Workshop report

189th ENMC International workshop
Complex I deficiency: Diagnosis and treatment
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1. Introduction

An ENMC meeting was held in Naarden, the Netherlands (20–22 April 2012) of 17 clinical and basic scientists and a patient representative to discuss the diagnosis and treatment of mitochondrial respiratory chain complex I deficiency. Participants work in six member countries (Finland, France, Germany, Italy, the Netherlands and the UK) and three non-member countries (Australia, Canada and Israel). Mitochondrial complex I (NADH:ubiquinone oxidoreductase) is the largest enzyme in the inner mitochondrial membrane and provides the main entry point into the respiratory chain for electrons derived from fuel oxidation. Complex I deficiency is the most commonly observed biochemical defect in childhood-onset mitochondrial disease, accounting for \( \sim 25\% \) of cases \[1\]. The enzyme has essential functions in electron transfer and proton pumping, generating \( \sim 40\% \) of the proton motive force that is eventually harnessed by complex V to synthesise ATP. However, despite its fundamental role in mitochondrial energy generation, complex I remains the least well understood of the respiratory chain enzyme complexes. Little is known about the function of many of the \( 45 \)\textsuperscript{1} subunits, and the factors necessary for and mechanisms of assembly of this macromolecular complex remain largely unknown.

2. Meeting outcomes

2.1. Advances in knowledge of complex I structure and function

The last six years have witnessed remarkable breakthroughs in our knowledge of the structure of complex I, with the elucidation of key structural data including most recently the crystal structure of the entire complex I holoenzyme \[2\]. These X-ray crystallographic data have allowed a structural picture of complex I to be formed. The complex I holo-enzyme is an L-shaped macromolecule, with matrix and membrane arms comprised of 4 functional modules: an N module responsible for NADH oxidation, a Q module that reduces ubiquinone, and proximal PP and distal PD modules for proton translocation. The matrix arm contains the N and Q modules, whilst the P modules are located in the membrane arm. X-ray structures of the oxidized and reduced hydrophilic (matrix) domain of complex I from \textit{Thermus thermophilus} (at 3.1 Å resolution) revealed the mechanism of complex I interaction with NADH to involve hydrophobic interactions between the nicotinamide ring of NADH stacked above flavin rings of bound flavin mononucleotide (FMN) \[3\]. Electrons are transferred from the primary electron acceptor FMN through seven conserved iron-sulfur (FeS) clusters to the quinone-binding site near the interface with the

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\textsuperscript{1} The exact number of complex I subunits has recently been challenged \[45\]. The precise subunit composition will become clear once the final crystal structure of the enzyme has been characterised.

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membrane domain. The head group of the quinone moiety was higher than anticipated, and the quinone tail has not yet been precisely localized. Structural data from intact complex I subsequently demonstrated three antiporter-like subunits linked by a lateral helix. It is thought that each of the antiporter subunits (ND2, ND4 and ND5) transports one proton, but this has not been definitively established. Because of the 4 proton-2 electron stoichiometry of complex I, a putative fourth proton channel has been postulated.

Systems for investigating the function of complex I include studies on isolated enzyme (this system is not useful for studying proton translocation); isolated complex I incorporated into proteoliposomes; submitochondrial particles (SMPs); and intact mitochondria (the last is a highly complicated system that is difficult to control). Biophysical studies of the flavin site reaction in purified complex I from bovine heart, using artificial electron acceptors such as FeCN, have shown that energy transfer from NADH to complex I is very fast compared to most dehydrogenases and that NADH oxidation is reversible and thermodynamically efficient [4]. The FeS clusters are difficult to study but experiments in a liquid helium spectrophotometer have suggested that only 5 FeS clusters are reduced from NADH, i.e. 3 clusters do not appear to be reduced. Transfer of electrons through the FeS clusters down to NADH, i.e. 3 clusters do not appear to be reduced. 

