], c.1464+1 G>A	comp het	Diagnosis of Mucopolysaccharidosis type IIIC (Sanfilippo C) confirmed	Single gene mutation analysis	None
19	<1 year	Failure to thrive, hepatomegaly. Biochemical diagnosis of Wolman disease.	*LIPA* (whole gene deletion)	hom	Diagnosis Wolman disease confirmed.	Single gene mutation analysis	None
Single sub-panel tested: (OA, VIT)							
20	1-11 years	Vomiting and respiratory distress. Metabolic acidosis with elevated methylmalonic acid in urine and blood. Biochemical diagnosis of methylmalonic acidaemia.	*MMAA* c.433C>T [p.(Arg145Ter)]	hom	Diagnosis of cblA type methylmalonic aciduria achieved	^Skin biopsy, fibroblast culture, complementation assays and sequencing of specific gene, or sequential mutation analysis of multiple genes	None
21	1-11 years	Vomiting and weight loss, with macrocytic anaemia and severe vitamin B12 deficiency. Suspected cobalamin transport abnormality.	*GIF* c.1155C>A [p.(Tyr385Ter)], c.685G>A [p.(Ala229Thr)]	comp het	Diagnosis of Intrinsic Factor deficiency achieved. Genetic testing of sibling undertaken and found to be affected.	^Sequential analysis of multiple genes and/or Schilling test	None
22	1-11 years	Encephalopathy and cognitive deficit with elevated homocysteine and urinary methylmalonic acid. Suspected cblC disease.	*MMACHC* c.394C>T [p.(Arg132Ter)]	hom	Diagnosis of cblC type methylmalonic aciduria and homocystinuria, confirmed. Genetic testing of sibling	^Skin biopsy, fibroblast culture, complementation assays and sequencing of specific gene, or sequential mutation	None

					undertaken and found to be affected.	analysis of multiple genes	
23	<1 year	Elevated methylmalonic acid in urine. Suspected methylmalonic acidaemia.	*MUT* c.1207C>T [p.(Arg403Ter)], c.52C>T [p.(Gln18Ter)]	comp het	Diagnosis of mut(0) type methylmalonic acidaemia achieved.	^Skin biopsy, fibroblast culture, complementation assays and sequencing of specific gene, or sequential mutation analysis of multiple genes	None
24	1-11 years	Developmental delay with myoclonic-astatic epilepsy. Elevated homocysteine, normal methionine, no MMA.	*MTHFR* c.1129C>T [p.(Arg377Cys)]	hom	Diagnosis of homocystinuria due to MTHFR deficiency achieved. Genetic testing of sibling undertaken and found to be affected.	^Skin biopsy, fibroblast culture, enzyme assay in fibroblasts and single gene mutation analysis	None
25	1-11 years	Long standing developmental delay and encephalopathy	*MTHFR* c.1530G>A [p.(Lys510Lys)]**	hom	Diagnosis of homocystinuria due to MTHFR deficiency achieved	^Skin biopsy, fibroblast culture, enzyme assay in fibroblasts and single gene mutation analysis	None
26	<1 year	Lactic acidosis with methylmalonic aciduria and elevated plasma homocysteine. Poor feeding and lethargy. Suspected cblC disease.	*MMACHC* c.271dupA [p.(Arg9lLysfsTerl4)]	hom	Diagnosis of cblC type methylmalonic aciduria and homocystinuria, confirmed	^Skin biopsy, fibroblast culture, complementation assays and sequencing of specific gene, or sequential mutation analysis of multiple genes	None
Single sub-panel tested: (AA, NT)							
27	1-11 years	Macrocephaly, elevated cystine and lysine in urine. Suspected cystinuria or lysinuric protein intolerance.	*SLC7A9* c.614dupA [p.(Asn206GlufsTer3)]	het	Carrier finding or possible diagnosis of cystinuria (but no second variant found)	Single gene mutation analysis	None
28	<1 year	Hypotonia and poor feeding on day 1 of life, hiccups, apnoeas, absent corpus callosum, burst suppression EEG. Elevated CSF glycine. Suspected non-ketotic hyperglycinaemia.	*GLDC* c.1545G>C [p.(Arg515Ser)], whole gene deletion	hemizygous	Diagnosis of non-ketotic hyperglycinaemia due to GLDC deficiency achieved.	^Liver biopsy, measurement of specific enzyme activity and/or sequential mutation analysis of multiple genes	None
29	<1 year	Seizures. Elevated CSF:plasma glycine ratio. Suspected non-ketotic hyperglycinaemia.	*GLDC* c.1629delT [p.(Asn543LysfsTer10)]	hom	Diagnosis of non-ketotic hyperglycinaemia due to GLDC deficiency achieved.	^Liver biopsy, measurement of specific enzyme activity and/or sequential mutation analysis of multiple genes	None

30	<1 year	Biochemical diagnosis of 6-pyruvoyl-tetrahydropterin synthase (PTS) deficiency. Hyperphenylalananinaemia.	*PTS* c.84-323A>T, whole gene deletion	hemizygous	Diagnosis of Tetrahydrobiopterin-deficient hyperphenylalaninemia confirmed	^Hospital admission for tetrahydrobiopterin loading test (serial phenylalanine and tyrosine analyses) Single gene mutation analysis	None
31	1-11 years	Developmental delay, seizures, muscular hypotonia. Elevated cerebrospinal fluid:plasma glycine ratio.	*GLDC* c.1108C>A [p.(Gln370Lys)], c.2885G>C [p.(Arg962Pro)]	comp het	Diagnosis of non-ketotic hyperglycinaemia due to GLDC deficiency achieved.	^Liver biopsy, measurement of specific enzyme activity and/or sequential mutation analysis of multiple genes	None
32	<1 year	Pyridoxine responsive seizures. Elevated urine AASA.	*ALDH7A1* c.1364T>C [p.(Leu455Pro)]	hom	Diagnosis of pyridoxine-dependant epilepsy confirmed	Single gene mutation analysis	None
33	1-11 years	Developmental delay, central hypotonia. 4-hydroxybutyric aciduria. Suspected succinic semialdehyde dehydrogenase deficiency	*ALDH5A1* c.612G>A [p.(Trp204Ter)], c.375 378delGAAG [p.(Lys126GlyfsTer4)]	comp het	Diagnosis of succinic semialdehyde dehydrogenase deficiency confirmed. Prenatal testing undertaken, fetus unaffected	^Specific enzyme assay in leucocytes Single gene mutation analysis	None
34	1-11 years	Hyperammonaemia in the neonatal period, raised ornithine. Suspected ornithine aminotransferase deficiency.	*OAT* c.461G>A [p.(Argl54His)]	hom	Diagnosis of ornithine aminotransferase deficiency confirmed	^Skin biopsy, fibroblast culture, specific enzyme activity in fibroblasts and single gene mutation analysis	None
Single sub-panel tested: (CHO)							
35	1-11 years	Motor delay, mild myopathy, hepatomegaly. Moderately elevated triglycerides, total cholesterol, ALT, GGT and lactate. Suspected Glycogen storage disease (GSD)	*PHKA2* c.3614C>T [p. (Pro1205Leu)]	hemizygous	Diagnosis of GSD IX confirmed	^Sequential mutation analysis of multiple genes	None
36	1-11 years	Failure to thrive, multiple malformations. Severe congenital neutropenia. Suspected Dursun syndrome.	*G6PC3* c.882_903dup22 [p.(His302GlyfsTer92)]	hom	Diagnosis of G6PC3 deficiency (Dursun syndrome) confirmed.	Single gene mutation analysis	None
Single sub-panel tested: (PER)							
37	<1 year	Death in early infancy (before 2 months). Elevated very long chain fatty acids. Suspected Zellweger spectrum disorder.	*PEX12* c.604C>T [p.(Arg202Ter)]	hom	Diagnosis of peroxisome biogenesis disorder 3A (Zellweger) achieved.	^Skin biopsy, fibroblast culture, peroxisomal metabolite analysis in fibroblasts, immuno(cyto/bio)chemical assays, enzyme activity measurements, pathway analyses, complementation studies and mutation analysis of specific gene and/or sequential mutation analysis of multiple genes	None
38	<1 year	Developmental delay with failure to thrive,	*PEX1* c.2T>C [p.(Met1?)]	hom	Diagnosis of peroxisome	^Skin biopsy, fibroblast culture, peroxisomal	None

