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232nd ENMC international workshop: Recommendations for treatment of mitochondrial DNA maintenance disorders. 16 – 18 June 2017, Heemskerk, The Netherlands[1]

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1. Introduction

Mitochondrial DNA (mtDNA) maintenance disorders, also known as mtDNA depletion and deletions syndrome (MDDS), encompass diseases characterized by the presence of mtDNA alterations (depletion, multiple deletions, and somatic point mutations) in affected tissues. Over the last 20 years, important advances in preclinical research have provided pathophysiological insights leading to novel therapy approaches for specific subtypes. **Table 1** displays the genes with pathogenic identified variants.

The 232nd ENMC International Workshop took place in Heemskerk, The Netherlands in June 2017, with the aim to identify actions needed to advance the clinical recognition, diagnosis, and treatment of patients suffering from disorders of mtDNA maintenance, with focused discussion on two of these diseases: thymidine kinase 2 (TK2) deficiency (TK2d) and mitochondrial neurogastrointestinal encephalomyopathy (MNGIE).

Twenty-four participants, including two relatives of patients and two members of biopharmaceutical companies, came from nine countries: Finland, France, Israel, Italy, Netherlands, Spain, Sweden, United Kingdom and United States.

Although ENMC workshop reports are typically published about one year after the meeting, this article is now published after a considerable delay for reasons beyond of the control of the participants of this workshop. Despite this late publication, the topic of the meeting, as well as the discussions and conclusions reached at that time are timely and, in fact, the importance of this subject has been reinforced by further translational advances over the past years. Some of the new concepts have been consolidated in a final short section summarising the new developments and recent publications that are directly related to the aims content of the workshop.

2. Background on mtDNA replication disorders

The first session was dedicated to background information on mtDNA replication disorders, including detailed introduction to these diseases as well as presentations focused on the molecular and biochemical mechanisms involved, such as the basic biology of mtDNA replication and deoxyribonucleoside triphosphate (dNTP) metabolism and mitochondria. Dr. Rahman presented a brief history of disorders in which mtDNA replication is primarily involved since the first report of multiple mtDNA deletions in muscle in a family with autosomal dominant progressive external ophthalmoplegia (adPEO) in 1989 and the initial description of profound depletion of mtDNA presenting as infantile myopathy, hepatopathy and nephropathy in 1991. Since then, more than 30 genes have been linked to these diseases (**Table 1**). Those genes encode proteins which can be classified into the following four functional categories: (1) mtDNA replication; (2) deoxyribonucleotide metabolism; (3) mitochondrial dynamics;

[1] A full list of the 232nd ENMC participants can be found at the end of the manuscript. All participants gave their approval to the final version of the manuscript.

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Table 1
Genes with pathogenic variants identified as causes of mtDNA maintenance diseases.

Category	Gene	Protein	Protein function / pathway	Clinical Features	Type of inheritance	Type of mtDNA aberration	OMIM # (gene)	Refs. *(year)
mtDNA replication machinery	*POLG*	DNA polymerase subunit gamma-1	Polymerase-catalytic subunit	Alpers-Huttenlocher syndrome / ataxia / PEO	AR / AD	D / MD / PM	174763	[59] (2001)
	TWNK	Twinkle	Helicase	Perrault syndrome / PEO / ataxia / encephalopathy / IOSCA	AD / AR	D / MD / PM	606075	[60] (2001)
	POLG2	DNA polymerase subunit gamma-2	Polymerase-ancillary subunit	PEO / hepatic failure	AD / AR	MD / D	604983	[61] (2006)
	MGME1	Mitochondrial genome maintenance exonuclease 1	Exonuclease	PEO / emaciation	AR	D / MD	615076	[62] (2013)
	DNA2	DNA replication ATP-dependent helicase/nuclease DNA2	Helicase / nuclease	PEO / myopathy / Seckel syndrome	AD	MD	601810	[63] (2013)
	RNASEH1	Ribonuclease H1	Ribonuclease	PEO / muscle weakness / dysphagia / spino-cerebellar signs	AR	D / MD	604123	[64] (2015)
	TFAM	Mitochondrial transcription factor A	Transcription factor	Neonatal liver failure	AR	D	600438	[65] (2016)
	TOP3A	DNA topoisomerase 3 alpha	Topoisomerase	PEO	AR	MD	601243	[66] (2018)
	SSBP1	Mitochondrial single strand binding protein	ssDNA stabilization	Optic atrophy / kidney failure / neurological syndrome / retinopathy	AD / AR	D	600439	[67] (2019)
	LIG3	Ligase III	Mitochondrial DNA ligase	MNGIE-like / MELAS-like encephalopathy	AR	D	600940	[68] (2021)
dNTP metabolism	*TYMP*	Thymidine phosphorylase	Nucleoside catabolism	MNGIE	AR	D / MD / PM	603041	[11] (1999)
	TK2	Thymidine kinase 2	dNTP anabolism	Myopathy / PEO	AR	D / MD	188250	[9] (2001)
	DGUOK	Deoxyguanosine kinase	dNTP anabolism	Neurohepatopathy / myopathy / PEO	AR	D / MD	601465	[10] (2001)
	RRM2B	p53-subunit of ribonucleotide reductase	dNTP anabolism	Encephalomyopathy / PEO / MNGIE / KSS / neuropathy / deafness / tubulopathy	AR / AD	D / MD	604712	[6] (2007)
Mitochondrial dynamics	*OPA1*	Dynamin-like 120 kDa protein, mitochondrial	GTPase / mitochondrial fusion	Optic atrophy / Behr syndrome	AD	MD	605290	[69] (2008)
	MFN2	Mitofusin-2	GTPase / mitochondrial fusion	Optic atrophy / myopathy / axonal neuropathy / Charcot-Marie-Tooth	AR / AD	D / MD	608507	[70] (2012)
	SPG7	Paraplegin	Subunit of m-AAA protease	PEO / spastic paraplegia / optic atrophy	AR	MD	602783	[71] (2014)
	AFG3L2	AFG3-like protein 2	Subunit of m-AAA protease	PEO / ataxia / optic atrophy	AD	MD	604581	[72] (2015)
	MSTO1	Protein misato homolog 1	Mitochondrial fusion	Muscular dystrophy with cerebellar involvement / myopathy / ataxia	AR	D	617619	[73] (2017)
	MICOS13	MICOS complex subunit MIC13	Maintenance of cristae structure	Hepato-encephalopathy	AR	D	616658	[74] (2019)

