Advantages and pitfalls of an extended gene panel for investigating complex neurometabolic phenotypes

Emma S. Reid,1 Apostolos Papandreou,1,2 Suzanne Drury,3 Christopher Boustred,3 Wyatt W. Yue,4 Yehani Wedatilake,1 Clare Beesley,3 Thomas S. Jacques,5,6 Glenn Anderson,5 Lara Abulhoul,7 Alex Broomfield,7 Maureen Cleary,7 Stephanie Grunewald,1,7 Sophia M. Varadkar,2 Nick Lench,3 Shamima Rahman,1,7 Paul Gissen,1,7,* Peter T. Clayton1,* and Philippa B. Mills1*

*These authors contributed equally to this work.

Neurometabolic disorders are markedly heterogeneous, both clinically and genetically, and are characterized by variable neurological dysfunction accompanied by suggestive neuroimaging or biochemical abnormalities. Despite early specialist input, delays in diagnosis and appropriate treatment initiation are common. Next-generation sequencing approaches still have limitations but are already enabling earlier and more efficient diagnoses in these patients. We designed a gene panel targeting 614 genes causing inborn errors of metabolism and tested its diagnostic efficacy in a paediatric cohort of 30 undiagnosed patients presenting with variable neurometabolic phenotypes. Genetic defects that could, at least partially, explain observed phenotypes were identified in 53% of cases. Where biochemical abnormalities pointing towards a particular gene defect were present, our panel identified diagnoses in 89% of patients. Phenotypes attributable to defects in more than one gene were seen in 13% of cases. The ability of in silico tools, including structure-guided prediction programmes to characterize novel missense variants were also interrogated. Our study expands the genetic, clinical and biochemical phenotypes of well-characterized (POMGNT1, TPP1) and recently identified disorders (PGAP2, ACSF3, SERAC1, AFG3L2, DPYS). Overall, our panel was accurate and efficient, demonstrating good potential for applying similar approaches to clinically and biochemically diverse neurometabolic disease cohorts.

1 Genetics and Genomics Medicine Programme, UCL Institute of Child Health, London, UK
2 Neurology Department, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK
3 North East Thames Regional Genetics Service, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK
4 Structural Genomics Consortium, University of Oxford, Oxford, UK
5 Histopathology Department, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK
6 Developmental Biology and Cancer Programme, UCL Institute of Child Health, London, UK
7 Metabolic Medicine Department, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK

Correspondence to: Dr Philippa B. Mills, PhD, Genetics and Genomic Medicine Programme, UCL Institute of Child Health, 30 Guildford Street, London, WC1N 1EH, UK
E-mail: p.mills@ucl.ac.uk

Keywords: neurometabolic disorders; inborn errors of metabolism; heterogeneity; next-generation sequencing; gene panel
Introduction

Inborn errors of metabolism (IEM) are markedly heterogeneous, both clinically and genetically, with more than 600 genes known to cause disease. In the presence of neurological dysfunction, which is not only common in IEM but also often the most prominent phenotypic feature, these patients are frequently labelled as having ‘probable neurometabolic disease’, especially if suggestive neuroimaging or laboratory findings co-exist. The challenges when diagnosing neurometabolic disorders are largely attributable to the clinical and genetic heterogeneity (including often non-specific or atypical presentations early on in the disease course) and lack of clinical awareness of rare entities. Patients with suspected neurometabolic disease are frequently referred to specialist centres and undergo extensive and often invasive diagnostic testing. Despite this, diagnostic delays or difficulties establishing a definitive diagnosis are commonly encountered, with many such patients attending secondary and tertiary neurology clinics remaining undiagnosed (Verity et al., 2010).

Timely diagnosis of neurometabolic disease is crucial, especially for those disorders that are treatable or manageable, with early initiation of treatment often resulting in improved outcomes. Next-generation sequencing (NGS) has revolutionized the diagnostic approach to such conditions (Nemeth et al., 2013; Martin et al., 2014) and helped to reduce the number of tests required for a diagnosis to be established. However, despite the continuous progress made in the field, there are still limitations to the approach, including access to NGS technology (especially in a non-specialist setting), costs, incomplete coverage of candidate genes and generation of large amounts of data that are difficult to interpret. Whole-exome sequencing (WES) and whole-genome sequencing (WGS) studies are primarily offered either in research laboratories or in a commercial setting, and have not yet been fully integrated into the clinical genetics services of many healthcare systems worldwide. An alternative NGS method, gene panel testing, has recently become available in clinical services and offers targeted testing of candidate genes. An extended genetic panel approach to investigating IEM might be advantageous (Saudi Mendelioome Group, 2015) due to reduced times required for data processing and increased coverage depth compared to WES and WGS. Our objective was to investigate the utility of this approach by designing an IEM gene panel and applying it to patients presenting with a wide array of neurometabolic phenotypes. We discuss the panel’s effectiveness in establishing a diagnosis, the clinical implications of its use as well as potential pitfalls of using broad-scale genetic testing. We also consider the predictive value of in silico tools commonly used for characterization of novel variants and investigate whether mapping of detected variants to known 3D protein structures can help further elucidate their significance.