		hypotonia and hepatosplenomegaly. Elevated very long chain fatty acids, normal phytanic acid. Suspected peroxisomal biogenesis disorder.			biogenesis disorder 1A (Zellweger) achieved.	metabolite analysis in fibroblasts, immuno(cyto/bio)chemical assays, enzyme activity measurements, pathway analyses, complementation studies and mutation analysis of specific gene and/or sequential mutation analysis of multiple genes	
39	<1 year	Bilateral cataracts, pulmonary stenosis, patent ductus arteriosus. Feeding difficulties. Low C16, C18 plasmalogens. Suspected rhizomelic chondrodysplasia punctata	*PEX7* c.649G>A [p.(Gly2I7Arg)], c.875T>A [p.(Leu292Ter)]	comp het	Diagnosis of type 1 rhizomelic chondrodysplasia punctata achieved. Prenatal testing undertaken, fetus unaffected.	^Skin biopsy, fibroblast culture, peroxisomal metabolite analysis in fibroblasts, immuno(cyto/bio)chemical assays, enzyme activity measurements, pathway analyses, complementation studies and mutation analysis of specific gene and/or sequential mutation analysis of multiple genes	None
40	1-11 years	Developmental delay, hypotonia, severe bilateral hearing loss, retinitis pigmentosa, poor weight gain, low set ears. Elevated VLCFA and phytanic acid, normal plasmalogens. Suspected infantile Refsum disease	*PEX1* c.2097dupT [p.(Ile700TyrfsTer42)], c.2528G>A [p.(Gly843Asp)]	comp het	Diagnosis of infantile Refsum disease confirmed	^Skin biopsy, fibroblast culture, peroxisomal metabolite analysis in fibroblasts, immuno(cyto/bio)chemical assays, enzyme activity measurements, pathway analyses, complementation studies and mutation analysis of specific gene and/or sequential mutation analysis of multiple genes	None
Combination of sub-panels tested: (CHO) (AMN, FAOD, KET)							
41	1-11 years	Hypoketotic hypoglycaemia. Elevated C2 acylcarnitine, borderline elevated C5 acylcarnitine. Feeding difficulties. Suspected ketogenesis defect.	*HMGCS2* c.430G>T [p.(Val144Leu)]	hom	Diagnosis of HMG-CoA synthase-2 deficiency achieved and confirmed by enzyme activity analysis.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles	Expression and characterization of the mutated enzyme
42	1-11 years	Recurrent hypoketotic hypoglycaemia, diagnostic fast normal. Short stature. Suspected disorder of carbohydrate metabolism.	*PYGL* c.1900G>C [p.(Asp634His)]	het	Carrier finding or possible diagnosis of Glycogen storage disease VI (but no second variant found)	^Sequential mutation analysis of multiple genes	None
Combination of sub-panels tested: (OA, VIT) (CHO) (AMN, FAOD, KET)							
43	1-11 years	Recurrent ketotic hypoglycaemia. Mild	*GYS2* c.1553A>C	comp het	Diagnosis of Glycogen storage	^Glucose loading tests with serial glucose	None

		developmental delay, gastro-oesophageal reflux. Suspected glycogen synthase deficiency.	[p.(Glu518Ala)], c.729C>A [p.(Cys243Ter)]		disease 0 confirmed	and lactate measurements, single gene mutation analysis	
Combination of sub-panels tested: (OA, VIT) (AA,NT)							
44	12-17 years	Failure to thrive, hyporeflexia, abnormal nerve conduction. Vitamin B12 responsive megaloblastic anaemia, methylmalonic aciduria. Suspected transcobalamin II deficiency.	*TCN2* c.497_498delTC [p.(Leu166ProfsTer7)]	hom	Diagnosis of Transcobalamin II deficiency confirmed	^Single gene mutation analysis and/or skin biopsy, fibroblast culture and studies of transcobalamin synthesis in fibroblasts	None
Combination of sub-panels tested: (OA, VIT) (AA, NT) (AMN, FAOD, KET)							
45	1-11 years	Renal failure, cardiomyopathy. Elevated methylmalonic acid in urine, abnormal acylcarnitine profile. Suspected methylmalonic acidaemia.	*MMAA* c.433C>T [p.(Arg145Ter)], c.970-2A>C	comp het	Diagnosis of cblA type methylmalonic aciduria achieved	^Skin biopsy, fibroblast culture, complementation assays and sequencing of specific gene, or sequential mutation analysis of multiple genes	None
Combination of sub-panels tested: (OA, VIT) (AMN, FAOD, KET)							
46	1-11 years	Hypoketotic hypoglycaemia, hyperammonaemia, supraventricular tachycardia and Reye-like illness. Suspected carnitine palmitoyltransferase IA (CPT1A) deficiency.	*CPT1A* c.733C>T [p.(Arg245Ter)]	hom	Diagnosis of CPT 1A confirmed	Single gene mutation analysis	None
All sub-panels tested							
47	Adult (18+ years)	Generalised epilepsy and learning difficulties. Hydrocephalus diagnosed at 6 weeks. Mildly coarse facies, short stature. White cell enzymes normal.	*FAH* c.648C>G [p.(Ile216Met)]	hom	Possible incidental diagnosis of Tyrosinaemia type I (no biochemical evidence)	None	None
48	<1 year	Gastroschisis. Investigated for conjugated hyperbilirubinaemia, urine organic acids revealed methylmalonic aciduria and raised malonic acid levels. Suspected methylmalonic acidaemia.	*ACSF3* c.782G>A [p.(Gly261Glu)], c.1672C>T [p.(Arg558Trp)]	comp het	Diagnosis of Combined malonic and methylmalonic aciduria achieved	^Skin biopsy, fibroblast culture, complementation assays and sequencing of specific gene, or sequential mutation analysis of multiple genes	None
49	<1 year	Jaundice, hepatosplenomegaly, growth failure, features of haemophagocytic lymphohistiocytosis. Elevated ALT, coagulopathy, reduced leukocyte LAL activity.	*LIPA* c.824C>T [p.(Ser275Phe)]	hom	Diagnosis of Wolman disease confirmed. Prenatal testing undertaken, fetus unaffected	Single gene mutation analysis	None
50	1-11 years	Global developmental delay, cerebral atrophy, thin corpus callosum, hypertonia, delayed visual maturity. Low CSF 5-methyltetrahydrofolate. Suspected cerebral folate deficiency	*FOLR1* c.493+2T>C	het	Possible diagnosis of cerebral folate transport deficiency (no second variant found)	Single gene mutation analysis	None
51	1-11 years	Global developmental delay, bilateral retinal dystrophy, unilateral cataract, umbilical hernia, hepatosplenomegaly, hypothyroidism, prolonged hyperbilirubinaemia.	*ACOX1* c.443G>A [p.(Arg148Gln)] *GALK1* c.479C>T	hom hom	Possible diagnosis of peroxisomal acyl-CoA oxidase deficiency	None None	Further confirmatory biochemical testing could not be performed as

			[p.(Ser160Leu)]		Possible diagnosis of galactokinase deficiency with cataracts		patient lost to follow up. May have required skin biopsy, fibroblast culture, peroxisomal specific enzyme activity, galactose and galactitol in blood and urine, galactokinase activity in blood.

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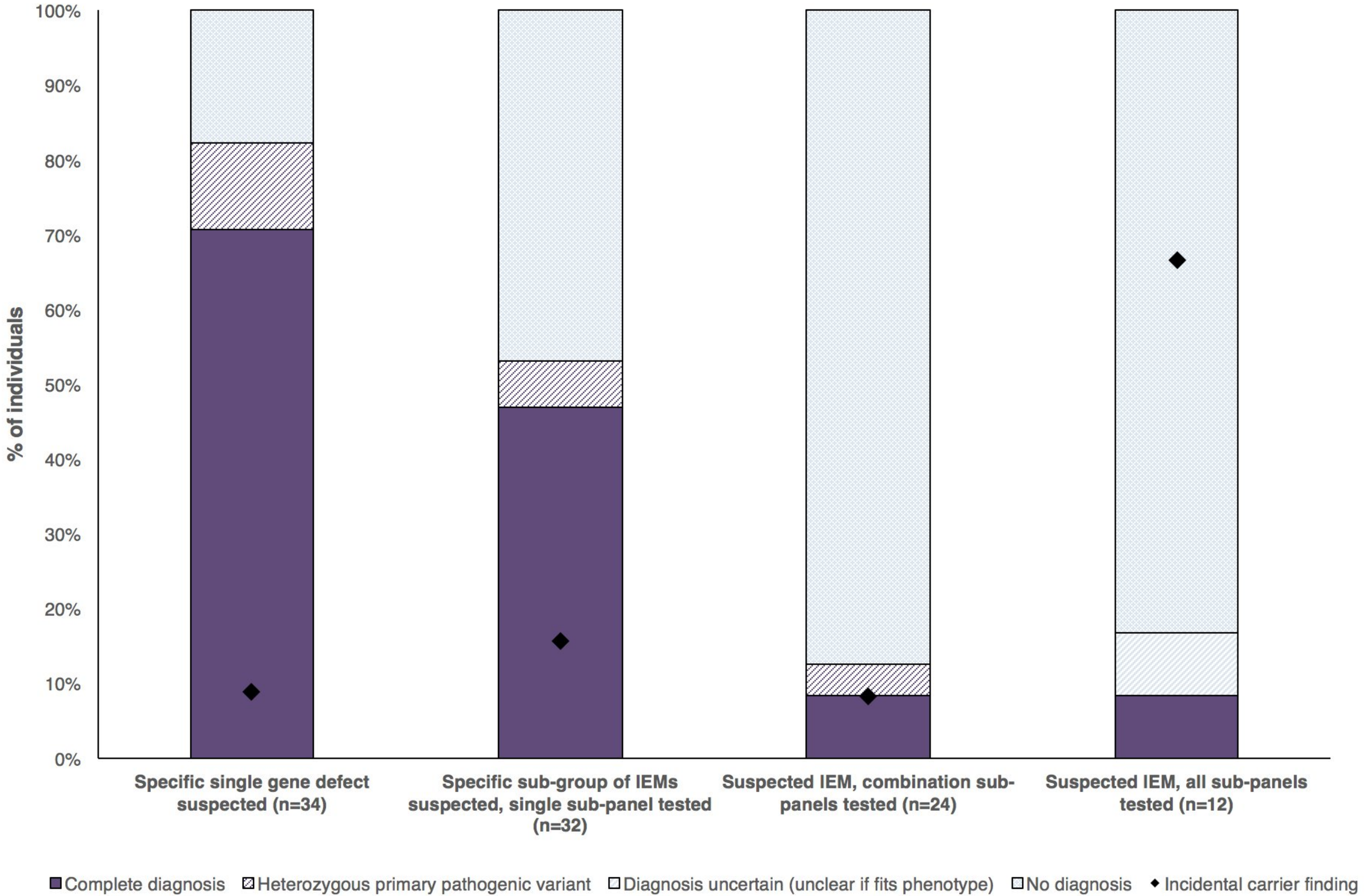
FIGURE LEGENDS