(continued on next page)

Table 1
(*Continued*).

Category		Gene	Protein	Protein function / pathway	Clinical Features	Type of inheritance	Type of mtDNA aberration	OMIM # (gene)	Refs. *(year)
Unknown pathomechanism	Membrane channels	*SLC25A4*	Adenine nucleotide translocator 1	ADP / ATP carrier	PEO / cardiomyopathy / myopathy	AD / AR	MD	103220	[15] (2000)
		MPV17	Mpv17 protein	Membrane channel / unknown	Neurohepatopathy / neuropathy / leukoencephalopathy / Charcot-Marie-Tooth	AR	D / MD	137960	[27] (2006)
		SLC25A21	Mitochondrial 2-oxodicarboxylate carrier	Transmembrane transporter	Spinal muscular atrophy-like	AR	D	607571	[75] (2018)
		SLC25A10	Mitochondrial dicarboxylate carrier	Transmembrane transporter	Epileptic encephalopathy	AR	D	606794	[76] (2018)
	Other function / unknown function	*SUCLA2*	β-subunit, Succinate-CoA ligase	Krebs cycle	Encephalomyopathy	AR	D	603921	[16] (2005)
		SUCLG1	α-subunit, Succinate-CoA ligase	Krebs cycle	Encephalomyopathy	AR	D	611224	[17] (2007)
		GFER	Growth factor, augmenter of liver regeneration	Growth factor	Progressive myopathy / congenital cataract / sensorineural hearing loss / developmental delay	AR	MD	600924	[77] (2009)
		AGK	Acylglycerol kinase	Lipid metabolism / signaling	Congenital cataract / hypertrophic cardiomyopathy / skeletal myopathy and lactic acidosis / Sengers syndrome	AD	D	610345	[14] (2012)
		FBXL4	F-box/LRR-repeat protein 4	Protein homeostasis	Encephalomyopathy	AR	D	605654	[78] (2013)
		ABAT	4-aminobutyrate aminotransferase	Aminotransferase	Encephalomyopathy	AR	D	137150	[41] (2015)
		MRM2	rRNA methyltransferase 2	Mito rRNA maturation	MELAS-like	AR	D	606906	[79] (2017)
		C1QBP	Complement component C1q binding protein	Inflammation / nuclear transcription / mitoribosome biogenesis / apoptosis	Cardiopathy-multisystemic / PEO-myopathy	AR	MD	601269	[80] (2017)
		GMPR	GMP reductase 1	Ribonucleotide metabolism (GMP deamination to IMP)	PEO	AD	MD	139695	[13] (2020)

AD: autosomal dominant; AR: autosomal recessive; D: depletion; MD: multiple deletions; PM: point mutations; PEO: progressive external ophthalmoplegia.