Materials and methods

Patients

This study was approved by the National Research Ethics Service (NRES) Committee London – Bloomsbury (REC reference: 13/LO/0168). We recruited patients from a single UK tertiary centre’s neurometabolic disease clinics presenting with a range of neurological features such as developmental delay, macro or microcephaly, neurological regression, ataxia, epilepsy and/or organomegaly with or without other diagnostic indicators [including suggestive biochemical marker(s) or neuromaging abnormalities]. All participants had undergone extensive previous investigations including multiple standard and specialized biochemical tests, invasive procedures (e.g. muscle and/or skin biopsy, lumbar puncture) and targeted gene testing but lacked a definitive molecular diagnosis. Thirty patients were included (Tables 1–3). First, we recruited 21 patients with suspected IEM but absence of specific clinical findings or biochemical pointers towards a particular disorder. Additionally, we included nine cases where biochemical findings indicated a particular disorder or group of disorders, not only to investigate the utility of this approach in more specific presentations but also because similar biochemical abnormalities could result from mutations in multiple genes. Finally, for panel validation purposes, we additionally recruited 13 patients with a known genetic diagnosis (Supplementary Table 1). Written informed consent was obtained in all cases.

Gene capture, sequencing and variant analysis

A custom HaloPlex target enrichment system (Agilent) was used to capture 614 genes, covering 16 broad classes of IEM (Supplementary material). Sequencing was performed using the HiSeq 2500 platform (Illumina). Sequence variants with putatively deleterious effects were confirmed by Sanger sequencing (Supplementary Table 4). To interrogate for potential pathogenicity in identified variants, we investigated whether variants had been reported previously as pathogenic, their frequency in the population, segregation within the family (where samples were available) and predicted functional impact utilizing SIFT (http://sift.bii.a-star.edu.sg/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and Combined Annotation Dependent Depletion (CADD) (http://cadd.gs.washington.edu/). Where possible, missense variants were mapped to known 3D protein structures and compared to in silico findings (Supplementary Table 5).
| Patient | Age (years) | Gender | Primary clinical phenotype | Other phenotypic features | Relevant specialist investigations | MRI head | Diagnosis | Gene |
|---------|-------------|--------|----------------------------|--------------------------|----------------------------------|----------|-----------|------|
| B1      | 4           | M      | Macrocephaly, intermittent squint | Short stature, asthma, development unremarkable | Elevated 3-methylcrotonylglycine, methylcitrate and 3-hydroxyisovalerate in urine. Normal biotinidase activity | Not performed | Holocarboxylase synthetase deficiency | HLCS |
| B2      | 1           | M      | Sibling born prematurely and passed away due to hyper-ammonaemia. No clinical concerns | No | Moderately elevated orotic acid in urine between 9.48 \( \mu \)mol/mmol creatinine (ref: 0-5) | Not performed | Orotic aciduria | UMPS |
| B3      | 15          | M      | At 3 1/2 years: lethargy, vomiting, alkalosis and hyper-ammonaemia. Learning and behavioural difficulties | Day 2 of life: to lethargy and irritability- presumed sepsis but cultures negative. \( NH_3 \) not measured | Plasma aminoacids: raised glutamine. Low carbomylphosphate synthase activity in liver biopsy (0.15 mmol/hr/mg protein) | Not performed | Carbomoyl phosphate synthetase I deficiency | CPS1 |
| B4      | 17          | F      | Short stature, obesity, distended abdomen at 3 years | Hepatomegaly. No documented learning difficulties | Low glycogen debranching enzyme activity of 0.07 \( \mu \)mol/min/g protein (ref: 0.3-3.0) | Not performed | Glycogen storage disease type III | AGL |
| B5      | 9           | F      | Galactosaemia, picked up through newborn screening, treated early | Normal development apart from difficulties in mathematics | Elevated galactose-1-phosphate activity | Not performed | Galactosaemia | GALT |
| B6      | 5           | M      | Global delay. One of monozygotic twins | Hypotonia, brachycephaly, long face | Elevated plasma lysine ranging between 439-449 \( \mu \)mol/l (ref: 100-300). Elevated CSF lysine of 67 \( \mu \)mol/l (ref: 10-32) | Delayed myelination, lack of white matter bulk | Hyperlysinaemia | AASS |
| B7      | 5           | M      | Global delay, epilepsy, one of monozygotic twins | Hypotonia, brachycephaly, long face | Elevated plasma lysine ranging between 440-780 \( \mu \)mol/l (ref: 100-300). Elevated CSF lysine of 92 \( \mu \)mol/l (ref: 10-32) | Not performed | Hyperlysinaemia | AASS |
| B8      | 20          | F      | Sensorineural hearing loss, ataxia, neurological regression | Scoliosis, constipation | Bile acid analysis and skin fibroblast studies suggestive of a peroxisomal biogenesis defect | Leukodystrophy | Peroxisome biogenesis disorder | PEX6 |
| U1      | 11          | F      | Developmental delay, ataxia, horizontal nystagmus | Microcephaly, retinal dystrophy | CSF: low 5-MTHF and high HVA and BH4. Blood: Elevated prolactin, alanine, intermittently high CK and plasma lactate. Muscle: Normal RCE | Normal | Muscular dystrophy-dystroglycanopathy | POMGNT1 |
| U2      | 2           | M      | Microcephaly, developmental delay | Dysplastic kidneys | Neonatal lact acidosis, high plasma triglycerides, elevated urine thymidine and uracil, low plasma urate and detectable tynine | Normal | Dihydropyrimidinase deficiency | DPYS |
| U3      | 6           | M      | Neonatal jitteriness, developmental delay, autism | Joint hypermobility | Persistent methylmalonic and malonic aciduria | Not performed | Combined malonic and methylmalonic aciduria | ACSF3 |
| U4      | 9           | M      | Congenital ataxia, diplegia; drop attacks, no obvious EEG correlate | No | Plasma: mildly raised alanine, normal lactate. CSF: low 5-HIAA | Abnormal bilateral caudate and lentiform nuclei signal | Spino cerebellar ataxia 28 / Autosomal recessive spastic ataxia 5 | AFG3L2 |
| U5      | 4           | M      | Developmental delay, subsequent regression with progressive sensorineural deafness | Sensorineural deafness | Raised 3-methylglutaconic acid level with normal 3-methylglutaric levels | Basal ganglia high T2 signal, cerebellar atrophy | 3-methylglutaconic aciduria with deafness | SERAC1 |