2.2. Advances in understanding complex I assembly

Human complex I consists of 38 different nuclear-encoded and 7 mitochondrial DNA (mtDNA)-encoded structural proteins, one FMN and 8 FeS clusters, all of which are assembled together in an intricate process. Comparatively little is understood about the assembly process of complex I. Various models have been suggested, but the current consensus is that the ND1 core subunit anchors an early Q subassembly to the inner membrane, then further subunits and/or subassemblies are added to both the Q and P modules, and finally the N module is added to form the complex I holo-enzyme [5]. The precise order of subunit incorporation into the nascent enzyme, and the nature and number of additional factors required for integrity of the assembly process, remain unknown. It has recently been proposed that complex I assembly is a dynamic multidirectional process that may include direct subunit exchange into pre-existing mature enzyme. This model was not challenged during the workshop, suggesting that a general consensus has been reached. Putative roles for some assembly factors have been proposed in the early, middle or late stages of complex I assembly [6], but their precise functions remain obscure.

2.3. Clinical recognition of complex I deficiency

Patients with complex I deficiency have heterogeneous clinical phenotypes, including Leigh syndrome (subacute necrotising encephalomyelopathy), leukoencephalopathy, myopathy, fatal infantile lactic acidosis, hypertrophic cardiomyopathy and multisystem diseases [7]. Two systematic reviews of the clinical phenotypes associated with nuclear-encoded complex I defects were presented at the workshop: one including 130 patients and the other 172 patients [1,8]. These systematic reviews aimed to establish, by collating published cases from across the world, whether there are any clinical, biochemical or radiological clues that may expedite genetic diagnoses in individual cases. About two-thirds of the patients studied had structural nuclear subunit mutations, whilst the remaining third had assembly defects. There was a male preponderance of 1.4:1 for the whole group, but this increased to 1.7:1 if only nuclear structural subunit mutations were considered (the M:F ratio was 1:1 for assembly factor defects) [1]. The median ages of onset and death were 4 months and 10 months, respectively [8]. Twenty-six percent of cases presented in the neonatal period, 59% in infancy, 11% in early childhood, and 3% in late childhood. Twenty-five percent of cases died before 6 months of age, 47% below 12 months, 74% below 24 months, and 85% had died by 10 years. Hubert Smeets reported that the oldest surviving patient, who has ACAD9 mutations, is 56 years old and still alive. Survival appeared to be similar for structural vs assembly defects, but was shorter in patients with mutations in core subunits compared to those with non-core subunit mutations [8].

MRI brain scans are probably not very discriminatory for complex I deficiency except in the case of NDUFAF2 mutations, which are associated with a predilection for the mamillothalamic tracts, substantia nigra/medial lemniscus, medial longitudinal fasciculus and spinothalamic tracts and relative sparing of the basal ganglia [9]. Twenty-four percent of cases had leukoencephalopathy, associated with mutations in the NDUSF1, NDUFV1 and NDUSF8 subunits, and the NDUFAF3 and NUBPL assembly factors [1]. ACAD9 and NDUFAF2 defects appear to have the strongest genotype/phenotype correlations but the overall impression was that it is difficult to stratify patients based on their genetic defect.
2.4. Laboratory investigation of complex I deficiency: biochemical testing

2.4.1. Spectrophotometric assays

A general discussion about the diagnosis of complex I deficiency using enzyme assays debated the use of fresh vs frozen tissue; tissue vs cells as a first line investigation; and the sole use of an enzyme assay vs an additional global measurement of respiration. Practice varies considerably between centres around the world, but there was an agreement that the combined complex I + III assay is unreliable, at least in frozen tissues, leading to large numbers of apparently false positives. Most experienced centres assay complex I using a short-chain ubiquinone analog such as CoQ1 or decylbenzylquinone as electron acceptor. Anne Lombes reported the results of consensus spectrophotometric assays for the diagnosis of mitochondrial disease, which were agreed by a network of 8 French centres. Two thousand samples have been assayed in a 15 year period. Of these, 178 had complex I activity below the 5th percentile, with citrate synthase and complex II above the 5th percentile. Two adult patients with Leigh syndrome, one with an ND3 mutation and the other with an ND5 mutation, had complex I activity >5th centile, whilst a patient with an ND6 mutation also had reduced complex III activity. It was noted that isolated complex I deficiency, particularly in liver, may be seen with antiretroviral therapy. The importance of monitoring for "batch" effects (such as a cluster of complex I deficient cases in a short time interval) was also emphasized. In such events, the possibility of artefactual lowering of complex I by a contaminant should be considered, as was found with some cheap blue plastic pipette tips and some batches of 96 well plates containing excess NP-10 (Tergitol) [10].