and (4) other functions. The prevalence of mutations in different genes is variable: *POLG* is the most frequently affected gene, followed by *TWNK, RRM2B* and *TK2*. Although initially, specific genes were preferentially associated with either mtDNA depletion or with multiple mtDNA deletions, as the number of reported cases has increased it has become clear that mutations in the same gene can cause mtDNA depletion, multiple deletions, or both in individual patients. In fact, the phenotypes of these disorders should be regarded as a continuous clinical spectrum with variable degrees of severity, from fatal multisystemic infantile presentation to mild adult forms of myopathy. To advance knowledge on these disorders, to optimize diagnosis, and to make progress towards evidence-based therapies, translational research should be aimed to: (i) define and apply clinical diagnostic algorithms based on available knowledge, in line with what has been proposed for Leigh syndrome [1], (ii) conduct prevalence and natural history studies, (iii) determine outcome measures and biomarkers, (iv) identify and assess candidate therapies *in vitro* and *in vivo*, (v) conduct randomized clinical trials to demonstrate efficacy, and (vi) explore potential application of precision medicine, in the context of the wide clinical variability observed in these diseases. All these concepts were documented by Dr. Rahman with several examples that illustrate the importance of preclinical research together with evidence-based medicine.

Next, Dr. Copeland provided a detailed description of the molecular elements involved in mtDNA replication, including POLG and POLG2 (polymerase gamma subunits), TWNK (mtDNA helicase), SSBP (stabilisation of single stranded mtDNA during replication) and MGME1 (exonuclease). It has been long known that antiviral nucleoside analogue drugs can cause side-effects due to inhibition of POLG. Mutations in *POLG* lead to a wide clinical spectrum. Progressive External Ophthalmoplegia, (PEO) and Alpers-Huttenlocher syndrome were the first two disorders associated with mutations in *POLG*, but myoclonic epilepsy myopathy sensory ataxia (MEMSA), sensory ataxic neuropathy dysarthia ophthalmoplegia (SANDO), and mitochondrial encephalomyopathy lactic acidosis stroke-like episodes (MELAS) syndromes have also been linked to POLG mutations. More than 300 mutations in *POLG* spanning the entire gene have been reported to lead to disease (https://tools.niehs.nih.gov/polg/index.cfm), both recessive and dominant, and their combined prevalence in the population is as high as 2%. In particular, p.Tyr955Cys is the most frequent POLG mutation leading to adPEO. This mutation dramatically increases the POLG K_M, therefore, an increased pool of dNTPs may have therapeutic benefits. Baruffini and colleagues partially rescued the p.Tyr955Cys defect in yeast by enhancing ribonucleotide reductase (RNR) activity, which increased the levels of mitochondrial dNTPs. [2] In addition, increased dNTP pool may also facilitate stability of Twinkle helicase conformation. After purification, only 9% of Twinkle molecules were in the active hexamer state; however, in the presence of higher dNTP levels, both total amount of Twinkle and number of molecules in hexamers were increased. Nevertheless, not all the mutations in POLG impair affinity of the polymerase to dNTPs; some pathogenic variants affect processivity or catalytic activity of the enzyme. For example, the p.Ala467Thr mutation affects k_{cat} [3]. For such mutations, increased levels of dNTPs may not be beneficial.

Dr. Rampazzo provided an overview of the complex subject of homeostasis of the dNTP building blocks of mtDNA by introducing the basics of dNTP synthesis through *de novo* and the salvage pathways, as well as the catabolic counterparts, highlighting the contributions of the different pathways throughout the cell cycle. In dividing cells, the *de novo* pathway accounts for the majority of dNTP synthesis. dNTPs are then transported to the mitochondria through the bidirectional transporter PNC1 while nucleosides are transported through the ENT transporters (ENT1, ENT2 and ENT3). In quiescent cells, mtDNA synthesis and maintenance are fed mostly by the salvage pathway, which is rate-limited by TK1 and dCK activities in the cytosol and TK2 and dGK in mitochondria. Hydrolysis of cytosolic dNTPs by SAMHD1 and further transport of deoxyribonucleosides through the ENT transporters also feed the mitochondrial dNTP pools, while the compartmentalized salvage pathway within mitochondria prevents dNTPs from being degraded by SAMHD1. In fact, *in vitro* cultured fibroblasts from patients with mutations in *DGUOK* encoding dGK showed mtDNA depletion, which was partially corrected when SAMHD1 was silenced [4]. This observation highlights: (1) the role of SAMHD1 in hydrolysing dNTPs in the nucleus especially outside the S-phase when dNTP demand is decreased, and (2) the role of dGK in recycling deoxyribonucleosides from catabolic pathways for incorporation into mtDNA in quiescence. RNR, a key enzyme in the *de novo* pathway, is another example of how nucleotide metabolism is differentially regulated during the cell cycle. CDP reduction activity of RNR is 8.6 pmol/min in cycling cells, but only 0.2 pmol/min in quiescent cells.[5]. RNR is composed of two different subunits: R1 is present during the whole cell cycle, while R2 is limited to the S-phase. Outside the S-phase, R2 is replaced by p53R2 and both R1 and p53R2 are imported into the nucleus for the synthesis of dNTPs that are also imported into mitochondria. Thus, not surprisingly, mutations in *RRM2B* encoding p53R2 lead to mtDNA maintenance disorders [6].