(continued)
Results

Panel validation

Nineteen of 20 pathogenic sequence variants were identified in the 13 genetically diagnosed control samples (Supplementary Table 1). These included seven heterozygous and five homozygous missense, two heterozygous splice site mutations, a heterozygous single base insertion and four deletions ranging in size from 2 bp to ~6 kbp. The homozygous 37-amino acid deletion in Patient D6 was not identified. Seven of 20 variants had not been previously reported in the literature.

Clinical characteristics of undiagnosed cohort

Age ranged from 1 to 20 years (mean 7.2 years, median 6 years). Only 9/30 patients (Patients B1–B9, Tables 1–3) had abnormal biochemistry suggestive of an underlying genetic diagnosis, despite previous extensive testing in all cases. Our panel identified 21 variants in 16 patients, of which only seven had previously been reported in the literature (Reichardt et al., 1991; 1992; Shen et al., 1996; Wohlers et al., 1999; Aoshima et al., 2001; Yoshida et al., 2001; Santer et al., 2005). Ten variants were classified as pathogenic, 10 as likely pathogenic and one of uncertain significance (Richards et al., 2015) (Supplementary Table 5). Variants included 15 missense, two nonsense, three insertions/deletions and one splice site mutation. Identified variants could at least partially explain the observed clinical phenotype in all cases.

Of nine patients with previous biochemical testing pointing towards a diagnosis, identification of pathogenic variants was possible for eight (88.8%). Parental DNA to check segregation within families was not available. We were unable to identify any potential pathogenic variants in Patient B9, whose biochemical profile suggested hyperprolinaemia type II and, in whom, a homozygous complex insertion/deletion event resulting in a frameshift and premature stop codon in ALDH4A1 was subsequently identified via Sanger sequencing. Otherwise, in most other cases, two pathogenic variants were identified in each candidate gene.

We were also able to attain a molecular genetic diagnosis in 8/21 (38%) of patients without a biochemical marker pointing towards a specific genetic diagnosis (Tables 1–3). Two pathogenic (or likely pathogenic) variants were identified for each candidate gene. All variants were confirmed by Sanger sequencing in probands and family members where possible. Detailed clinical descriptions of these patients are given in the Supplementary material. In Patients B6, B7 and U2, the identified variants could explain the biochemical abnormalities but not other clinical features observed, indicating the presence of other, as yet unidentified gene defects. Additionally, Patient U7
| Patient | Gene | Nucleotide change | Amino acid | Segregation confirmed | SIFT | PolyPhen-2 | CADD score | Reference | Clinical phenotype explained | Biochemical phenotype explained |
|---------|------|-------------------|------------|----------------------|------|------------|------------|-----------|--------------------------------|----------------------------------|
| B1      | HLCS | c.2126C>T         | p.Pro709Leu | No                   | Deleterious | Probably damaging | 20.9       | Novel     | Yes                                           | Yes                             |
| B2      | UMPS | c.451G>A          | p.Val151Met | No                   | Deleterious | Possibly damaging | 24.8       | Novel     | Yes                                           | Yes                             |
| B3      | CPS1 | c.1010A>G         | p.His337Arg | No                   | Deleterious | Probably damaging | 26.3       | Aoshima et al. 2001 | Yes<sup>a</sup>                  | Yes<sup>a</sup>                  |
| B4      | AGL  | c.2590C>T         | p.Arg864Ter | No                   | Deleterious | Possibly damaging | 38.0       | Shen et al. 1996  | Yes                                           | Yes                             |
| B5      | GALT | c.563A>G          | p.Gln188Arg | No                   | Deleterious | Probably damaging | 25.3       | Reichardt et al. 1991 | Yes                                           | Yes                             |
| B6      | AASS | c.965G>A          | p.Arg322His | No                   | Deleterious | Benign          | 26.1       | Reichardt et al. 1992 | Yes                                           | Yes                             |
| B7      | AASS | c.965G>A          | p.Arg322His | No                   | Tolerated   | Probably damaging | 29.1       | Novel     | No                                             | Yes                             |
| B8      | PEX6 | c.965G>A          | p.Arg322His | No                   | Tolerated   | Probably damaging | 29.1       | Novel     | No                                             | Yes                             |
| U1      | POMGNT1 | c.373C>G         | p.Arg12Thr  | No                   | Deleterious | Probably damaging | 34.0       | Novel     | Yes                                           | Yes                             |
| U2      | DPYS | c.144_151dupGCTGCGGG | p.Arg12Thr  | -                    | Benign      | -              | 21.7       | Yoshida et al. 2001 | Yes                                           | Yes                             |
| U3      | ACSF3 | c.1453A>C         | p.Leu356Arg | No                   | Deleterious | Probably damaging | 24.2       | Novel     | Yes                                           | Yes                             |
| U4      | AFG3L2 | c.1067T>G        | p.Leu356Arg | -                    | Deleterious | Probably damaging | 29.9       | Novel     | Yes                                           | Yes                             |
| U5      | SERAC1 | c.1850delinsCA    | p.Leu356Arg | -                    | Deleterious | Probably damaging | 35.0       | Novel     | Yes                                           | Yes                             |
| U6      | PGAP2 | c.560C>T          | p.Ala187Val | Yes                  | Deleterious | Possibly damaging | 23.3       | Novel     | Yes                                           | Yes                             |
| U7      | TPPI | c.887G>A          | p.Gly296Asp | No                   | Deleterious | Possibly damaging | 24.6       | Novel     | Yes                                           | No                              |
| U8      | GALE | c.280G>A          | p.Val94Met  | No                   | Deleterious | Possibly damaging | 29.9       | Wehlers et al. 1999 | Yes                                           | Yes                             |