2.4.2. Oxygen consumption in small biopsies or cell lines

Measurement of oxygen consumption has been used for decades to assess the functional capacity of mitochondria. The traditional approach using a Clark electrode typically required 0.5 g of muscle and has largely been supplanted by methods that either use more sensitive electrodes [11] or microtitre plate systems with fluorescent probes. The latter approach, often referred to as microscale oxygraphy, is emerging as a new method to assess cells with defects in complex I or other complexes. Such techniques offer the advantage over spectrophotometric enzyme assays of interrogating global respiratory capacity in intact living cells in a physiological environment, and can be performed on small numbers of cells in a relatively high throughput context. Microscale oxygraphy typically requires only 20,000 cells per well, compared to ~1 million for traditional enzyme assays. Fibre optic probes are used to measure the rates of oxygen consumption (OCR) and extracellular acidification (ECAR), which can be altered by the addition of different substrates or pharmacological blockers of components of the OXPHOS system.

Valeria Tiranti reported the use of microscale oxygraphy to interrogate respiratory function of 19 genetically characterized patient fibroblast samples, including 6 with complex I deficiency (3 with mtDNA mutations and 3 with nuclear mutations) [12]. Statistically significant differences in basal respiration and respiration after the addition of oligomycin and FCCP were observed in all 6 samples from patients with complex I deficiency, including 3 fibroblast samples with normal spectrophotometric complex I activity. However there are some limitations of this technique: interpretation of results is difficult, and many reagents are cell impermeable, although new permeabilising reagents have shown promising results. It is important to use similar passage (P) numbers of fibroblasts, preferably below P5, since OCR and ECAR decrease substantially after P15. Finally, although this method can detect impaired respiration in patient samples it cannot identify which OXPHOS complex is defective. The general discussion emphasized that this currently remains a research tool. It is particularly useful in the evaluation of novel genetic defects, especially in assessing the results of rescue experiments. Demonstrating a defect in fibroblasts using microscale oxygraphy may also support the need for a muscle biopsy in a patient with suspected mitochondrial disease. However it is important to note that a role for this technique in clinical diagnostics has not yet been validated.

2.4.3. Blue native gel electrophoresis (BNGE)

BNGE has become established as a technique for assessment of native membrane complexes such as the OXPHOS complexes. Complex I in-gel activity (after incubation with NADH), steady-state levels and assembly can be assessed in one-dimensional Blue native gels. Complex I assembly involves the formation of multiple assembly intermediates and an important question is whether subgroups of patients with complex I deficiency have a characteristic assembly pattern or 'fingerprint' that suggests specific candidate genes, as has been observed for some subgroups of complex IV deficiency. So far, it appears that patients with complex I deficiency caused by mutations in the NDUF54, NDUF56, NDUF52 and NDUF10 subunits [5] or NDUF11 assembly factor [13] do accumulate specific subassemblies, and this technique could therefore be useful to screen for defects in these genes. However with other genetic defects there may be very low or even normal steady state levels of the complex I holo-enzyme, with no evidence of subassemblies; in these cases BNGE does not help to prioritise candidate disease genes. The development of new antibodies may help to improve the diagnostic utility of BNGE.

One-dimensional gels can subsequently be cut out and run in a second dimension, for improved detection of subassemblies via Western blotting. Second dimension metabolic labelling can be achieved by 35S labelling of
2.5. Genetics of complex I deficiency

Complex I deficiency is genetically heterogeneous and may arise from mutations in the 45\textsuperscript{th} structural subunits of the enzyme or in any of an unknown number of assembly factors.