Dr. Wang provided an overview of the enzymatic properties of TK2, a key enzyme in the mitochondrial pyrimidine salvage pathway. TK2 substrates are both thymidine (dT) and deoxycytidine (dC). Regulation of TK2 is very complex, with negative cooperativity (binding of one molecule of substrate decreases affinity for the second molecule of the same substrate) for the phosphorylation of dT but not of dC, which follows classic pure Michaelis-Menten kinetics. As a consequence, TK2 has a higher catalytic activity for dT at low dN concentrations as compared with a non-cooperative TK2 with the same K_M. TK2 also shows substrate competition with dT being a competitive inhibitor of dC phosphorylation and dC being a non-competitive inhibitor of dT phosphorylation [7]. Both dTTP and dCTP inhibit TK2 (negative feedback). TK2 activity shows some cell-cycle dependency, with highest activity during stationary phase, in contrast to dGK (the rate limiting enzyme of the purine salvage pathway within mitochondria), which has constitutive activity irrespective of cell cycle phases. In tissues, TK2 is poorly expressed in skeletal muscle. A recent study by Dr. Wang shows the presence of low residual TK2 activity in cytosolic fractions of muscle [8]. Interestingly, the authors suggest that skeletal muscle relies on both the salvage pathway (TK2) and the *de novo* pathway (p53R2) for the synthesis of dTTP for mtDNA replication, which may also explain the predominant mitochondrial myopathy phenotype induced by mutations in both *TK2* and *RRM2B*.

The last talk of this session was devoted to collection of general and specific data on how dNTP availability plays an important role on some mtDNA replication diseases. Dr. Martí reviewed the classification of the genes involved in these disorders into 4 categories, one of them defined by those genes directly involved in dNTP metabolism: *TK2* [9], *DGUOK* [10], *RRM2B* [6] and *TYMP* [11]. The first 3 genes encode enzymes that catalyse anabolic reactions for dNTPs synthesis. Therefore mutations in these genes directly compromise the supply of mitochondrial dNTPs needed for mtDNA replication. In contrast, the last gene encodes thymidine phosphorylase, which, when mutated, causes MNGIE. This is a catabolic enzyme whose dysfunction leads to dTTP expansion. Although not obvious how an excess of a given dNTP leads to mtDNA depletion, experimental evidence indicates that the mitochondrial dTTP expansion caused by thymidine phosphorylase deficiency is associated with concomitant dCTP depletion [12] due

to the fact that mitochondrial phosphorylation of dC by TK2 is inhibited by the excess of dT and dTTP, as previously discussed by Dr. Wang [7]. dCTP depletion induced by excess of dT has been observed in multiple reports. Therefore, the common biochemical explanation for reduced mtDNA copy number in this subgroup of diseases (i.e. those caused by impaired dNTP metabolism) is a limited availability of one or more substrates for mtDNA replication.

In 2020, a mutation in *GMPR*, encoding the cytosolic enzyme guanosine monophosphate reductase 1, was reported as a cause of adPEO, but the authors did not find any dNTP imbalances or other disturbances in dNTP homeostasis [13]. Therefore, although *GMPR* participates in ribonucleotide metabolism, it is not considered part of this group.

3. Clinical and molecular diagnosis

This session started with Dr. Lombès providing a list of affected genes leading to mtDNA maintenance disorders, focusing her discussion on their clinical spectrum, describing the main clinical features and syndromes associated to the different MDDS forms, as summarized in Table 1. She also raised relevant points of discussion, such as the possibility of genetic diagnostic bias leading to an overrepresentation of frequent mutations in the Caucasian population, and the need for functional assessments to confirm pathogenicity of mutations. Among additional questions needing further investigation, some generally assumed pathomechanisms still lack proper demonstration. For example, are mutations in *AGK* [14] or *SLC25A4* [15] interfering with mtDNA replication primarily or secondarily, for example *via* ATP decrease?. Similarly, are mutations in *SUCLA2* and *SUCLG1* [16,17] encoding subunits of the Krebs cycle enzyme succinyl-CoA synthetase, causing a MDDS or a primary Krebs cycle dysfunction?.