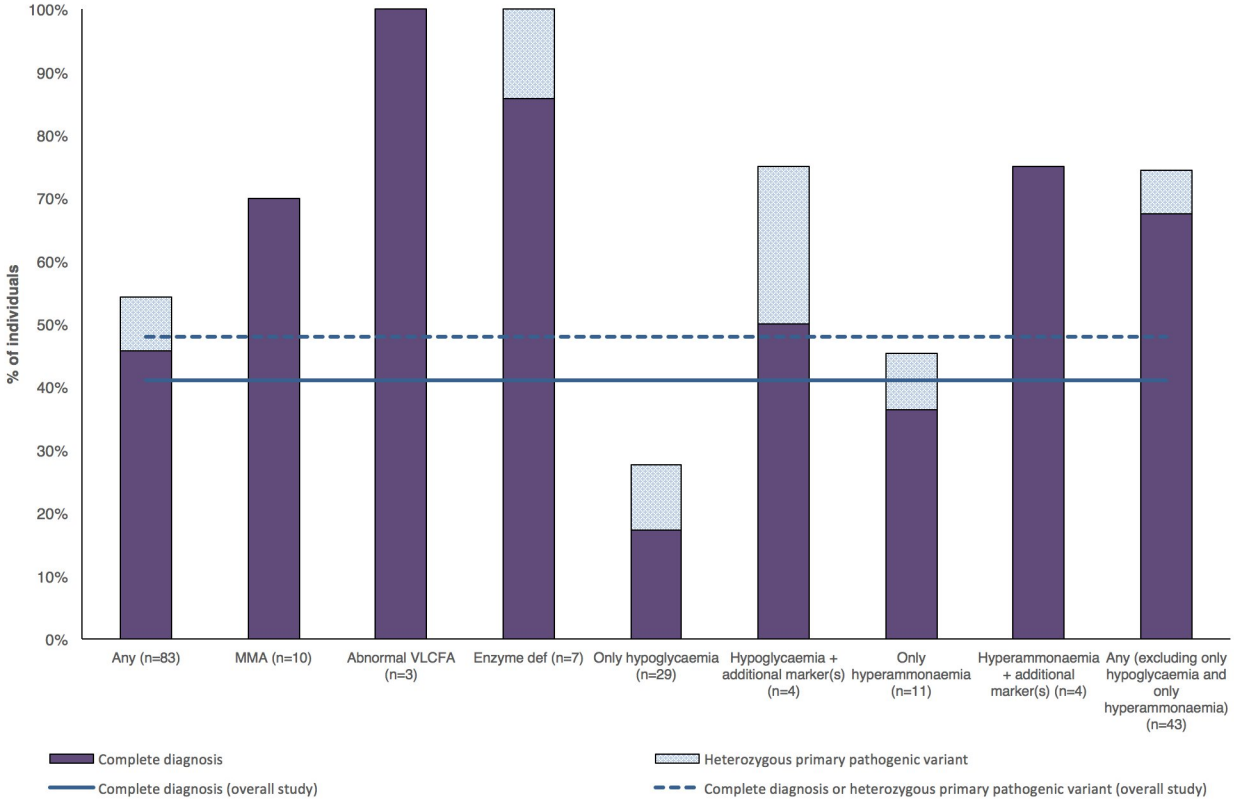
Figure 1: Frequency of diagnosis by sub-panels and biochemical markers:
(A) Proportion of individuals in whom primary pathogenic variants and incidental carrier findings were identified according to whether specific gene defects or sub-groups of IEMs were suspected and sub-panels requested. 'Specific sub-group of IEM suspected, single sub-panel tested' refers to individuals in whom the differential diagnosis included more than one gene but only a single sub-panel was requested. 'Combination sub-panels' refers to any combination of sub-panels other than all six sub-panels. The frequency of a confirmed genetic diagnosis was significantly higher when a single specific gene defect or a specific sub-group of IEM was suspected, in comparison with suspected IEM tested by requests for combination or all sub-panels ($p<0.0001$), underlining the complementary role of metabolic and NGS investigations in clinical diagnostics.

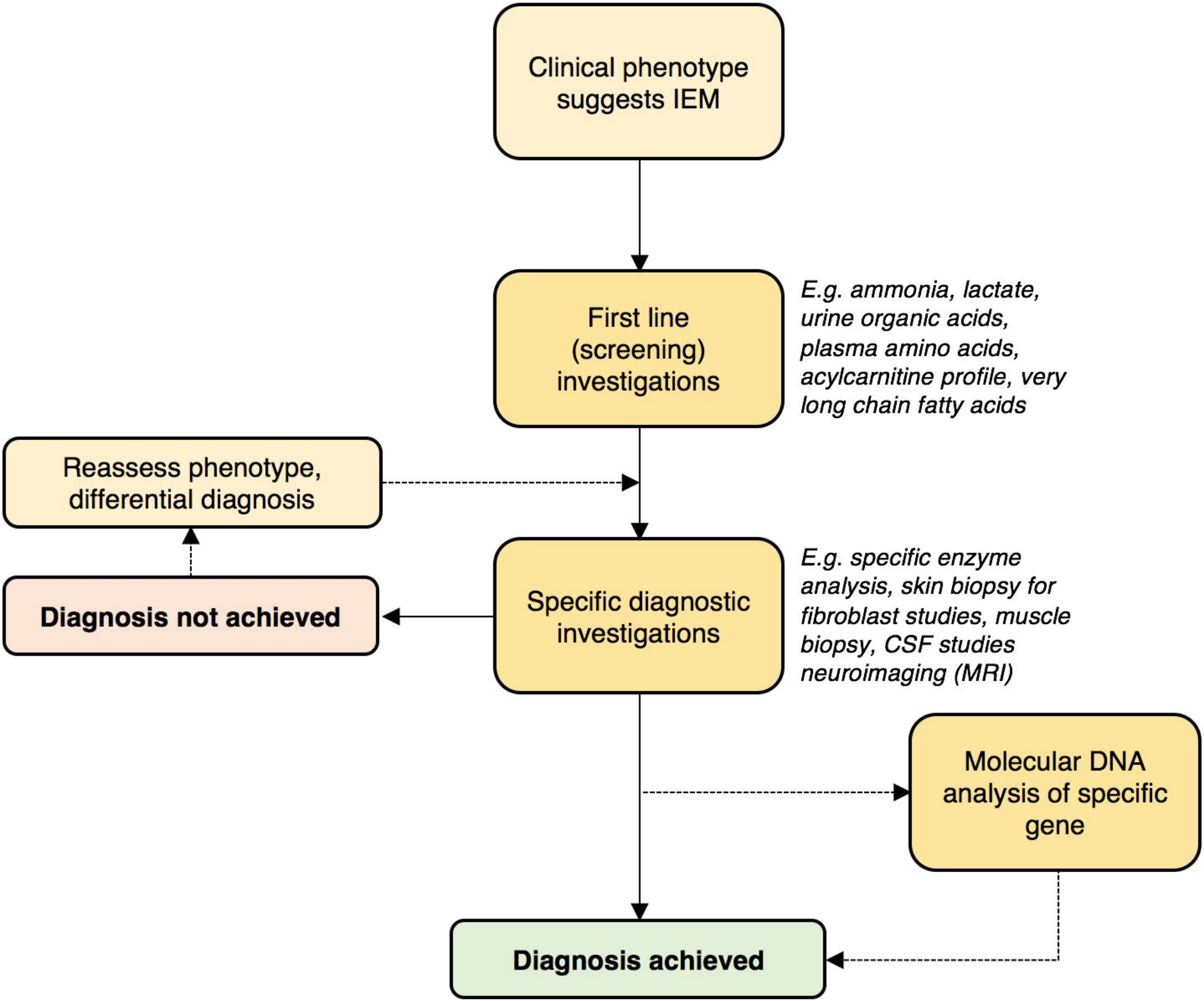
(B) Proportion of individuals with primary pathogenic variants according to abnormal biochemical markers detected in screening investigations. The diagnostic yield was much higher for metabolic markers such as MMA, abnormal VLCFA and specific enzyme deficiencies. It was lower for hypoglycaemia and hyperammonaemia, especially if no additional marker was identified in the previous biochemical testing. This suggests that many of these individuals perhaps do not have a Mendelian genetic disorder or many genes influencing glucose and ammonia levels in children are yet to be discovered. 'Any' refers to any biochemical marker(s) that were suggestive of a specific diagnosis or group of disorders. 'Only hypoglycaemia' and 'only hyperammonaemia' refer to the absence of additional markers such as abnormal plasma amino acids, acylcarnitine profile or urine organic acids that suggested a specific diagnosis or group of disorders.