<sup>a</sup>Parental DNA was unavailable but Sanger sequencing identified the same homozygous mutation in a similarly affected sister.
<sup>b</sup>Parental DNA was unavailable but Sanger sequencing identified the same homozygous mutation in a brother who also had an abnormal type I transferrin isoelectric focusing pattern.
<sup>c</sup>Second mutation not identified. CADD = Combined Annotation Dependent Depletion.
had pathogenic variants identified in ALDOB and TPP1, while the clinical and biochemical phenotype was consistent with simultaneous presence of mutations in both genes (Supplementary material).

3D structure analysis

3D structural analysis of identified variants was performed using the ICM-Pro software (Molsoft LLC), when structural data were available for the proteins (Patients B2, U7 and U8) or for ‘close homologues/orthologues’ (Patients B8 and U4) (Supplementary Table 6). The impact of the amino acid substitution for six missense variants, all predicted to be deleterious and probably/possibly damaging by SIFT and PolyPhen-2, was determined by mapping them onto the wild-type structures and inspecting potential changes in bonding interactions, packing and secondary structures due to the amino acid substitution. In all cases, our structure-guided findings concurred with in silico prediction software, further supporting variant pathogenicity.

Discussion

In our study, we investigated the utility of an extended gene panel in diagnosing patients with neurometabolic disorders. Due to the marked clinical, biochemical and genetic heterogeneity encountered in neurometabolic disease, targeted gene testing is often not advantageous, economical or efficient. The panel described in our study was shown to be a powerful tool that enhances the diagnostic ability in the clinical setting. It covers 614 genes, including the vast majority of genes currently known to cause neurometabolic disease, hence sharing similarities with WES approaches but with the added advantage of more optimal coverage of targeted areas (Kammermeier et al., 2014). Indeed, coverage of targeted areas was similar or superior to that reported in other gene panels despite the large number of genes covered (Nemeth et al., 2013; Yohe et al., 2015). Moreover, the diagnosis rate in our study was comparable to, or higher than, that reported in similar approaches recently applied in other patient groups exhibiting phenotypic heterogeneity (Kammermeier et al., 2014; Sommen et al., 2016; Trump et al., 2016).

We investigated patients with a wide array of, and often non-specific, neurometabolic symptomatology and were able to identify disease-causing mutations in a large number of cases. We interrogated 30 cases with no definitive molecular diagnosis despite having had all the pathology laboratory (including metabolic biochemistry) tests and imaging modalities that a tertiary referral metabolic centre considered might lead to a diagnosis. Of the 21/30 patients lacking pointers towards an underlying molecular diagnosis, pathogenic variants that explained all the clinical and biochemical findings were identified in seven (33%) and some of the phenotypic features in one (5%); demonstrating the effectiveness of this approach in a clinically heterogeneous, diagnostically challenging cohort. In these patients, there was no clear phenotypic or biochemical feature associated with higher or lower diagnostic rates on our panel, although study numbers preclude further conclusions. Additionally, where suggestive biochemical abnormalities existed, our panel efficiently led to a definitive genetic diagnosis in 8/9 cases. However, it is important to note that our cohort was recruited through a single tertiary referral centre, which may lead to selection bias. Therefore, further studies using large cohorts of patients consecutively enrolled from multiple metabolic medicine centres are warranted to establish the exact sensitivity and specificity of our panel. Nevertheless, we demonstrate that our extended panel approach, with subsequent focus on candidate gene(s), can be an initial relatively cost-effective approach to investigate patients with suspected neurometabolic disorders. Moreover, although applied to a paediatric cohort, our approach would arguably be even more useful in adult populations, where neurometabolic phenotypes can be even more atypical, presentations more variable and biochemical phenotypes even more subtle. Indeed, many lysosomal storage, mitochondrial, peroxisomal and other metabolic disorders present atypically in adults. For example, adrenoleukodystrophy can present as early-onset dementia (Kumar et al., 1995). Patients with urea cycle disorders, organic acidemias and Niemann Pick type C can also exhibit psychiatric manifestations (Sedel et al., 2007). Thus, a comprehensive panel approach can have high utility in patients presenting with unexplained/atypical psychiatric or neurological manifestations.