Next, Dr. Garone briefly presented the phenotypic progression of these disorders as well as potential therapeutic approaches. She highlighted the importance of defining the natural history of these diseases to reliably assess whether the treatment is providing benefits without a placebo group, which is critical for rare disorders. Stages of a natural history study include any feature of the presymptomatic phase and the clinical, symptomatic phase without treatment intervention, as illustrated by Duchenne Muscular Dystrophy. She then presented her retrospective study of the natural history of TK2d [18]. The phenotypic spectrum of TK2d can be classified in three major subgroups: infantile-onset myopathy, childhood-onset myopathy and late-onset myopathy. They differ not only in disease onset, but also in severity. The infantile form is the most severe, with rapid progression leading to early mortality (median post-onset survival of 1 year), while the childhood-onset form has a moderate-to-severe progression and a median post-onset survival of 13 years. This study has helped to identify outcome measures to reliably evaluate the benefits of treatment with dT/dC in patients with TK2d (clinical trials NCT03639701, NCT03845712 and NCT03701568). Finally, Dr. Garone also provided information on potential molecular biomarkers such as creatine kinase (CK), GDF-15 and FGF-21. Recent works authored by some of the meeting participants have shown both GDF-15 and FGF-21 as excellent mitochondrial disease biomarkers and suggest that they need to be assessed together with muscle biopsy during diagnosis [19–21]. The study by Maresca and colleagues showed that both FGF-21 and GDF-15 are good biomarkers of mitochondrial tRNA mutations [21], while the study by Domínguez-González and colleagues focused in TK2d and found that GDF-15 is a better biomarker than FGF-21 for this particular disease [19]. These observations reveal the potential application of these biomarkers as surrogate outcome measures in clinical trials to test therapeutic benefits of new treatments.

Dr. Saada gave an overview of the diagnostic procedure for mtDNA maintenance disorders. Diagnosis is based on biochemical, histological and molecular findings. Biochemical findings include the presence of metabolites in human fluids such as alanine, lactate, pyruvate, ketones in blood, and ketones, tyrosine metabolites, tri-carboxylic acid cycle metabolites and 3-methylglutaconic acid in urine. The presence of decreased free carnitine with elevated acylcarnitines may also be indicative of a mitochondrial disorder. In particular, mutations in *SUCLA2* and *SUCLG1* lead to elevated C3- and C4DC-carnitines, but fatty acid oxidation defects must be ruled out. Biochemical and molecular investigation in muscle biopsy, although invasive, is often informative and frequently confirms the presence of a mitochondrial disorder. Enzymatic assays of electron transport chain complex activities showing decreased complexes I,III,IV and V with normal complex II could be indicative of a mtDNA maintenance defect [9,10], as well as molecular findings of mtDNA (depletion and/or deletions). Nevertheless, these assays share the quandary of identifying “normal” control samples to compare measurements from patient samples. The final confirmation of mtDNA maintenance disorder diagnosis is finding the causative mutation, for which there are currently panels for Next Generation Sequencing (NGS). The increased availability of clinical WES and WGS testing, frequently replaces the need for muscle biopsy for the identification of known mtDNA maintenance disorders.

4. Animal models

This session started with Dr. Hirano and Dr. Karlsson describing *Tk2* knock-in [22] and knock-out [23] mouse models, respectively. Both TK2d mouse models share many similarities: apparently normal development during the first week of life, followed by rapidly progressive weakness and mtDNA depletion in multiple organs, including brain, liver, heart and skeletal muscle. In both cases, homozygous mutant mice die by fourth week of life. Thus, these two mouse models recapitulate many features observed in TK2d patients and represent excellent tools to study pathomechanisms and potential therapies for TK2d.

Dr. Karlsson further showed that TK2 deficiency in the KO mouse model can be rescued by expression of a *Drosophila melanogaster* deoxyribonucleoside kinase (Dm-dNK). Dm-dNK expressing mice were able to live up to 20 months on average with no mtDNA depletion. Dr. Karlsson also showed preliminary data on two new mouse models. The first was a CKm-Cre KO *Tk2*, which would allow conditional KO of the *Tk2* gene in skeletal muscle and heart. These mice showed weight plateau at postnatal week 6, but they are still alive at postnatal week 8. The second new mouse model Dr. Karlsson presented was a *Dguok* knockout mouse [24], which shows loss of body weight after 6 weeks and severe mtDNA depletion in several organs after 8 weeks, including brain and skeletal muscle, although liver was the most affected tissue, recapitulating the phenotype of human dGK deficiency. In contrast to the *Tk2* KO mouse model, expression of the Dm-dNK only partially rescues the phenotype, prolonging the lifespan of *Dguok* KO mice.

Dr. Suomalainen continued this session presenting mouse models for several mtDNA maintenance disorders: PEO-mouse model (“deletor” mouse, *Twnk*), infantile onset spinocerebellar ataxia (IOSCA)-mouse model, adult-onset mitochondrial recessive ataxia syndrome (MIRAS)-mouse model (*Polg*) and Alpers-Huttenlocher syndrome mouse model (conditional *Twnk*-KO in postnatal forebrain neurons or astrocytes). Study of *Polg* in mice has led to the identification of a distant regulatory enhancer locus with non-coding RNAs co-regulated with *POLG* that explain the neurological manifestations of mutations in *POLG* as well as the recognition of important alterations in the folate cycle in

the pathomechanism of this disease [25]. Likewise, experiments with the deletor and IOSCA mouse models showed that different Twinkle defects can lead to either increased, unbalanced or decreased dNTP pools, and alterations of the folate driven one-carbon cycle [26].