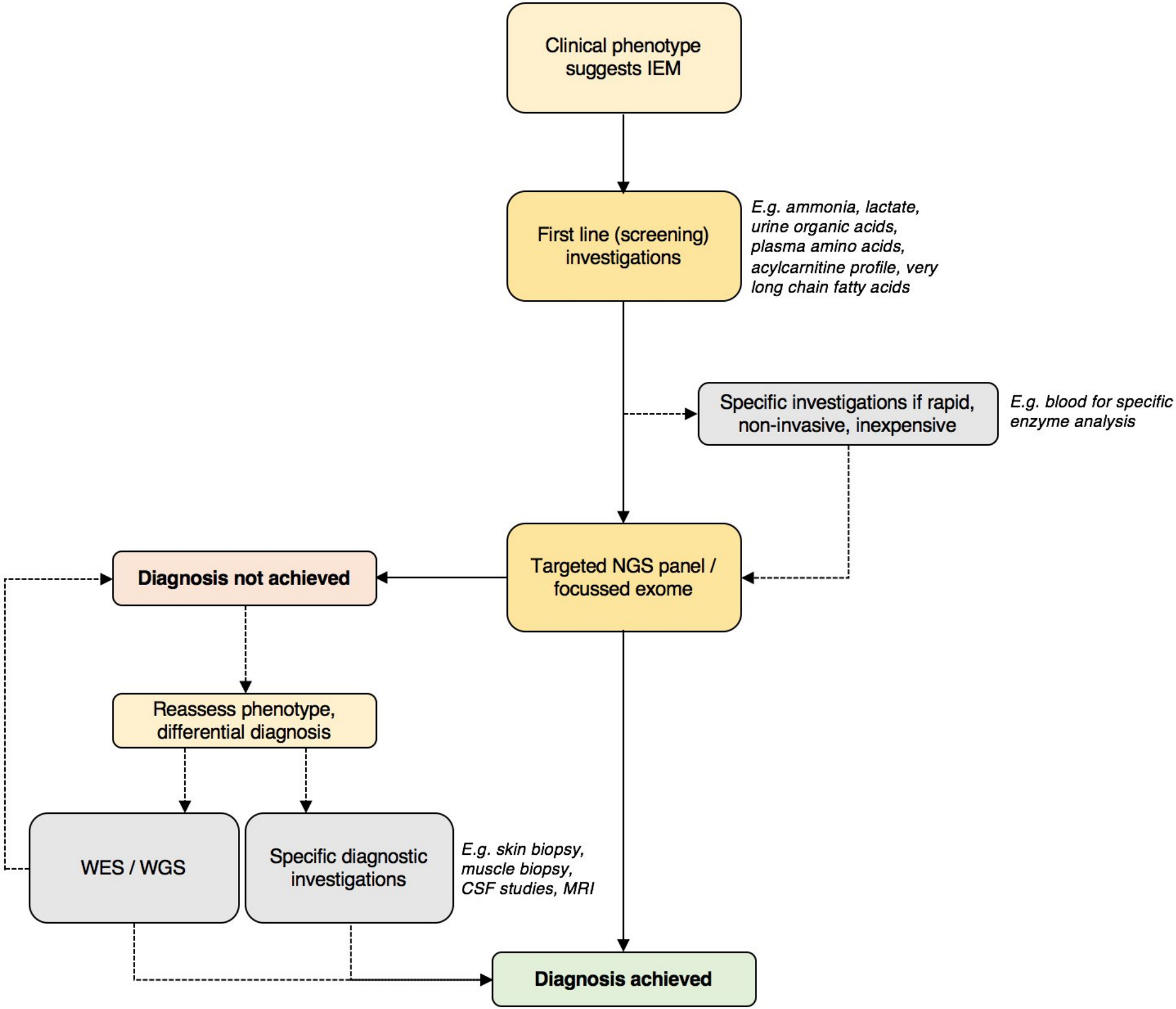
'Heterozygous primary pathogenic variant' refers to individuals in whom a mutation was identified in a heterozygous state in a gene relevant to the reported phenotype, which may reflect a carrier finding or a partial diagnosis.
Key – VLCFA very long chain fatty acids, def deficiency, MMA methylmalonic aciduria

Figure 2: (A) 'Traditional' approach to diagnosis of IEMs. **(B)** Incorporation of NGS techniques into diagnostic algorithm of IEMs.
Key – WES whole exome sequencing, WGS whole genome sequencing


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of individuals
Specific single gene defect suspected (n=34)
Specific sub-group of IEMs suspected, single sub-panel tested (n=32)
Suspected IEM, combination sub-panels tested (n=24)
Suspected IEM, all sub-panels tested (n=12)
Complete diagnosis
Heterozygous primary pathogenic variant
Diagnosis uncertain (unclear if fits phenotype)
No diagnosis
Incidental carrier finding


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of individuals
Any (n=83)
MMA (n=10)
Abnormal VLCFA (n=3)
Enzyme def (n=7)
Only hypoglycaemia (n=29)
Hypoglycaemia + additional marker(s) (n=4)
Only hyperammonaemia (n=11)
Hyperammonaemia + additional marker(s) (n=4)
Any (excluding only hypoglycaemia and only hyperammonaemia) (n=43)
Complete diagnosis
Heterozygous primary pathogenic variant
Complete diagnosis (overall study)
Complete diagnosis or heterozygous primary pathogenic variant (overall study)


Clinical phenotype suggests IEM
First line (screening) investigations
E.g. ammonia, lactate, urine organic acids, plasma amino acids, acylcarnitine profile, very long chain fatty acids
Reassess phenotype, differential diagnosis
Specific diagnostic investigations
E.g. specific enzyme analysis, skin biopsy for fibroblast studies, muscle biopsy, CSF studies neuroimaging (MRI)
Diagnosis not achieved
Molecular DNA analysis of specific gene
Diagnosis achieved


Clinical phenotype suggests IEM
First line (screening) investigations
E.g. ammonia, lactate, urine organic acids, plasma amino acids, acylcarnitine profile, very long chain fatty acids
Specific investigations if rapid, non-invasive, inexpensive
E.g. blood for specific enzyme analysis
Targeted NGS panel / focussed exome
Diagnosis not achieved
Reassess phenotype, differential diagnosis
WES / WGS
Specific diagnostic investigations
E.g. skin biopsy, muscle biopsy, CSF studies, MRI
Diagnosis achieved

Diagnosing Childhood-onset Inborn Errors of Metabolism by Next Generation Sequencing

CLINICAL PROFORMA FOR MANCHESTER METABOLIC NGS PANELS

Patient Name:

Sex: Male ☐ Female ☐ Date of Birth (D/M/Y): Reference Number:

Clinical Information	
Clinical features	
Age of onset	
Details of relevant biochemical testing	
Likely mode of inheritance	Dominant ☐ X-linked ☐ Recessive ☐ Sporadic ☐ Information not available ☐
Relevant family history (draw brief pedigrees if needed) and any other relevant information	Parental consanguinity - Yes ☐ No ☐ Information not available ☐
Possible or likely diagnosis or disease group	
Is the patient known to any Consultants in Manchester Genetics department? (If yes, give name)	

Gene panel request		
AA + NT	☐	**Key:** ***AA*** *Disorders of amino acid metabolism and cerebral organic acid disorders;* ***NT*** *Disorders of neurotransmission;* ***AMN*** *Disorders associated with hyperammonaemia;* ***FAOD*** *Fatty acid oxidation defects;* ***KET*** *Disorders of ketogenesis or ketolysis;* ***OA*** *Organic acidaemias, including disorders of branched chain amino acid catabolism, 3-methylglutaconic acidurias;* ***VIT*** *Folate and cobalamin defects, also riboflavin transport defects, and biotin-responsive disorders;* ***CHO*** *Disorders of carbohydrate metabolism;* ***LSD*** *Lysosomal disorders;* ***NCL*** *Neuronal ceroid lipofuscinoses;* ***PER*** *Peroxisomal biogenesis disorders and disorders of single peroxisomal enzymes*
AMN + FAOD + KET	☐	
OA + VIT	☐	
CHO	☐	
LSD + NCL	☐	
PER	☐	
Full Panel	☐	For full list of genes in each panel please see www.mangen.co.uk

Signature	Department
	Hospital
Name	Phone
Date	Email

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www.mangen.org.uk

Manchester Metabolic NGS Panel Test Consent Form

I, _____ (DOB _____)

agree for myself/_____(DOB_____)

to undergo genetic testing for the Manchester Metabolic NGS Panel.

I understand:

- The genetic test looks at many different metabolic genes.
- The test may or may not find the cause of the metabolic disorder.
- The test may find changes in other genes that are not causing the metabolic disorder.
- The test may have implications for my wider family.
- The test may reveal a change in a gene that has other health implications in addition to the metabolic disorder.
- The test may reveal a change that cannot be interpreted. In these cases, family studies may be helpful.
- The genetic doctor / counsellor will explain the results of the test and its implications for me and my family as far as current knowledge allows.

I agree to my sample being stored in case future relevant tests become available and that my test result may be used by health care professionals to help other family members.
I agree for my GP and other health professionals to be informed of the test result.
I have been given an information sheet entitled 'Manchester Metabolic NGS panel testing: Information for patients'

Signed  Date _____

Relationship with the patient - Patient/Father/Mother/Guardian/Other (please specify _____)

Clinician signature _____ Date _____

Supplementary Table S1: Genes reported since 2013 to be implicated in mitochondrial disorders or congenital disorders of glycosylation demonstrating that the genetic heterogeneity of these group of disorders remains to be resolved.