2.5.1. Mitochondrial DNA mutations

Ten years ago it was thought that mtDNA mutations cause only 5–10\% of complex I deficiency. However it is now clear that mutations in the 7 mtDNA-encoded (MT-ND) subunits account for ~25\% of complex I deficiency [14]. More than 130 MT-ND variants have been listed as ‘disease-causing’ in the MitoMap database (www.mitomap.org), but robust evidence for pathogenicity has not been established for many of these. Some may be secondary mutations or rare single nucleotide polymorphisms (SNPs). Systematic screening of MT-ND genes in several cohorts of patients with isolated complex I deficiency have suggested a consistent relative frequency: 20\% in Paris [15], 26\% in Milan [16], 29\% in Melbourne [14] and 15\% in Nijmegen [17]. Although patients with related parents are more likely to have autosomal recessive inheritance, pathogenic MT-ND5 and MT-ND6 mutations have been reported in consanguineous pedigrees [18], and so comprehensive mtDNA sequence analysis should be performed in all patients with isolated complex I deficiency, regardless of family structure. Some recurrent mtDNA mutations have been reported in affected children, particularly associated with Leigh syndrome.

2.5.2. Nuclear subunit mutations

Mutations in 17 of the 38\' nuclear-encoded subunits have been implicated in a further 20\% of cases [1], and these were discussed during the workshop. It was agreed that differences in nomenclature between species is problematic, with different names for the bovine, human, Escherichia coli and Yarrowia lipolytica complex I subunits. A uniform system of nomenclature would be beneficial, but in practice this might be very difficult to achieve. Mutations affecting the NDUFS1, NDUFS2, NDUFS5 and NDUFS4 subunits appear to be relatively prevalent [1]. Patients with NDUFS4 mutations frequently have mild reduction of complex III in both skeletal muscle and fibroblasts, possibly due to a supercomplex effect.

2.5.3. Assembly factor mutations

Fewer than half of patients with isolated complex I deficiency have mutations in structural subunits; mutations in assembly factors are thought to account for the majority of cases. Mutations in 9 different assembly factors (NDUFAF1, NDUFAF2, NDUFAF3, NDUFAF4, NDUFAF5 (C20ORF7), NDUFAF6 (C8orf38), FOXRED1, NUBPL and ACAD9) have been associated with human complex I deficiency to date [1,19] but, given the complexity of the enzyme, many more factors are likely to be needed for its assembly. Diseases related to the 9 known assembly factors were discussed. NDUFAF1 mutations have been described in two patients. Both presented with cardiomyopathy in the first two years of life, with one dying soon after and the other still alive in his twenties, with mild to moderate intellectual disability [20,13]. Both cases had a characteristic complex I assembly defect on BNAGE profiling. Patients with NDUFAF2 mutations appear to present later [21], with a form of Leigh syndrome affecting highly specific nuclei within the brain stem, whilst those with NDUFAF3 and NDUFAF4 mutations presented in the neonatal period and had severe defects with very low residual complex I activity [22,23].

In some cases more than one diagnostic approach led to identification of the same gene. Phylogenetic profiling implicated both NDUFAF6 (C8orf38) and NDUFAF5 (C20ORF7) in complex I assembly, and both genes were subsequently linked to human disease, by candidate gene analysis and homozygosity mapping respectively [24,25]. Their protein products are thought to be involved at an early stage of complex I assembly, leading to a specific defect in ND1 synthesis or stability. Mutations in FOXRED1 were identified by homozygosity mapping [26] and targeted next generation sequencing (NGS) [27]. Three patients have been reported in total, with broadly similar clinical courses. The functional role of FOXRED1 remains obscure. Three centres had identified mutations in ACAD9 using different approaches: homozygosity mapping [28], functional studies [29] and whole exome sequencing [30]. More than 15 patients have now been identified, suggesting that this is a relatively frequent defect. Moreover these patients appear to respond to riboflavin (vitamin B2), emphasizing the clinical importance of this condition. Three patients with NUBPL mutations were reported [27].