Our study expands the genotypic and phenotypic spectrum of several disorders but also re-emphasizes the complexity of diagnosing patients with IEM. Patient U1 presented with a multi-system disorder and significant myopathy; however, due to unremarkable brain imaging and a non-diagnostic muscle biopsy (Supplementary Fig. 1), the diagnosis of POMGNT1-related dystroglycanopathy was delayed. Although uncommon, normal glycosylated α-dystroglycan immunofluorescence staining has been reported previously in POMGNT1 patients (Clement et al., 2008). Patient U7 had neurodevelopmental difficulties and hyperreflexia, hence representing a mild TPP1-related phenotype compared to those typically reported in the literature (Breedveld et al., 2004; Sun et al., 2013), whereas his abnormal transferrin isoelectric focusing was attributable to the ALDOB mutations. Indeed, following variant identification, tripeptidyl peptidase I activity in patient leucocytes was found to be at the upper boundary of the affected range. The above cases demonstrate the spectrum of severity associated with IEM and how common it is for clinicians investigating neurometabolic disorders to be misguided by investigation results, with resulting diagnostic delays. For example, an abnormal transferrin pattern combined with neurological dysfunction would prompt investigations for congenital disorders of glycosylation (Scott et al., 2014), which was the case in Patient U8 in whom variants in GALE were identified and UDP-galactose
### Table 3 Patients with no diagnosis identified though the genetic panel

| ID | Gender | Age (years) | Primary neurological phenotype | Other phenotypic features | Relevant specialist investigations | MRI head | Eventual diagnosis |
|----|--------|-------------|--------------------------------|---------------------------|----------------------------------|----------|------------------|
| B9 | F      | 6           | Developmental delay, absence seizures | Bilateral sensorineural deafness | Grossly elevated plasma proline. Elevated n-pyruvate-2-carboxyglycine confirming hyperprolinemia type II | Not performed | Hyperprolinemia type II (novel homozygous deletion in ALDH4A1) |
| U9 | F      | 7           | Learning difficulties. Delayed motor milestones, reduced exercise tolerance, responsive to intra-muscular vitamin B12 injections | Joint hypermobility | Intermittently elevated plasma lactate but normal CSF lactate, low plasma manganese | Normal | Not yet reached |
| U10 | M     | 8           | Global developmental delay, neurological regression, dysphagia, epilepsy | Alopecia, reflux, neutropenia, platelet dysfunction | Normal acylcarnitines, plasma amino acids. Slightly low fructose-1,6-bisphosphatase activity | Not performed | Not yet reached |
| U11 | F     | 5           | Episodes of severe ketotic hypoglycaemia with seizures | No | No other abnormalities | Delayed myelination | Multiple mutations, only one of which picked up by gene panel CUBN (p.Ala2194Val) |
| U12 | F     | 3           | Developmental delay and regression, dysphagia | Low vitamin B12 | Delayed myelination | Not yet reached |
| U13 | F     | 5           | One of similarly affected siblings. Parental consanguinity. Developmental delay, reduced exercise tolerance, joint hypermobility | Dysmorphic features. Pancreatic insufficiency and fat malabsorption | Several raised plasma amino acids. Muscle histology suggestive of mitochondrial disorder but RCE normal | Normal | Not yet reached |
| U14 | F     | 8           | One of similarly affected siblings. Parental consanguinity. Developmental delay, reduced exercise tolerance, joint hypermobility | Pancreatic insufficiency and fat malabsorption | Several raised plasma amino acids. | Not performed | Not yet reached |
| U15 | F     | 6           | Global delay, microcephaly. Movement disorder with chorea and non-epileptic myoclonic jerks | Previous faltering growth. Renal tubular acidosis on NaHCO₃ supplements | No other abnormalities | Delayed myelination | Not yet reached |
| U16 | M     | 12          | Global delay, seizures, dysphagia. Sibling with similar features | Dymorphism | Persistent low arginine but normal lactate, carnitine profile and urine organic acids | Leukodystrophy | Unconfirmed NDUFS1 deletions |
| U17 | M     | 5           | Global delay, retinal dystrophy, dystonic extensor spasms, epilepsy | Reflux, hip dislocation, scoliosis | EEG features consistent with Electrical Status Epilepticus in Sleep (ESES) | Progressive cerebral and cerebellar atrophy | Microarray: deletion at 7q36.2 - de novo change which includes DPP6 gene, known to be associated with neurological disorders. DDD ongoing KANSL1 c.1635-3T>C; Koolen-de Vries syndrome. Diagnosis made by geneticists |
| U18 | F     | 15          | Developmental delay, paroxysmal episodes of gasping, opisthotonus and discomfort related to food ingestion | Distinct facial features. Abnormal maculae on OCT and slightly swollen optic discs | Abnormal visual evoked potential (VEP/electroretinogram (ERG)) | Non-progressive ventricular dilatation | |
| ID | Gender | Age (years) | Primary neurological phenotype | Secondary neurological phenotype | MRI head | Other phenotypic features | Relevant specialist investigations | Eventual diagnosis | Eventual investigations |
|----|--------|-------------|-------------------------------|---------------------------------|----------|------------------------|---------------------------------|---------------------|----------------------|
| U19 | M      | 6           | Global delay, 4-limb motor disorder, slight dysarthria | Variable growth retardation | High CK. Very long chain fatty acids; moderately raised C26 | Loss of white matter, delayed myelination | Autosomal recessive, EARS2 mutation identified through the panel. | Not yet reached | | |
| U20 | M      | 1           | Global delay, 4-limb motor disorder, slight dysarthria | Variable growth retardation | Normal | Normal | Autosomal recessive, EARS2 mutation identified through the panel. | Not yet reached | | |
| U21 | M      | 7           | Global delay, acquired microcephaly | Variable growth retardation | Normal | Normal | Autosomal recessive, EARS2 mutation identified through the panel. | Not yet reached | | |