Gene	Disorder	Reference
COA-X	Mitochondrial	Legati (2016)
CYP2U1	Mitochondrial	Legati (2016)
E4F1	Mitochondrial	Legati (2016)
CLPB	Mitochondrial	Wortmann (2015a)
PARS2	Mitochondrial	Sohou (2015)
NARS2	Mitochondrial	Sohou (2015)
FBXL4	Mitochondrial	Gai (2013)
TMEM126B	Mitochondrial	Sanchez-Cabellero (2016)
NAXE	Mitochondrial	Spiegel (2016)
NDUFB11	Mitochondrial	van Rahden (2015)
LYRM7	Mitochondrial	Invernizzi (2013)
TTC19	Mitochondrial	Atwal (2014)
GYG2	Mitochondrial	Imagawa (2014)
PET100	Mitochondrial	Lim (2014)
NUDFA4	Mitochondrial	Pitceathly (2013)
VARS2	Mitochondrial	Taylor (2014)
GARS	Mitochondrial	Taylor (2014)
FLAD1	Mitochondrial	Taylor (2014)
PTCD1	Mitochondrial	Taylor (2014)
SLC25A42	Mitochondrial	Shamseldin (2016)
CHKB	Mitochondrial	Castro-Gago (2014)
CAD	Congenital disorder of glycosylation	Ng (2015)
STT3A	Congenital disorder of glycosylation	Shrimal (2013)
STT3B	Congenital disorder of glycosylation	Shrimal (2013)
NUS1	Congenital disorder of glycosylation	Park (2014)
SSR4	Congenital disorder of glycosylation	Losfeld (2014)
MAN1B1	Congenital disorder of glycosylation	van Scherpenzeel (2014)
SLC35A1	Congenital disorder of glycosylation	Mohamed (2013)
TMEM165	Congenital disorder of glycosylation	Zeevaert (2013)
SLC35A2	Congenital disorder of glycosylation	Kodera (2013)
PGM3	Congenital disorder of glycosylation	Zhang (2014)
CCDC115	Congenital disorder of glycosylation	Jansen (2016a)
SLC39A8	Congenital disorder of glycosylation	Boycott (2015)
TMEM199	Congenital disorder of glycosylation	Jansen (2016b)

sub-panels.

Key – AA Disorders of amino acid metabolism including phenylketonuria, and cerebral organic acid disorders; AMN Disorders associated with hyperammonaemia; CDS Coding DNA Sequence; CHO Disorders of carbohydrate metabolism; FAOD Fatty acid oxidation defects including multiple acyl-CoA dehydrogenase deficiency; KET Disorders of ketogenesis or ketolysis; LSD Lysosomal disorders: includes lysosomal storage disorders, transport defects and protease defects; NCL Neuronal ceroid lipofuscinoses; NT Disorders of neurotransmission (includes pterins, tyrosinaemia); OA Organic acidaemias, including disorders of branched chain amino acid catabolism, 3-methylglutaconic acidurias; PER Peroxisomal biogenesis disorders and disorders of single peroxisomal enzymes; ROI Regions of Interest, VIT Folate and cobalamin defects, also riboflavin transport defects, and biotin-responsive disorders;

Note that some genes are included in more than one sub-panel.

Gene	CDS Size	Number of Exons	ROI Size	Gene OMIM #	Sub-panel	Additional sub-panel
OAT	1320	10	2320	613349	AA, NT	AMN, FAOD, KET
SLC7A7	1536	11	2636	603593	AA, NT	AMN, FAOD, KET
PTS	438	6	1038	612719	AA, NT	
QDPR	735	7	1435	612676	AA, NT	
GCH1	753	6	1353	600225	AA, NT	
PCBD1	315	4	715	126090	AA, NT	
FAH	1260	15	2760	613871	AA, NT	
ALDH5A1	1647	11	2747	610045	AA, NT	
GCDH	1317	12	2517	608801	AA, NT	
ALDH18A1	2388	18	4188	138250	AA, NT	
PAH	1359	13	2659	612349	AA, NT	
TAT	1365	12	2565	613018	AA, NT	
HPD	1182	14	2582	609695	AA, NT	
HGD	1338	14	2738	607474	AA, NT	
CBS	1698	18	3498	613381	AA, NT	
MAT1A	1188	9	2088	610550	AA, NT	
GNMT	888	6	1488	606628	AA, NT	
AHCY	1299	10	2299	180960	AA, NT	
CTH	1218	12	2418	607657	AA, NT	
SUOX	1638	4	2038	606887	AA, NT	
L2HGDH	1392	10	2392	609584	AA, NT	
D2HGDH	1566	10	2566	609186	AA, NT	
ASPA	942	7	1642	608034	AA, NT	
NAT8L	909	3	1209	610647	AA, NT	
AMT	1212	9	2112	238310	AA, NT	
GLDC	3063	25	5563	238300	AA, NT	
GCSH	522	5	1022	238330	AA, NT	
SLC3A1	2058	10	3058	104614	AA, NT	
SLC7A9	1464	13	2764	604144	AA, NT	
SLC6A19	1905	12	3105	608893	AA, NT	
SLC36A2	1452	10	2452	608331	AA, NT	
ABAT	1548	16	3148	137150	AA, NT	
GLRA1	1374	9	2274	138491	AA, NT	
GABRG2	1548	11	2648	137164	AA, NT	
SLC25A22	972	10	1972	609302	AA, NT	
TH	1587	14	2987	191290	AA, NT	
DDC	1443	15	2943	107930	AA, NT	
DBH	1854	12	3054	609312	AA, NT	
MAOA	1584	15	3084	309850	AA, NT	
SPR	786	3	1086	182125	AA, NT	
SLC6A3	1863	15	3363	126455	AA, NT	
ALDH7A1	1620	18	3420	107323	AA, NT	
PNPO	786	7	1486	603287	AA, NT	
ETFA	1002	12	2202	608053	AMN, FAOD, KET	
ETFB	1041	5	1541	130410	AMN, FAOD, KET	
ETFDH	1854	13	3154	231675	AMN, FAOD, KET	
NAGS	1605	7	2305	608300	AMN, FAOD, KET	
CPS1	4521	39	8421	608307	AMN, FAOD, KET	
OTC	1065	10	2065	300461	AMN, FAOD, KET	
ASS1	1239	15	2739	603470	AMN, FAOD, KET	
ASL	1395	17	3095	608310	AMN, FAOD, KET	
ARG1	993	8	1793	608313	AMN, FAOD, KET	
SLC25A13	2031	18	3831	603859	AMN, FAOD, KET	
SLC25A15	906	7	1606	603861	AMN, FAOD, KET	
GLUD1	1677	13	2977	606762	AMN, FAOD, KET	
SLC22A5	1746	11	2846	603377	AMN, FAOD, KET	
CPT1A	2322	19	4222	600528	AMN, FAOD, KET	
SLC25A20	906	9	1806	613698	AMN, FAOD, KET	
CPT2	1977	5	2477	600650	AMN, FAOD, KET	
ACADVL	2037	21	4137	609575	AMN, FAOD, KET	
HADHA	2292	20	4292	600890	AMN, FAOD, KET	
HADHB	1425	16	3025	143450	AMN, FAOD, KET	
ACADM	1365	13	2665	607008	AMN, FAOD, KET	
ACADS	1239	10	2239	606885	AMN, FAOD, KET	
ACAD9	1866	18	3666	611103	AMN, FAOD, KET	
DECR1	1008	10	2008	222745	AMN, FAOD, KET	
ACAT1	1284	12	2484	607809	AMN, FAOD, KET	
ACAT2	1281	9	2181	100678	AMN, FAOD, KET	
HMGCL	978	9	1878	613898	AMN, FAOD, KET	
HMGCS2	1527	10	2527	600234	AMN, FAOD, KET	
OXCT1	1563	17	3263	601424	AMN, FAOD, KET	
G6PC	1074	5	1574	613742	CHO	
SLC37A4	1356	12	2556	602671	CHO	
AGL	4599	34	7999	610860	CHO	
GBE1	2109	16	3709	607839	CHO	
PYGL	2544	20	4544	613741	CHO	
PHKA2	3708	33	7008	300798	CHO	
PHKB	3282	31	6382	172490	CHO	
PHKG2	1233	10	2233	172471	CHO	
PHKA1	3723	33	7023	311870	CHO	
PHKG1	1260	11	2360	172470	CHO	
GYS2	2112	16	3712	138571	CHO	