The *Mpv17* KO mouse model was presented by Dr. Spinazzola. MPV17 is a protein from the mitochondrial inner membrane whose very function remains unknown. In humans, *MPV17* mutations [27] lead to either infantile hepato-cerebral syndrome with liver mtDNA depletion and dysfunction, adult-onset neuropathy or multisystemic disease. The *Mpv17* KO mouse model [28] recapitulates several aspects of the human disease, including the early onset tissue-specific mtDNA depletion, which affects the liver at 8-10 weeks, but not kidney and brain. Only in the liver, dTTP and dGTP are decreased, and consequently, mtDNA replication is slow, which explains the mtDNA depletion. However, multiple tissues of *Mpv17* KO mice show a higher rate of GTP (ribonucleotide) incorporation to mtDNA [29]. While the contribution of altered ribonucleotide incorporation to mtDNA stability and integrity is not yet fully understood, only the tissue with the highest level of GMP, the liver, displayed low mitochondrial dGTP and mtDNA depletion, suggesting a possible threshold effect. Important for the translational aspect, quiescent fibroblasts from *MPV17*-mutant patients also show decreased dNTP levels and mitochondrial DNA depletion, and *in vitro* deoxyribonucleoside supplementation prevents and rescues the mtDNA loss. Thus, the results from mouse and cell culture models of MPV17 deficiency indicate a role of this protein in the metabolism of mitochondrial purines, and suggest that ribonucleotide misincorporation in mtDNA is a new type of mtDNA abnormality that may have pathological consequences [30].

5. Treatment with nucleosides/nucleotides: preclinical evidence

As previously described, supplementation with either deoxyribonucleosides or deoxyribonucleotides can rescue certain mtDNA maintenance defects. Preclinical studies are necessary to determine whether a deoxyribonucleos(t)ide-based treatment have therapeutic potential in patients. Dr. Horvath gave examples of *in vitro* studies assessing the efficacy of deoxyribonucleoside monophosphates for different mtDNA maintenance disorders. Supplementation with dAMP/dGMP rescued mtDNA copy number in myotubes from *DGUOK* deficiency patients but the rescue was only partial in myotubes from *POLG* patients [31]. Selection of an appropriate cell model is critical, because diverse cell types behave in different ways *in vitro*. Skin biopsies, for instance, are convenient sources of cells from patients requiring only a minimally invasive method, but very frequently fibroblasts do not recapitulate molecular features of patients. Therefore, development of alternative cell models is often necessary. Myotubes derived from patients showed mtDNA depletion but not OXPHOS defects after serum deprivation [31]. Inhibition of mtDNA replication using ethidium bromide led to severe mtDNA depletion and OXPHOS defects, but dAMP/dGMP supplementation did not rescue mtDNA copy number, which highlights the necessity of appropriate cell models [31]. Induction of mtDNA depletion using nucleoside reverse transcriptase inhibitors in myotubes obtained from MyoD-transduced patient fibroblasts was a useful model to show rescue of mtDNA copy number from two *POLG* patients after supplementation with dAMP/dGMP [32]. Dr. Horvath also presented a zebrafish *DGUOK* KO model [33] showing mtDNA depletion that was rescued with dAMP/dGMP. Supplementation with only dGMP reduced mtDNA copy number in both mutant and wild type juvenile fish, in contrast to a study showing rescue of mtDNA copy number in fibroblasts from dGK deficient patients with only dGuo [34].

Deoxyribonucleoside monophosphate and deoxyribonucleoside treatment have also been tested in two mouse models of TK2d (KI and KO). Dr. López-Gómez presented results with the *Tk2* KI mouse. The first approach was a bypass therapy, with oral supplementation of the products from the reaction catalysed by TK2, dTMP and dCMP [35]. The treatment demonstrated therapeutic effect, prolonging the lifespan of mutant mice and delaying the onset of the disease and mtDNA depletion. Nevertheless, dTMP and dCMP were undetectable in mice after treatment. Instead, increased levels of their catabolic products, dT, dC and dU, were identified. These results led the team to the idea of enhancing residual TK2 activity by increasing substrate bioavailability (substrate enhancement therapy). In fact, oral treatment with dT and dC prolonged lifespan and delayed disease onset in the *Tk2* KI mice to a similar degree as dTMP and dCMP [36]. Nevertheless, brain remained severely affected probably accounting for the death of mice at ages 6-9 weeks. TP activity in intestine, which increases dramatically from postnatal day 13 to postnatal day 29, is thought to decrease dT/dC bioavailability, which would ultimately reduce therapeutic effect [37,38]. This finding raised the question whether intravenous injection of dT/dC might be more efficacious than oral treatment. In fact, parenteral (intraperitoneal) treatment in mice produced much higher levels of dT and dC in blood and slightly higher levels in brain and liver. However, parenteral treatment did not increase mtDNA copy number in brain and survival did not improve relative to oral treatment. The tissue-specific response to dT/dC may depend, not only on substrate bioavailability, but also on the presence of cytosolic enzymes, TK1 and dCK, that metabolize deoxyribonucleosides into deoxyribonucleoside monophosphates [38]. TK1 is expressed in small intestine, while it is dramatically down-regulated in brain, which may explain the different therapeutic response of those tissues.