After gene panel analysis, some of the patients in our cohort had diagnoses established via comparative genomic hybridization array or targeted gene testing. Patient U29 had EARS2 mutations identified through the panel which would be consistent with the clinical phenotype. However, as the same variants were also encountered in unrelated non-affected individuals, their significance is still being investigated through functional studies (data not shown). Patient U25 had NDUFS1 mutations identified through the panel which would be consistent with the clinical phenotype. However, as the same variants were also encountered in unrelated non-affected individuals, their significance is still being investigated through functional studies (data not shown).

Apart from expanding the phenotypic spectrum of ‘well-described’ disorders, our results help expand the genotypic and phenotypic spectrum of recently described genetic conditions including PGAP2 (Hansen et al., 2013; Krawitz et al., 2013), ACSF3 (Sloan et al., 2011), DPYS (van Kuilenburg et al., 2010), AFG3L2 (Pierson et al., 2011) and SERAC1 (Wortmann et al., 2012). Hence, panel approaches enable clinicians to establish diagnoses in (and increase awareness of) ever broadening phenotypes and recently-described disorders, while at the same time circumventing problematic heterogeneity issues and potentially shortening the time to establish a definitive diagnosis for some patients.

Some patients with IEM have defects in more than one gene contributing to observed phenotypes. Patient U7 had mutations in ALDOB and TPP1. While mutations in ALDOB have been associated with abnormal transferrin patterns (Adamowicz et al., 2007), the majority of clinical features seen in this case are likely attributable to the TPP1 mutation (Breedveld et al., 2004; Sun et al., 2013). Similarly, Patients B6 and B7 had mutations in AASS, which would explain the hyperlysinaemia seen in both plasma and CSF but not the presence of developmental delay, microcephaly, hypotonia and epilepsy (Houten et al., 2013). Patient U2 had mutations in DPYS, which are associated with abnormal purine and pyrimidine metabolites but not with dysplastic kidneys, eczema, microcephaly and developmental delay (van Kuilenburg et al., 2010). The phenotypic features in these patients are most likely attributable to other, yet unidentified, genetic defects. The existence of pathogenic variants at two genetic loci in one patient is not surprising, as individuals have ~3.5 million variants in their genome (Gonzaga-Jauregui et al., 2012). A recent genetic study showed that 4.6% of participants had blended phenotypes resulting from two single gene defects (Yang et al., 2014). The above issues further complicate the diagnosis of IEM and highlight the utility of NGS, especially in highly heterogeneous disorders while emphasizing the need for diagnosticians to perform elaborate clinical phenotyping and not over-rely on sequencing results, especially when identified gene defects do not account fully for the observed clinical picture.
3 x), an intronic area or a promoter region. More research including WES or WGS in mutation-negative cases is warranted to reach further conclusions. Overall, our findings agree with previous studies indicating that, when analysed by NGS, targeted genetic regions can be inconsistently covered at read depths sufficient for comprehensive variant analysis (Dewey et al., 2014). Additionally, although able to identify deletions, we were unable to detect the homozygous 111 bp deletion in Patient D6 or insertion/deletion event in Patient B9, which highlights the challenges of using NGS to detect copy number variants (Mullaney et al., 2010). Indeed, some common pathogenic alleles can be missed by conventional sequencing approaches, including targeted NGS, unless methods are specifically adapted or additional assays are included to capture them. These can include deep intronic splice variants as in leukencephalopathy with brainstem and spinal cord involvement and lactate elevation (van Berge et al., 2014) or whole gene deletions and duplications as in Pelizaeus-Merzbacher disease (Lee et al., 2006).