PGAM2	782	3	1082	612931	CHO	
LDHA	1086	8	1886	150000	CHO	
ALDOA	1257	16	2857	103850	CHO	
ENO3	1305	12	2505	131370	CHO	
PGM1	1743	11	2843	171900	CHO	
GYG1	1053	8	1853	603942	CHO	
GYS1	2214	16	3814	138570	CHO	
PRKAG2	1710	16	3310	602743	CHO	
NHLRC1	1188	1	1288	608072	CHO	
EPM2A	996	4	1396	607566	CHO	
G6PC3	1041	6	1641	612541	CHO	
SLC2A1	1479	10	2479	138140	CHO	
SLC2A2	1575	11	2675	138160	CHO	
FBP1	1017	7	1717	611570	CHO	
GALT	1140	11	2240	606999	CHO	
GALE	1047	12	2247	606953	CHO	
GALK1	1269	8	2069	604313	CHO	
LAMP2	1236	9	2136	309060	CHO	
GAA	2859	21	4959	606800	LSD, NCL	
IDUA	2028	11	3128	252800	LSD, NCL	
IDS	1653	9	2553	300823	LSD, NCL	
SGSH	1509	8	2309	605270	LSD, NCL	
NAGLU	2232	6	2832	609701	LSD, NCL	
HGSNAT	1992	18	3792	610453	LSD, NCL	
GNS	1755	15	3255	607664	LSD, NCL	
ARSG	1578	12	2778	610008	LSD, NCL	
GALNS	1569	14	2969	612222	LSD, NCL	
GLB1	2178	17	3878	611458	LSD, NCL	
ARSB	1602	8	2402	611542	LSD, NCL	
GUSB	1956	12	3156	611499	LSD, NCL	
HYAL1	1308	4	1708	607071	LSD, NCL	
ASAH1	1236	14	2636	613468	LSD, NCL	
GLA	1290	7	1990	300644	LSD, NCL	
GBA	1611	11	2711	606463	LSD, NCL	
HEXA	1623	14	3023	606869	LSD, NCL	
HEXB	1671	14	3071	606873	LSD, NCL	
GM2A	582	4	982	613109	LSD, NCL	
GALC	2058	17	3758	606890	LSD, NCL	
ARSA	1530	9	2430	607574	LSD, NCL	
PSAP	1581	14	2981	176801	LSD, NCL	
SMPD1	1896	6	2496	607608	LSD, NCL	
LIPA	1200	10	2200	613497	LSD, NCL	
NPC1	3837	25	6337	607623	LSD, NCL	
NPC2	664	5	1164	601015	LSD, NCL	
SUMF1	1281	13	2581	607939	LSD, NCL	
CTSA	1497	15	2997	613111	LSD, NCL	
GNPTAB	3771	21	5871	607840	LSD, NCL	
GNPTG	918	11	2018	607838	LSD, NCL	
MCOLN1	1743	14	3143	605248	LSD, NCL	
AGA	1041	9	1941	613228	LSD, NCL	
FUCA1	1401	8	2201	612280	LSD, NCL	
MAN2B1	3036	24	5436	609458	LSD, NCL	
MANBA	2640	17	4340	609489	LSD, NCL	
NEU1	1248	6	1848	608272	LSD, NCL	
NAGA	1236	9	2136	104170	LSD, NCL	
CTNS	1203	13	2503	606272	LSD, NCL	
SLC17A5	1488	11	2588	604322	LSD, NCL	
CTSK	990	8	1790	601105	LSD, NCL	
GNE	2262	12	3462	603824	LSD, NCL	
CLN3	1317	17	3017	607042	LSD, NCL	
CLN5	1224	4	1624	608102	LSD, NCL	
CLN6	1032	7	1732	606725	LSD, NCL	
CLN8	861	3	1161	607837	LSD, NCL	
DNAJC5	597	5	1097	611203	LSD, NCL	
MFSD8	1557	13	2857	611124	LSD, NCL	
PPT1	921	9	1821	600722	LSD, NCL	
TPP1	1692	13	2992	607998	LSD, NCL	
CTSD	1239	9	2139	116840	LSD, NCL	
IVD	1281	12	2481	607036	OA, VIT	AMN, FAOD, KET
PCCA	2187	24	4587	232000	OA, VIT	AMN, FAOD, KET
PCCB	1713	16	3313	232050	OA, VIT	AMN, FAOD, KET
MUT	2253	13	3553	609058	OA, VIT	AMN, FAOD, KET
MMACHC	849	4	1249	609831	OA, VIT	AMN, FAOD, KET
MMADHC	993	9	1893	611935	OA, VIT	AMN, FAOD, KET
MMAA	1257	7	1957	607481	OA, VIT	AMN, FAOD, KET
MMAB	753	9	1653	607568	OA, VIT	AMN, FAOD, KET
BCKDHA	1440	9	2340	608348	OA, VIT	
BCKDHB	1179	11	2279	248611	OA, VIT	
DBT	1449	11	2549	248610	OA, VIT	
PPM1K	1119	7	1819	611065	OA, VIT	
MCEE	531	3	831	608419	OA, VIT	
MCCC1	2178	19	4078	609010	OA, VIT	
MCCC2	1692	17	3392	609014	OA, VIT	
AUH	1020	10	2020	600529	OA, VIT	
TAZ	879	11	1979	300394	OA, VIT	
OPA3	543	2	743	606580	OA, VIT	
DNAJC19	351	6	951	608977	OA, VIT	
SERAC1	1965	17	3665	614725	OA, VIT	
TMEM70	783	3	1083	612418	OA, VIT	
LMBRD1	1623	16	3223	612625	OA, VIT	
GIF	1254	9	2154	609342	OA, VIT	
TCN1	1302	9	2202	189905	OA, VIT	
CUBN	10872	67	17572	602997	OA, VIT	
AMN	1362	12	2562	605799	OA, VIT	
TCN2	1284	9	2184	613441	OA, VIT	
CD320	849	5	1349	606475	OA, VIT	
MTRR	2178	15	3678	602568	OA, VIT	
MTR	3798	33	7098	156570	OA, VIT	
ABCD4	1821	19	3721	603214	OA, VIT	
SLC46A1	1380	6	1980	611672	OA, VIT	

DHFR	564	6	1184	126060	OA, VIT	
MTHFR	2094	13	3394	607093	OA, VIT	
FTCD	1719	15	3219	606806	OA, VIT	
MTHFD1	3063	27	5763	172460	OA, VIT	
DHFRL1	564	2	764		OA, VIT	
HCFC1	6243	26	8843	300019	OA, VIT	
SLC52A1	1347	5	1847	607883	OA, VIT	
SLC52A3	1410	5	1910	613350	OA, VIT	
SLC52A2	1338	5	1838	607882	OA, VIT	
HLCS	2181	12	3381	609018	OA, VIT	
BTD	1638	4	2038	609019	OA, VIT	
SLC19A3	1491	7	2191	606152	OA, VIT	
ACSF3		10	-	614265	OA, VIT	
SUCLG1		9	-	245400	OA, VIT	
SUCLA2		11	-	603922	OA, VIT	
PEX1	3852	24	6252	602136	PER	
PEX2	918	4	1318	170993	PER	
PEX3	1122	12	2322	603164	PER	
PEX5	1983	16	3583	600414	PER	
PEX6	2943	17	4643	601498	PER	
PEX10	1041	6	1641	602859	PER	
PEX12	1080	3	1380	601758	PER	
PEX13	1212	4	1612	601789	PER	
PEX14	1134	9	2034	601791	PER	
PEX16	1041	11	2141	603360	PER	
PEX19	900	8	1700	600279	PER	
PEX26	918	6	1518	608666	PER	
PEX7	972	10	1972	601757	PER	
ABCD1	2238	10	3238	300371	PER	
ACOX1	1983	14	3383	609751	PER	
HSD17B4	2286	25	4786	601860	PER	
AMACR	1185	6	1785	604489	PER	
GNPAT	2043	16	3643	602744	PER	
AGPS	1977	20	3977	603051	PER	
PHYH	1017	9	1917	602026	PER	
AGXT	1179	11	2279	604285	PER	
CAT	1584	13	2884	115500	PER	
DNM1L	2250	21	4350	603850	PER	

Supplementary Table S3: Details of individuals in whom no primary pathogenic variants were identified

Results are grouped by sub-panels - (AMN, FAOD, KET), (LSD, NCL), (OA, VIT), (AA, NT), (CHO), (PER), followed by combinations.

Key – AA Disorders of amino acid metabolism including phenylketonuria, and cerebral organic acid disorders; AMN Disorders associated with hyperammonaemia; CHO Disorders of carbohydrate metabolism; FAOD Fatty acid oxidation defects including multiple acyl-CoA dehydrogenase deficiency; KET Disorders of ketogenesis or ketolysis; LSD Lysosomal disorders: includes lysosomal storage disorders, transport defects and protease defects; NCL Neuronal ceroid lipofuscinoses; NT Disorders of neurotransmission (includes pterins, tyrosinaemia); OA Organic acidaemias, including disorders of branched chain amino acid catabolism, 3-methylglutaconic acidurias; PER Peroxisomal biogenesis disorders and disorders of single peroxisomal enzymes; VIT Folate and cobalamin defects, also riboflavin transport defects, and biotin-responsive disorders.

^ Denotes invasive or hazardous or lengthy or expensive investigations that were avoided by using the NGS approach.

Subject #	Age at investigation	Key biochemical and clinical features	Investigations avoided by NGS (estimated cost given where specific investigations anticipated)
Single sub-panel tested: (AMN, FAOD, KET)			
52	<1 year	Hyperammonaemia	^Sequential mutation analysis of multiple genes and/or liver biopsy and enzyme activities in liver tissue
53	<1 year	Death at 2 days of age. Suspected FAOD.	^Sequential mutation analysis of multiple genes
54	<1 year	Prematurity, severe lactic acidosis and profound hyperammonaemia with mildly elevated citrulline, normal arginine. Possible urea cycle disorder or organic acidaemia.	^Sequential mutation analysis of multiple genes and/or liver biopsy and enzyme activities in liver tissue
55	1-11 years	Common acute lymphoblastic leukaemia. Encephalopathy one week following high dose methotrexate. Hyperammonaemia with low citrulline and low orotate. Possible carbamoyl phosphate synthetase (CPS) or N-acetylglutamate synthase (NAGS) deficiency.	^Sequential mutation analysis of multiple genes and/or liver biopsy and enzyme activities in liver tissue
56	1-11 years	Sudden unexplained death in infancy, steatosis in liver post-mortem	^Sequential mutation analysis of multiple genes
57	1-11 years	Recurrent ketotic hypoglycaemia	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
58	1-11 years	Sudden onset of intractable seizures, extensive lipid accumulation in multiple organs on post-mortem. Low free carnitine. Suspected carnitine transport defect.	Further clinical and biochemical phenotyping
Single sub-panel tested: (LSD, NCL)			
59	1-11 years	Developmental regression with myoclonic epilepsy and cherry red spots. MRI brain normal. Normal lysosomal enzymes.	Further clinical and biochemical phenotyping
60	1-11 years	Short stature, coarse features, splenomegaly, cardiomyopathy, dysostosis multiplex. Failure to thrive and developmental delay. Elevated urine dermatan sulfate and heparan sulfate but no MPS disorder revealed on enzyme testing.	^Sequential mutation analysis of multiple genes
61	12-17 years	Joint contractures, autistic spectrum disorders. Mildly reduced leukocyte alpha-mannosidase activity. Suspected alpha-mannosidosis.	Single gene mutation analysis