Several of the complex I assembly defects appear to be associated with defects of other respiratory chain enzyme complexes, possibly because of effects on the supercomplex (respirasome). For example, Ann Saada reported that several patients with complex I deficiency also had reduction of complex V by ~40–50\%, which possibly represented a secondary phenomenon. Furthermore an Israeli patient with an NDUFAF5 mutation had combined deficiency of complexes I and IV, although most reported cases with NDUFAF5 mutations had isolated complex I deficiency.
2.6. Approach to genetic diagnosis of complex I deficiency

Given the paucity of strong genotype-phenotype correlations in complex I deficiency, establishing a genetic diagnosis for patients with complex I deficiency remains challenging. However, the different modes of inheritance, including autosomal recessive (the majority), X-linked (e.g. NDUFA1 mutations) and maternal (mtDNA point mutations) mean that a precise genetic diagnosis is an essential prerequisite for accurate prognostic and genetic counselling advice regarding recurrence risks, and to guide reproductive options. The first line genetic investigation is currently complete mitochondrial genome sequence analysis, but this only has a maximum diagnostic yield of ~25%, as discussed above (see Section 2.5). Sequential analysis of mtDNA and relevant nuclear genes by Sanger sequencing is inefficient, costly and fails to target unrecognised genes.

Sequencing of whole exomes or targeted sequencing of large numbers of candidate genes using NGS technologies offers the prospect of faster and more cost-effective genetic diagnosis of complex I deficiency [27,35]. NGS is a particularly attractive option for OXPHOS disorders, since there are large numbers of candidate genes and very few common mutations with private mutations occurring in most affected families.

Targeted versus whole exome NGS approaches were discussed during the workshop. The Mito 10k project analysed ~100 genes implicated in complex I structure/assembly in 103 patients [27], but the rate of change in this field is so rapid that this approach is now considered antiquated. A more recent targeted NGS approach examined a high quality defined list of candidate genes: the known mitochondrial proteome or ‘MitoExome’ [36]. A total of 1034 genes (the entire mtDNA and all MitoCarta genes) were sequenced in 42 infants with various mitochondrial respiratory chain defects (i.e. not just complex I deficiency). In this study a rare variant was defined as having a frequency of <0.005 in any of the SNP databases, and 371 whole exome sequences from the Broad Institute were used as a control dataset. Mutations in novel candidate disease genes were confirmed by lentiviral rescue in some cases. The diagnostic yield was 23% for known disease genes, 36% including validated candidate disease genes, and 57% if all likely pathogenic variants were included. Reasons for failure to find the causative gene defect in at least 43% of patients in this study were discussed. Possible explanations are that the pathogenic variants were missed in the MitoExome sequencing; that they were detected but not prioritized (e.g. de novo dominant or variants with frequency >0.005); that they are in non-targeted genes or gene regions (e.g. introns or regulatory sequences); or that complex modes of inheritance are involved.

Holger Prokisch reported the whole exome sequencing strategy employed in Munich. Approximately 1500 exomes (mainly from Caucasian samples) have been sequenced to date, including 90 patients with isolated complex I deficiency, or complex I deficiency combined with other respiratory chain defects. There is typically 8–12 Gb of sequence per exome, with at least 20-fold coverage for >93% of the exome. Approximately 10,000 synonymous and 10,000 nonsynonymous variants are identified per exome, with ~300 rare nonsynonymous variants in Caucasian samples, and many more in other ethnic groups. For example, 2500 rare nonsynonymous variants were identified in an African patient due to missing control samples. Many filters are used in the data analysis, including known disease alleles; rare variants; assumption of autosomal inheritance; and comparison with other family members [35]. Pathogenicity is confirmed by transducing cells with the candidate wild type gene, followed by functional studies such as OCR and ECAR measurements using microscale oxygraphy (Seahorse analyser) and BN-PAGE assembly studies. Larger exome studies allow a combined analysis. An example is that searching for genes with two rare variants predicted to affect the corresponding protein in >4 families led to the identification of ACAD9 as a cause of complex I deficiency associated with lactic acidosis and cardiomyopathy [30]. Problems encountered include difficulties with SNP calling next to insertions or deletions and that the first exon of many genes is often not well covered due to their higher G/C content and PCR based amplification steps in the capture protocol. All these approaches have revealed enormous degrees of complexity, with many patients having potentially pathogenic SNVs in many genes.