Bypass therapy with monophosphates is limited by their charged nature which impedes passage of these molecules across biological membranes in the absence of devoted transporters. Furthermore, fast degradation by phosphatases and ectonucleotidases decreases their cellular availability. Dr. Cámara showed evidence that deoxyribonucleosides resulting from monophosphate degradation are the actual metabolites exerting the therapeutic effect in bypass therapy [34]. She reviewed all published pre-clinical evidence supporting deoxyribonucleoside supplementation for treating mtDNA maintenance disorders. Most results were obtained in cellular models of mtDNA maintenance disorders due to defective nucleotide metabolism: *DGUOK* [34], *RRM2B* [39], and MNGIE [12]. A common feature in these disorders is insufficiency of at least one dNTP, which limits replication of mtDNA. Supplementation with deoxyribonucleosides of the deficient dNTPs would then restore mtDNA synthesis. In addition, data in yeast [2] and patient-derived cells [31,32] has indicated that increasing dNTP pools could also be therapeutic for defects affecting POLG affinity for dNTPs. Dr. Cámara showed how deoxyribonucleoside supplementation allowed fibroblasts from different POLG-deficient patients to recover after ethidium bromide-induced mtDNA depletion. The treatment showed efficacy regardless of the specific POLG mutation [40]. These observations imply that increasing dNTP concentration by deoxyribonucleoside supplementation could enhance activity of enzymes at the replication fork independently of their associated genetic defect and therefore be of therapeutic use for mtDNA depletion beyond those caused by dNTP insufficiency. Experimental data supporting this idea was presented for models of MPV17 and ABAT defects, where deoxyribonucleoside or dNTP administration helped

Table 2
Patients with TK2d treated with nucleotides/nucleosides presented.

	Clinical evolution before treatment	Treatment regimen (doses in mg/kg/day)	Clinical evolution after treatment
P1	Onset at 12 mo, rapidly progressing weakness. Severe tetraparesis and ventilator and feeding tube dependent by age 17 mo.	Started at 21 mo, dTMP/dCMP at dose 100, gradually increased to dose 400 at age 3.75 yo. Changed to dT/dC at dose 260 titrated up to 400.	Still on a ventilator at age 6 yo. Regained functional use of the arms and legs, ability to sit with support and stand in a stander.
P2	Onset at 11 mo (weakness with falls). At age 4.2 yo, required assistance to walk with diffuse weakness against minimal resistance.	Started at age 4.2 yo, dCMP/dTMP at dose 100 for 23 months. Then dT/dC at dose 260 for 3 months and finally dose 400.	By age 7.1 yo: ability to walk, climb small steps and jump independently. Distance travelled in the 6MWT distance increased from 175 m pre-treatment to 371 m after 3 years of therapy.
P3	Onset at 18 mo (Gowers sign). Gradual progression of limb weakness that limited walking and restrictive lung disease. Required BiPAP nearly 24 h daily at age 24 yo.	Started at 24 yo, dT/dC at dose 130 for 6 months, then dose 260.	6MWT distance increased from 175 m at baseline to 340 m after 1 year of treatment. After 30 months BiPAP use decreased to 18 h/day. Gained abilities to climb up a few steps, take showers independently and lift arms overhead.
P4	Progressive weakness since age 25 yo. At 57 yo severe dysarthria and dysphagia with ability to drink only a few sips of coffee in the morning. Required gastrostomy feeds and BiPAP at night.	Started at 57 yo, dT/dC at dose 130 for 1 month then dose 260 for another month.	The patient reported feeling more energetic after 2 months of treatment.
P5	Onset at 16 mo. At 18 mo flaccid tetraplegia and >50% COX-negative fibres in muscle. Respiratory muscle weakness, required a cough assist machine. Nasal voice.	Started at 2.5 yo, dTMP/dCMP at dose 200. 11 months later, dT/dC at dose 400, divided in 8 doses/day.	6 months later EK score improved from 28 to 13. Use of cough assist machine reduced from 8 times/day to none, peak flow improved from 20 to 50 L/min.
P6	Onset at 18 mo. At 2.5 yo dysphagia, dysarthria, flaccid tetraplegia and neck muscle weakness with no head control. Nasal voice.	Started at 2.5 yo, dTMP/dCMP at dose 200. 11 months later, dT/dC at dose 400.	After treatment, able to lift the arms and sit unassisted, improved EK score. Use of the cough assist reduced from 30 times/day to none, increased peak flow from 20 to 85 L/min and FVC of 74%.
P7	Onset at 10 mo, weakness with rapid progression to flaccid tetraparesis, required mechanical ventilation 6 months after onset.	Started at 18 mo	1.5 years later, off the mechanical ventilation and able to lift the arms.
P8	Diagnosed at 20 yo: proximal weakness, aspiration pneumonia and a nasal voice. In retrospect, onset at 2 yo with a Gowers sign and ankle contractures.	Started at 29 yo dT/dC at dose 200. Gradually increased to dose 400 over 6 months.	FVC improved modestly from 18% to 23%.