62	12-17 years	Cognitive decline with ataxia and abnormal eye movements. Possible neuronal ceroid lipofuscinosis.	^Sequential mutation analysis of multiple genes
Single sub-panel tested: (OA, VIT)			
63	1-11 years	Developmental delay, failure to thrive, feeding difficulties, gastro-oesophageal reflux, maternal vitamin B12 deficiency. Low vitamin B12 and methylmalonic aciduria.	^Sequential mutation analysis of multiple genes and/or skin biopsy and complementation studies in fibroblasts
64	<1 year	Asymptomatic but elevated urinary methylmalonic acid.	^Sequential mutation analysis of multiple genes and/or skin biopsy and complementation studies in fibroblasts
65	12-17 years	Reactive lymphadenopathy, incidental finding of low vitamin B12	^Sequential mutation analysis of multiple genes
Single sub-panel tested: (AA, NT)			
66	<1 year	Previous diagnosis of Beckwith-Wiedemann syndrome with macroglossia. Upper limb hypotonia, ventricular septal defect, accessory nipples, thin upper lips. Elevated 2-hydroxyglutaric acid in urine.	^Sequential mutation analysis of multiple genes
Single sub-panel tested: (CHO)			
67	<1 year	Failure to thrive with recurrent lower respiratory tract infections and pulmonary hypertension. Neutropenia. Possible G6PC3 deficiency (Dursun syndrome)	Single gene mutation analysis
68	<1 year	Prematurity, frequent desaturations and apnoea. Hypoglycorrhacia (CSF : plasma glucose ratio <0.5). Suspected GLUT1 deficiency	Single gene mutation analysis
69	<1 year	Hepatomegaly, mouth ulcers, skin abscesses, recurrent upper respiratory tract infections, stenosing laryngitis. Hypoglycaemia with elevated lactate, triglycerides and urate. Suspected glycogen storage disease 1a or 1b.	^Sequential mutation analysis of multiple genes
Combination of sub-panels tested: (CHO) (AMN, FAOD, KET)			
70	1-11 years	Hypoglycaemia with and without ketosis. Global developmental delay and autism. Suspected FAOD, ketogenesis defect or glycogen synthase deficiency.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
71	1-11 years	Ketotic hypoglycaemia with elevated free fatty acids. Short stature, congenital heart disease, deafness, dysmorphism.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
72	1-11 years	Recurrent hypoglycaemia with severe ketosis. Seizures.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
73	1-11 years	Recurrent hypoglycaemia	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
74	1-11 years	Recurrent hypoglycaemia with ketoacidosis. Recurrent febrile seizures. Possible ketogenesis defect.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
75	1-11 years	Recurrent ketotic hypoglycaemia. Seizures.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
76	1-11 years	Recurrent unexplained hypoglycaemia	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles

77	1-11 years	Recurrent hypoglycaemia	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
78	<1 year	Developmental delay, microcephaly, hepatomegaly, muscle biopsy suggestive of glycogen storage disease (GSD VII). Episode of profound hypoglycaemia and metabolic acidosis.	^Sequential mutation analysis of multiple genes
79	1-11 years	Episodic weakness and ataxia. Ketoacidosis, hypoglycaemia and hyperammonaemia with mild elevation of 3-hydroxybutyrate. Suspected ketolytic disorder or glycogen storage disorder	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles, or ^sequential mutation analysis of multiple genes
80	1-11 years	Ketotic hypoglycaemia, gastrointestinal disturbance, joint laxity, hearing loss.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
81	1-11 years	Recurrent ketotic hypoglycaemia	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
82	1-11 years	Muscle weakness. Muscle biopsy nonspecific. Possible glycogen storage disorder.	^Sequential mutation analysis of multiple genes
83	<1 year	Poor feeding, hypoglycaemia and lactic acidosis. Cardiorespiratory arrest day 3 of life leading to hypoxic brain injury. Glycerol in urine. Suspected fructose 1,6-bisphosphatase deficiency	Single gene mutation analysis
84	1-11 years	Atypical ketotic hypoglycaemia. Known pancreatic exocrine insufficiency (aetiology uncertain). Neutropenia.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
Combination of sub-panels tested: (OA, VIT) (CHO) (AMN, FAOD, KET)			
85	1-11 years	Recurrent ketotic hypoglycaemia with elevated lactate, hypotonia.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
86	1-11 years	Recurrent hypoglycaemia. Mild developmental delay, hearing impairment.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
87	1-11 years	Two episodes of ketoacidosis, one with elevated lactate and one with hypoglycaemia. Suspected fructose-1,6-bisphosphatase deficiency or succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles, skin biopsy, fibroblast culture and enzyme analysis
88	<1 year	Recurrent hypoglycaemia. Short gut and liver disease.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
89	1-11 years	Recurrent ketotic hypoglycaemia, hypotonia, drooling, dyspraxia.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
Combination of sub-panels tested: (OA, VIT) (AA, NT)			
90	1-11 years	Developmental delay, severe vitamin B12 responsive	Single gene mutation analysis

		megaloblastic anaemia, mildly elevated homocysteine but no methylmalonic acid detected in urine. Suspected transcobalamin II deficiency.	
Combination of subpanels tested: (AA, NT) (AMN, FAOD, KET)			
91	<1 year	Failure to thrive, anaemia. Elevated methylmalonic acid in urine and homocysteine in blood. Suspected remethylation disorder.	^Sequential mutation analysis of multiple genes
92	<1 year	Cardiomyopathy, multiple organ failure. Severe hyperammonaemia and lactic acidosis.	^Sequential mutation analysis of multiple genes, or liver biopsy and enzyme analysis followed by single gene mutation analysis
All sub-panels tested			
93	<1 year	Respiratory distress, lethargy and poor feeding. Moderate hyperammonaemia with elevated lactate and ketonuria. Suspected urea cycle disorder or organic acidaemia.	^Sequential mutation analysis of multiple genes and/or liver biopsy and enzyme activities in liver tissue
94	1-11 years	Megaloblastic anaemia. Suspected cerebral folate deficiency	Single gene mutation analysis
95	Adult (18+ years)	Hepatosplenomegaly, chronic thrombocytopenia since 3 years of age, acute pancytopenia. Bone marrow examination normal. Lysosomal enzymes normal. Suspected Gaucher-like disorder.	Further clinical and biochemical phenotyping
96	Adult (18+ years)	Known *MTHFR* homozygous mutation. Epilepsy, autism, hypermobility, high arched palate. Symptoms since 3 years of age.	Further clinical and biochemical phenotyping
97	12-17 years	Muscle weakness, wasting and contractures. Symptoms since 5 years of age.	Further clinical and biochemical phenotyping
98	1-11 years	Ketotic hypoglycaemia, seizure secondary to hypoglycaemia.	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
99	12-17 years	Known myotonic dystrophy. Cardiomyopathy since 22 months, shortness of breath on exertion, hyper-IgM syndrome, intellectual disability, vasculitis, hypermobility, lower limb upper motor neurone signs. Abnormal white matter changes on brain MRI.	Further clinical and biochemical phenotyping
100	1-11 years	Bilateral optic atrophy, growth retardation, cerebral calcifications. Macrocytic anaemia.	Further clinical and biochemical phenotyping
101	1-11 years	Ketotic hypoglycaemia. Suspected FAOD or disorder of bile acid	^Hospital admission, diagnostic fast with serial glucose, lactate, ketone analyses and full metabolic/endocrine profiles
102	<1 year	Developmental regression, movement disorder, peripheral hypertonia, hypercalcaemia (suspected familial hypocalciuric hypercalcaemia), gastro-oesophageal reflux. Mildly raised HIAA and HVA levels. Suspected neurotransmitter disorders, non-ketotic hyperglycinaemia, serine synthase deficiency, or disorders of purine metabolism disorders especially *HPRT*.	^Sequential mutation analysis of multiple genes

Supplementary Table S4: Details of individuals in whom incidental carrier findings were identified

Results are grouped by sub-panels - (AMN, FAOD, KET), (LSD, NCL), (OA, VIT), (AA, NT), (CHO), (PER), followed by combinations. Grouping of sub-panels is indicated by parenthesis. Only individuals in whom incidental carrier findings were found are reported. Details of individuals in whom a genetic diagnosis was made are provided in Table 1 and details of individuals in whom no primary pathogenic variants were identified are provided in Table S2. Numbers of variants of unknown clinical significance are reported in Table S4.

Key – AA Disorders of amino acid metabolism including phenylketonuria, and cerebral organic acid disorders; AMN Disorders associated with hyperammonaemia; CHO Disorders of carbohydrate metabolism; FAOD Fatty acid oxidation defects including multiple acyl-CoA dehydrogenase deficiency; KET Disorders of ketogenesis or ketolysis; LSD Lysosomal disorders: includes lysosomal storage disorders, transport defects and protease defects; NCL Neuronal ceroid lipofuscinoses; NT Disorders of neurotransmission (includes pterins, tyrosinaemia); OA Organic acidaemias, including disorders of branched chain amino acid catabolism, 3-methylglutaconic acidurias; PER Peroxisomal biogenesis disorders and disorders of single peroxisomal enzymes; VIT Folate and cobalamin defects, also riboflavin transport defects, and biotin-responsive disorders.