2.7. Animal models of complex I deficiency

Two mouse models of complex I deficiency were described during the workshop: the Ndufs4 knockout mouse and the Ndufs6 gene trap mouse. The availability of these animal models allows investigation of complex I deficiency in different tissues, and the exciting prospect of conducting preclinical trials. These genetically well-defined models have the advantage that they more closely resemble human complex I deficiencies compared to pharmacologically induced models (such as the rotenone-treated rat) and naturally occurring mutant animals (such as the Harlequin mouse, which has a hypomorphic mutation in the Aif gene).
As in human disease, the *Ndufs4* knockout mouse has residual complex I activity in all tissues tested, ranging from 10% to 44%, providing support for the notion that complete complex I deficiency would most likely be lethal in utero [37]. Heterozygous *Ndufs4* knockout mice had normal complex I activity. Human *NDUFS4* defects are characteristically associated with an ~830 kDa subassembly, and a similar assembly defect was observed in all tissues investigated in the Ndufs4 mouse. This 830 kDa subassembly was lacking the N-module of the complex. Interestingly, a 200 kDa subassembly representing this module was found in the knockout mice. Moreover, fully assembled complex I could also be found in the knockout mice, but only when associated with complex III in a complex III-I supercomplex. The finding of an additional 830 kDa-complex III supercomplex assembly indicates that the nascent complex I may be incorporated into supercomplexes before assembly of the complex I holoenzyme is complete or that the N-module was lost after supercomplex formation.

An *Ndufs6* gene trap mouse has been studied in detail by the Melbourne group [38]. *NDUFS6* mutations appear to be a rare cause of human complex I deficiency and are usually associated with fatal neonatal lactic acidosis. The *Ndufs6* mutant mice were initially normal but from 6 months of age developed sudden weight loss, lethargy and cardiomegaly. Cardiac function was impaired; echocardiogram revealed a fractional shortening of only 15% in the mutant mice, compared to 47% in the wild type animals. The reason for these mice having a primarily cardiac phenotype was that other tissues contained small amounts of wild type *Ndufs6* mRNA generated by splicing over the gene trap insertion, so that the complex I defect was more severe in heart than other tissues. Biochemical phenotyping of the mutant animals revealed abnormal acylcarnitine profiles, with consistent elevation of C6 and C8 hydroxyacylcarnitines.

The tissue specificity of human and mouse complex I defects were debated. Possible explanations include intrinsic differences in complex I expression levels between tissues, and differential secondary effects. In brain, these secondary effects may include increased ROS production leading to oxidative damage to proteins, caspase 8 activation, altered dopamine metabolism and increased vulnerability to neurotoxins. At present there are insufficient experimental data to reach a consensus regarding the mechanisms underlying tissue specificity.

### 2.8. Treatment of complex I deficiency

Currently, treatment strategies for isolated complex I deficiency are extremely limited because of incomplete understanding of the underlying pathophysiology. However the discovery that patients with *ACAD9* mutations have riboflavin-responsive complex I deficiency has opened up the exciting prospect of a new era of therapy development for these currently untreatable disorders [28–30]. The final aim of this workshop was to discuss current knowledge regarding treatment of complex I deficiency, based on studies in cell and animal models of the disease together with clinical observations, and to explore the possibility of international clinical trials.