Finally, detection of variants of uncertain significance could pose a diagnostic and ethical issue, especially in patients with specific phenotypes where more targeted genetic testing could be a reasonable alternative. We firstly addressed this by following a ‘panel within a panel’ approach, initially interrogating genes in which mutations were likely to result in the observed phenotypes (e.g. MUT, MCEE, ACSF3, ALDH6A1, MMAA, MMAB, SUCLA2, LMBRD1, ABCD4, MMADHC and MMACHC in patients with methylmalonic aciduria) and expanding our search when no likely pathogenic variants were identified. Moreover, during the consenting process, we specifically counselled all study participants that they would not be informed about variants that were not deemed relevant to the clinical presentation. Utilizing expert phenotyping, current guidance on variant interpretation (Richards et al., 2015) and close collaboration between clinicians and scientists interrogating the data is crucial for the above to be successfully implemented. Nevertheless, our study shows that such approaches are feasible, even in patients with more specific clinical and/or biochemical phenotypes. This approach is particularly applicable in various neurometabolic conditions (such as the cases of peroxisomal biogenesis disorders and congenital disorders of glycosylation in our cohort), where mutations in a large number of genes could lead to similar biochemical abnormalities.

We also encountered difficulties when utilizing in silico tools for novel missense variant interpretation. When using SIFT and PolyPhen-2 interpretation, discordance was occasionally evident, not only for novel variants but also for common variants of established pathogenicity in ASL (Linnebank et al., 2002) and GALT (Reichardt et al., 1992) (Tables 2 and Supplementary Table 1). However, despite this discordance, CADD scores for these variants rank them more deleterious than 99.5% of all possible human single nucleotide variants. Additionally, SIFT, PolyPhen-2 and CADD suggested that a known pathogenic IDUA variant (Bach et al., 1993) was not likely to be deleterious (Supplementary Table 1). Inability of online prediction tools, particularly those using sequence-based algorithms, to predict pathogenicity of all variants analysed correctly has been evaluated previously (Castellana and Mazza, 2013; Dong et al., 2015; Walters-Sen et al., 2015). In silico tools remain invaluable in filtering large numbers of variants identified using NGS platforms; however, further evidence to support or refute pathogenicity should be sought (Richards et al., 2015), for example segregation analysis and enzymatic assays in appropriate patient tissues. In our study, we further characterized identified missense variants by mapping them to 3D protein structures where possible. All variants were predicted to be deleterious and probably/possibly damaging by SIFT and PolyPhen-2 and structural analysis supported these predictions in all cases, providing further evidence of pathogenicity. Should 3D structural information become available for larger parts of the human exome, this approach could become a valuable aid towards novel variant analysis (Yue et al., 2014).

Extended panel approaches have gained popularity and are used by many clinical laboratories in the investigation of a wide range of genetically heterogeneous conditions (http://www.labs.gosh.nhs.uk/media/759058/goshome_v7.pdf) including neurometabolic disease. With decreasing NGS costs and the advent of the Genomics England 100000 Genomes Project, WES and WGS will likely supersede the use of gene panels in the clinical diagnostic setting in the future. However, many challenges remain prior to this implementation, including difficulties in interpreting overwhelming amounts of data generated and uncertainties about clinically reportable findings (Dewey et al., 2014). Moreover, WES and WGS have proven invaluable in the identification of novel genes (Saito et al., 2013; Howard et al., 2014) but such findings are not currently actionable within the diagnostic setting. Elucidating the significance of these variants is not possible without functional characterization in appropriate settings and models, which is often expensive and beyond the capacity of most clinical diagnostic laboratories. Until such challenges are surpassed, gene panel approaches provide a rapid and cost-effective method of testing patients with neurometabolic disorders and enable more timely diagnosis and prompt treatment initiation in these conditions.

Funding

P.B.M. and P.T.C. received Great Ormond Street Hospital Children’s Charity (GOSHCC) Leadership awards (V2516 and V1254). P.G. is a Wellcome Trust Senior Research fellow. S.R. is supported by Great Ormond Street Hospital Children’s Charity (GOSHCC) Research Leadership Grant V1260. A.P. holds a joint Action Medical Research (AMR) and British Paediatric Neurology Association (BPNA) fellowship. This project
was funded by grants from the University College London Impact Award and GOSHCC Metabolic Fund and supported by the National Institute for Health Research Biomedical Research Centre at Great Ormond Street Hospital for Children NHS Foundation Trust and University College London.

Supplementary material
Supplementary material is available at Brain online.

References
Adamowicz M, Ploksi R, Rokicki D, Morava E, Gizewska M, Mierzewska H, et al. Transferrin hypoglycosylation in hereditary fructose intolerance: using the clues and avoiding the pitfalls. J Inherit Metab Dis 2007; 30: 407.

Aoshima T, Kajita M, Sekido Y, Kikuchi S, Yasuda I, Saheki T, et al. Novel mutations (H337R and 238-362del) in the CPS1 gene cause carbamoyl phosphate synthetase I deficiency. Hum Hered 2001; 52: 99–101.

Bach G, Moskwitz SM, Tieu PT, Matynia A, Neufeld EF. Molecular analysis of Hurler syndrome in Druze and Muslim Arab patients in Israel: multiple allelic mutations of the IDUA gene in a small geographic area. Am J Hum Genet 1993; 53: 330–8.

Breedveld GJ, van Wettum B, te Raa GD, Brusse E, van Swieten JC, Oostra BA, et al. A new locus for a childhood onset, slowly progressive autosomal recessive spinocerebellar ataxia maps to chromosome 11p15. J Med Genet 2004; 41: 858–66.

Castellana S, Mazza T. Congruency in the prediction of pathogenic missense mutations: state-of-the-art web-based tools. Brief Bioinform 2013; 14: 448–59.