Subject #	Sub-panels tested	Result	Zygosity	Interpretation
3	(AMN, FAOD, KET)	*ACADM* c.250C>T [p.(Leu84Phe)]	heterozygous	Carrier finding
16	(LSD, NCL)	*GAA* c.859-3C>G	heterozygous	Carrier finding
20	(OA, VIT)	*ACSF3* c.1672C>T [p.(Arg588Trp)]	heterozygous	Carrier finding
29	(AA, NT)	*PAH* c.688G>A [p.(Val230Ile)]	heterozygous	Carrier finding
38	(PER)	*CAT* c.1169G>A [p.(Gly390Asp)]	heterozygous	Carrier finding
47	All sub-panels	*ASL* c.392C>T [p.(Thr131 Met)]	heterozygous	Carrier finding
48	All sub-panels	*GNMT* c.529C>A [p.(His177Asn)]	heterozygous	Carrier finding
51	All sub-panels	*CUBN* c.2594G>A [p.(Ser865Asn)] *HPD* c.1005C>G [p.(Ile335Met)]	heterozygous heterozygous	Carrier finding Carrier finding
52	(AMN, FAOD, KET)	*SLC22A5* c.136C>T [p.(Pro46Ser)]	heterozygous	Carrier finding
67	(CHO)	*SLC2A2* c.497-2A>T	heterozygous	Carrier finding
85	(OA, VIT) (CHO) (AMN, FAO, KET)	*PCCB* c.1550C>T [p.(Ala517Val)]	heterozygous	Carrier finding
86	(OA, VIT) (CHO) (AMN, FAO, KET)	*MMAB* c.12C>A [p.(Cys4Ter)]	heterozygous	Carrier finding
93	All sub-panels	*MUT* c.1106G>A [p.(Arg369His)] *NAGS* c.785G>C [p.(Gly262Ala)] *GUSB* c.1121G>T [p.(Arg374Leu)]	heterozygous heterozygous heterozygous	Carrier finding Carrier finding Carrier finding
94	All sub-panels	*GALT* c.563A>G [p. (GIn188Arg)] *CLN8* c. 806A>T [p. (GIu269Val)]	heterozygous heterozygous	Carrier finding Carrier finding
95	All sub-panels	*PEX12* c.102A>T [p. (Arg34Ser)]	heterozygous	Carrier finding
96	All sub-panels	*ACSF3* c.1672C>T [p.(Arg558Trp)] *MTHFR* c.665C>T [p.(Ala222Val)]	heterozygous homozygous	Carrier finding Known diagnosis
97	All sub-panels	*GNE* c.2179G>T [p.(Val727Leu)]	heterozygous	Carrier finding
98	All sub-panels	*MMAB* c.394T>C [p.(Cys132Arg)] *HEXA* c.745C>T [p.(Arg249Trp)]	heterozygous heterozygous	Carrier finding Carrier finding

Supplementary Table S5: Number of variants of unknown clinical significance (VUS)

Results are grouped by sub-panels - (AMN, FAOD, KET), (LSD, NCL), (OA, VIT), (AA, NT), (CHO), (PER), followed by combinations. Grouping of sub-panels is indicated by parenthesis.

Key – AA Disorders of amino acid metabolism including phenylketonuria, and cerebral organic acid disorders; AMM Disorders associated with hyperammonaemia; CHO Disorders of carbohydrate metabolism; FAOD Fatty acid oxidation defects including multiple acyl-CoA dehydrogenase deficiency; KET Disorders of ketogenesis or ketolysis; LSD Lysosomal disorders: includes lysosomal storage disorders, transport defects and protease defects; NCL Neuronal ceroid lipofuscinoses; NT Disorders of neurotransmission (includes pterins, tyrosinaemia); OA Organic acidaemias, including disorders of branched chain amino acid catabolism, 3-methylglutaconic acidurias; PER Peroxisomal biogenesis disorders and disorders of single peroxisomal enzymes; VIT Folate and cobalamin defects, also riboflavin transport defects, and biotin-responsive disorders.

Sub-panels tested	**Total number of genes tested**	**Number of individuals with VUS (% of individuals tested)**	**Median number of VUS per individual (range)**
(AMN, FAOD, KET)	28	12 (71%)	1 (0-8)
(LSD, NCL)	50	12 (92%)	6 (0-15)
(OA, VIT)	50	8 (80%)	2 (0-12)
(AA, NT)	43	7 (78%)	1 (0-4)
(CHO)	32	4 (80%)	5 (0-7)
(PER)	23	2 (50%)	0.5 (0-2)
(CHO), (AMN, FAOD, KET)	60	15 (88%)	2 (0-4)
All sub-panels: (OA, VIT), (CHO), (AA, NT), (AMN, FAOD, KET), (PER), (LSD, NCL)	226	14 (93%)	12 (0-24)
(OA, VIT), (CHO), (AMN, FAOD, KET)	110	6 (100%)	8.5 (2-17)
(OA, VIT), (AA, NT)	93	2 (100%)	10 (7-13)
(OA, VIT), (CHO), (AA, NT), (AMN, FAOD, KET)	153	1 (100%)	2
(OA, VIT), (AA, NT), (AMN, FAOD, KET)	121	1 (100%)	5
(AA, NT), (AMN, FAOD, KET)	71	1 (100%)	6
(OA, VIT), (AMN, FAOD, KET)	78	1 (50%)	4

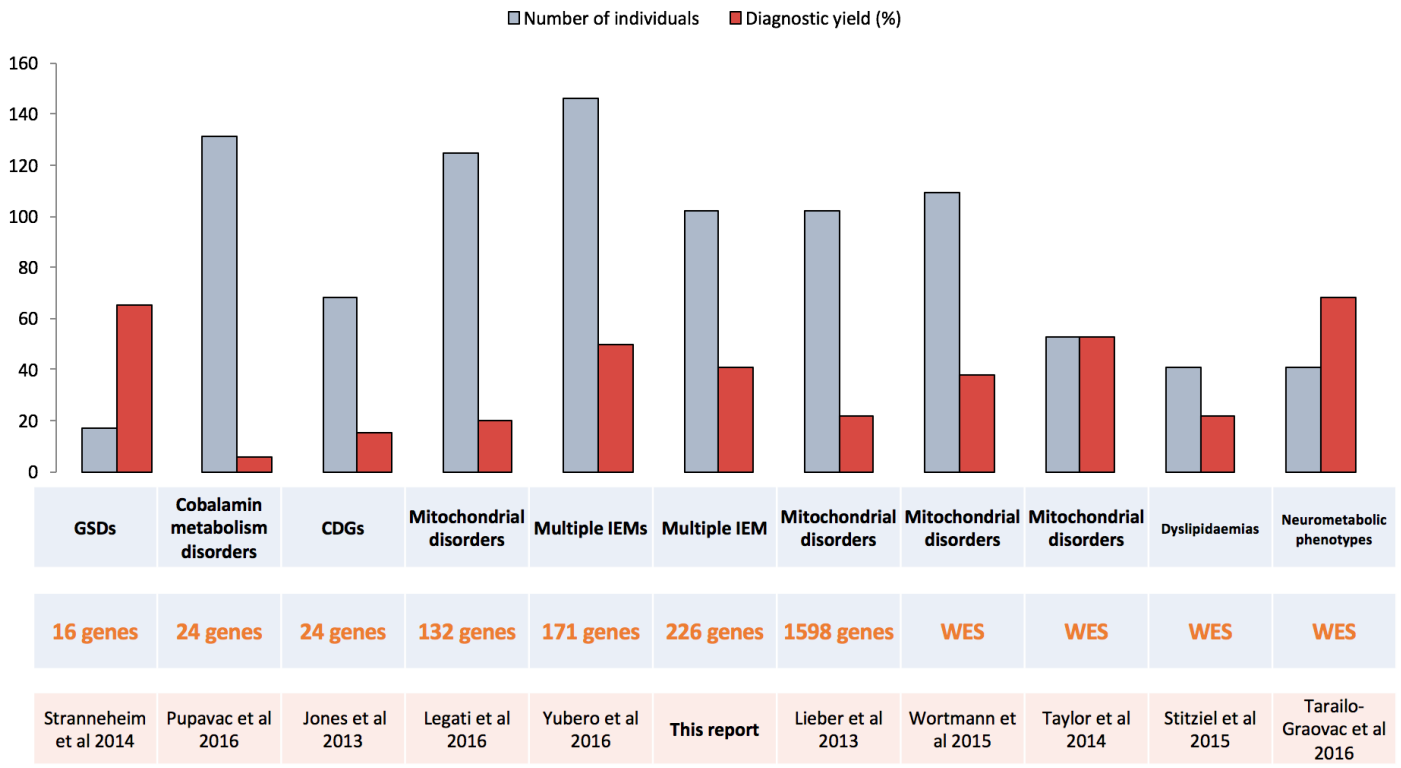
Supplementary Table S6: Comparison of NGS costs and estimated costs of investigations avoided by NGS. NGS costs based on £950 + £50 for each additional panel requested. 'Pre-NGS estimated cost' refers to estimated cost of additional investigations avoided by NGS approach. Where no pathogenic variants were identified, this refers to investigations that would have been undertaken had NGS not been available, and was only estimated where specific investigations could be anticipated e.g. single gene mutation analysis or specific biochemical tests.

Category	**Number of patients**	**NGS cost**	**Pre-NGS estimated cost**
Primary pathogenic variants identified	51	£47,550	£48,900
No primary pathogenic variants identified	51	£49,800	£32,500

Supplementary Figure S1: Summary of studies of use of NGS panels in IEM

Diagnostic yield of studies investigating NGS use in diagnosing IEMs, showing diagnostic yield (%), number of genes tested, and number of individuals tested. A targeted NGS panel approach was used in the Pupavac, Jones, Yubero and Stranneheim studies. Targeted exome sequencing was used in the Lieber study. Whole exome sequencing was used in the Taylor, Stitziel and Tarailo-Graovac studies. A two-step approach was used in the Legati study (targeted NGS proceeding to WES if no diagnosis) and in the Wortmann study (WES virtual panel proceeding to WES if no diagnosis).

Key – GSDs glycogen storage disorders, CDGs congenital disorders of glycosylation, WES whole exome sequencing



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