#### 2.8.1. In vitro treatment of cell models of complex I deficiency

Fatima Dhouadi treated 5 patient-derived cell cultures with complex I subunit mutations with bezafibrate, in order to up-regulate residual metabolic capacities. Cells were treated with 400 μM bezafibrate for 72 h, after which increased complex I activity was observed in 3 of the 5 cell lines. Bezafibrate led to stimulation of the transcription of various genes, probably due to the stimulation of PGC1α expression [39]. The pivotal role of PGC1α as the master regulator of mitochondrial biogenesis makes it a particularly attractive therapeutic target to induce stimulation of residual metabolic capacities in patients with respiratory chain deficiencies. Another recent study has demonstrated an effect of bezafibrate on homoplasmic cybrids carrying the most common mtDNA mutations [40]. Bezafibrate strongly induces PGC1α and TFAM mRNAs (two-three fold change) and increases oxygen consumption (32%) of fibroblasts in the presence of complex I substrates. Prokisch reported that in his experience cells with complex I activity below 20–30% of controls show no response to bezafibrate. Similarily Saada commented that bezafibrate increased ROS production in some cells but not others. Saada reported different responses of cell lines with different defects to various compounds, but that AICAR (5-amino-4-imidazole carboxamide riboside, an activator of AMPK) seems to be a particularly promising agent [41].

#### 2.8.2. Treatment of complex I deficient mouse models

MEFs from the Ndufs4 mice were treated with riboflavin, which appeared to stabilize electron transport into the electron acceptor part of the enzyme located at the tip of the peripheral arm, by increasing levels of the FMN co-factor which is noncovalently associated with the NDUFV1 subunit. This introduces the intriguing possibility that high dose riboflavin therapy may have some efficacy in human NDUFS4 defects. Therapeutic trials are in progress for the Ndufs6 mutant mice.

#### 2.8.3. Clinical observations

Complex I contains one FMN, which is derived from dietary riboflavin and is noncovalently bound to the NDUFV1 subunit. The potential role of riboflavin in the treatment of complex I deficiency was therefore discussed. The recommended daily intake of riboflavin is 1.1–1.8 mg, and it is required for 374 flavin-dependent proteins in the body, including 276 enzymes, 91% of which are oxidoreductases. Patients with NDUFV1 mutations were said not to respond to 30 mg/day of riboflavin supplementation. However, ACAD9 deficient
patients treated with 300 mg/day of riboflavin (i.e. approaching 300× the daily requirement) showed a good clinical response [28]. The rationale for using such high doses is not clear, but it appears that FAD derived from riboflavin stabilizes ACAD9. More information is needed; this may be provided by a randomized double-blind placebo-controlled clinical trial of riboflavin therapy in genetically confirmed patients. Eventually it may be possible to take a ‘personalized medicine’ approach in which an initial trial of riboflavin in patient-derived cells guides clinical management.

Approaches for stimulating mitochondrial biogenesis in patients with mitochondrial disease were discussed. Based on an observed response to the ketogenic diet in mice with late-onset myopathy caused by deleter mutations in the Peol1 gene [42], Anu Suomalainen is undertaking a pilot study of the modified Atkins diet (70% fat, 26% protein, 4% carbohydrate) in 6 adult patients with PEO (4 with autosomal dominant PEO and 2 with sporadic PEO and single mtDNA deletions) together with 14 aged-matched controls. The preliminary results indicate that diet has a major impact in mitochondrial disease patients, but differences between mice and humans are under examination.

3. Future prospects

3.1. Diagnostic criteria and biochemical diagnosis

Participants noted an expectation in some centres that diagnoses should be classified according to one of the known diagnostic criteria (e.g. Bernier or Nijmegen [43,44]), and that we should work toward a unified scheme. However, that was beyond the scope of this workshop and should encompass all respiratory chain disorders. Robust biochemical diagnoses have been the foundation of diagnoses in most experienced centres, but concerns remain about the veracity of enzyme diagnoses in some instances. The participants agreed on the following 10 aspects of diagnosing complex I deficiency:

(1) Diagnostic centres require strong links to a centre with clinical mitochondrial expertise.

(2) Assays of complex I using decylbenzylquinone or CoQ1 can provide a robust approach to identifying complex I enzyme defects in experienced hands. However, we regard it as a mandatory level of quality assurance that these assays require regular validation. In order to be confident in such results, the most effective way to do this is to audit biochemical diagnoses by comparison with molecular diagnoses in the patients diagnosed with complex I defects, and by comparison of enzyme activities identified in patients with specific molecular diagnoses. If this is impractical, then centres should be expected to demonstrate reliability of diagnoses in other ways. At a minimum, this could be by participating in regular sample exchanges with centres that do follow the biochemical/molecular comparative approach, or by participation in an external quality assurance programme measuring enzyme activity in tissues or cell lines from patients or from rodent models or other models with known genetic disorders.