Clement EM, Godfrey C, Tan J, Brockington M, Torelli S, Feng L, et al. Mild POMGnT1 mutations underlie a novel limb-girdle muscular dystrophy variant. Arch Neurol 2008; 65: 137–41.

Dewey FE, Grove ME, Pan C, Goldstein BA, Bernstein JA, Chab H, et al. Clinical interpretation and implications of whole-genome sequencing. JAMA 2014; 311: 1035–45.

Dong C, Wei P, Jain X, Gibbs R, Boerwinkle E, Wang K, et al. Comparison and integration of deleteriousness prediction methods for nonsynonymous SNVs in whole exome sequencing studies. Hum Mol Genet 2015; 24: 2125–37.

Gonzaga-Jauregui C, Lupski JR, Gibbs RA. Human genome sequencing in health and disease. Annu Rev Med 2012; 63: 35–61.

Hansen L, Tawamie H, Murakami Y, Mang Y, ur Rehman S, Bachert R, et al. Hypomorphic mutations in PGAP2, encoding a GPI-anchor-remodeling protein, cause autosomal-recessive intellectual disability. Am J Hum Genet 2013; 92: 575–83.

Hooten SM, Te Brinke H, Denis S, Ruiter JP, Knecht AC, de Klerk JB, et al. Genetic basis of hyperlysinemia. Orphanet J Rare Dis 2013; 8: 57.

Howard MF, Murakami Y, Pagnamenta AT, Daumer-Haas C, Fischer B, Hecht J, et al. Mutations in PGAP3 impair GPI-anchor maturation, causing a subtype of hyperphosphatasia with mental retardation. Am J Hum Genet 2014; 94: 278–87.

Kammermeier J, Drury S, James CT, Dzubak R, Oca I, Elawad M, et al. Targeted gene panel sequencing in children with very early onset inflammatory bowel disease—evaluation and prospective analysis. J Med Genet 2014; 51: 748–55.

Krawitz PM, Murakami Y, Riess A, Hietala M, Kruger U, Zhu N, et al. PGAP2 mutations, affecting the GPI-anchor-synthesis pathway, cause hyperphosphatasia with mental retardation syndrome. Am J Hum Genet 2013; 92: 584–9.
Sun Y, Almomani R, Breedveld GJ, Santen GW, Aten E, Lefeber DJ, et al. Autosomal recessive spinocerebellar ataxia 7 (SCAR7) is caused by variants in TPP1, the gene involved in classic late-infantile neuronal ceroid lipofuscinosis 2 disease (CLN2 disease). Hum Mutat 2013; 34: 706–13.

Trump N, McTague A, Brittain H, Papandreou A, Meyer E, Ngoh A, et al. Improving diagnosis and broadening the phenotypes in early-onset seizure and severe developmental delay disorders through gene panel analysis. J Med Genet 2016; 53: 310–7.

van Berge L, Hamilton EM, Linnankivi T, Uziel G, Steenweg ME, Isohanni P, et al. Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation: clinical and genetic characterization and target for therapy. Brain 2014; 137 (Pt 4): 1019–29.

van Kuilenburg AB, Dobritzsch D, Meier J, Meinsma R, Benoist JF, Assmann B, et al. Dihydropyrimidinase deficiency: phenotype, genotype and structural consequences in 17 patients. Biochim Biophys Acta 2010; 1802: 639–48.

Verity C, Winstone AM, Stellitano L, Will R, Nicoll A. The epidemiology of progressive intellectual and neurological deterioration in childhood. Arch Dis Child 2010; 95: 361–4.

Walters-Sen LC, Hashimoto S, Thrush DL, Reshmi S, Gastier-Foster JM, Astbury C, et al. Variability in pathogenicity prediction programs: impact on clinical diagnostics. Mol Genet Genomic Med 2015; 3: 99–110.

Wohlers TM, Christacos NC, Harreman MT, Fridovich-Keil JL. Identification and characterization of a mutation, in the human UDP-galactose-4-epimerase gene, associated with generalized epimerase-deficiency galactosemia. Am J Hum Genet 1999; 64: 462–70.

Wortmann SB, Vaz FM, Gardeitchik T, Vissers LE, Renkema GH, Schuurs-Hoeijmakers JH, et al. Mutations in the phospholipid remodeling gene SERAC1 impair mitochondrial function and intracellular cholesterol trafficking and cause dystonia and deafness. Nat Genet 2012; 44: 797–802.

Yang Y, Muzny DM, Xia F, Niu Z, Person R, Ding Y, et al. Molecular findings among patients referred for clinical whole-exome sequencing. JAMA 2014; 312: 1870–9.

Yohe S, Hauge A, Bunjer K, Kemmer T, Bower M, Schomaker M, et al. Clinical validation of targeted next-generation sequencing for inherited disorders. Arch Pathol Lab Med 2015; 139: 204–10.

Yoshida A, Kobayashi K, Manya H, Taniguchi K, Kano H, Mizuno M, et al. Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. Dev Cell 2001; 1: 717–24.

Yue WW, Froese DS, Brennan PE. The role of protein structural analysis in the next generation sequencing era. Top Curr Chem 2014; 336: 67–98.