(3) The NADH dehydrogenase assay is not a suitable method for diagnosis of complex I deficiency, but could be useful in subclassification of enzyme defects.

(4) The linked complex I + III assay is not suitable on its own for diagnosis in frozen tissues, but can be a useful assay in conjunction with decylbenzylquinone or CoQ1 assays.

(5) The histochemical NADH tetrazolium reductase stain does not have any relationship to complex I activity.

(6) Muscle histology and histochemistry play only a limited role in diagnosis of complex I defects in children, except in those rare cases with specific changes such as ragged-red fibres.

(7) Blue Native-PAGE western blotting and in-gel activity staining can be valuable as a front line diagnostic test or for subclassification, but are not in routine use in most centres at present.

(8) Functional assays such as microscale oxygraphy are not currently suitable as a frontline diagnostic assay but can be useful for confirmation and evaluation of the effect of novel mutations and to study animal models.

(9) Normal complex I activity does not completely exclude the possibility of a complex I genetic mutation, especially in the case of some mtDNA mutations.

(10) Muscle or another affected tissue should ideally be investigated in the first instance but if these are not available then fibroblasts may be used in some cases. However normal complex I activity in muscle or fibroblasts does not exclude the possibility of a complex I defect in another tissue.

3.2. Improving genetic diagnosis and treatment

Targeted sequencing of gene panels or whole exome sequencing by NGS offers the prospect of transforming molecular diagnosis of complex I deficiency and other mitochondrial disorders. These approaches are starting to become available in a clinical setting, prompting discussion of two potential pathways for molecular diagnosis. The first is to follow the traditional approach of clinical examination, noninvasive investigations (such as imaging and metabolic tests), followed by muscle biopsy to establish an enzyme diagnosis prior to NGS. An alternative approach would be to perform NGS prior
to, or in parallel with, any invasive biopsies. The more traditional approach has the advantage that an enzyme diagnosis is a great aid to prioritising genes with potential pathogenic variants. This facilitates rapid and correct molecular diagnosis using NGS. However, the latter approach could avoid muscle biopsy in patients found to have known pathogenic variants, whilst in other cases genetic findings may indicate muscle biopsy is needed to aid interpretation of results. At present, it is likely that most patients will need muscle biopsy in conjunction with NGS in order to interpret and validate diagnoses. This is likely to be a transitional phase and the “NGS first” approach will become increasingly useful as databases of validated pathogenic variants become more robust and comprehensive. In the interim, additional factors to consider regarding NGS prior to muscle biopsy are: (1) for patients with severe disease, the risk of death while waiting for NGS results could potentially mean missing the opportunity to obtain enzyme results needed to interpret NGS results; (2) whether additional molecular tests or tissue samples are needed to ensure reliable detection of mtDNA mutations and nuclear copy number variants. For NGS-based diagnosis to become efficient and robust, it will be important to develop better curated databases of pathogenic variants, linked to detailed clinical information, and to develop a collaborative scheme to share and access sequence information between international centres.

Additional mouse and other animal models of complex I deficiency are needed to understand the detailed pathogenic mechanisms linking genotype to phenotype. Careful validation of phenotypes is needed to determine the limitations of non-human models, for example in determining whether therapeutic trials in such models are directly relevant to human therapies. A major challenge to improving treatment of complex I deficiency is the genetic heterogeneity and difficulty of performing adequately powered clinical trials for rare diseases. More effort is needed to understand the natural history of these disorders using clinical databases or patient registries, so that an evidence-based approach can be taken towards developing new treatments. Validated clinical scales for documenting phenotypes and disease progression could enable “n of 1” trials where each patient can be studied in a way that allows subsequent aggregation of small treatment trials into larger series. Recent developments in this area suggest that the time is ripe for a workshop on discriminatory outcome measures and clinical trials for mitochondrial disease.

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References