6MWT: six-minute walk test; dC: deoxycytidine; dT: thymidine; EK: Egen Klassifikation scale; FVC: Forced Vital Capacity; mo: months old; yo: years old.

recovery of normal mtDNA copy number in otherwise depleted cells [30,41].

The rapid *in vivo* degradation of purine deoxyribonucleosides poses a major challenge when considering clinical implementation of deoxyribonucleoside-based therapies for mtDNA depletion. The possible use of inhibitors of nucleoside catabolic enzymes developed for chemotherapy, as a way to increase deoxyribonucleoside stability was discussed. However, mutations in some deoxyribonucleoside catabolic enzymes are associated with severe diseases. Hence, further research in disease models would be mandatory to prove efficacy and safety of chronic administration of inhibitory drugs.

6. Treatment with deoxyribonucleosides/deoxyribonucleotides: clinical experience

Dr. Hirano and Dr. Paradas presented several patients receiving treatment (Table 2). Dr. Hirano reported his initial experiences with dTMP+dCMP and dT+dC in four TK2d patients treated under expanded access at Columbia University Irving Center in New York, USA (P1 – P4). Then, Dr. Paradas followed by presenting a cohort of TK2d patients receiving treatment at the University Hospital Virgen del Rocío, in Seville, Spain (P5 – P8). All these patients but P4 belong to a cohort of patients reported in 2019 [42]. The presentation of these two cohorts of patients reinforced the opinion of the workshop participants that deoxyribonucleoside treatment is more effective in the infantile- and childhood-onset patients, compared to adulthood-onset patients.

The session continued with talks from Arturo Estopinan (USA) and Lander Nogués (Spain), two fathers of patients with TK2d. Their experiences reflect those of most TK2d patients with an initial phase of recognition of the abnormal behaviours/muscle weakness of their children, followed by a diagnostic odyssey which includes multiple blood tests and, in many cases, muscle biopsy, and ends with identification of *TK2* mutations. The time to access to treatment was very different for each presenter. In the late summer of 2012, Mr. Estopinan initially contacted Dr. Hirano, who, at that time, was assessing the therapeutic efficacy of dTMP/dCMP in the *Tk2* KI mouse model with Dr. Garone. The partial rescue of the phenotype in this mouse model encouraged them to submit an emergency IND application to the FDA, which gave approval for the compassionate use of dTMP/dCMP that was started in November, 2012. In the case of Mr. Nogués, by the time his daughter was diagnosed with TK2d, there were several cases of TK2d patients already enrolled in this therapy, also under compassionate use, therefore access to the deoxyribonucleoside treatment was easier. Nevertheless, he noted that, since it is an experimental treatment, there were bureaucratic procedures and

barriers to overcome before accessing the treatment. He presented videos of his daughter before and after the treatment. Although the improvements documented in the video were remarkable, Mr. Nogués pointed out that the real improvement perceived by the parents was even more evident. This led to the discussion about how to select proper outcome measures able to detect modest but clinically meaningful improvements to be used in a future clinical trial.

After the views of the parents of TK2d patients, the meeting had also the inputs from participants from 2 biotechnology companies (Peter Barber, Modis Therapeutics and Curtis Cui, Hongene Biotechnology). Curtis Cui described the company he works for and his experiences in providing Dr. Hirano's patients with deoxyribonucleotides and deoxyribonucleosides to perform both pre-clinical studies in the *Tk2* KI mouse model, as well as for the compassionate treatment of patients with TK2d. Peter Barber, founder of Modis Therapeutics, a company started by Aceras Life Sciences, described Modis and its work to develop deoxyribonucleotides and doexyribonucleosides for the treatment of TK2d. He shared his experience working with staff from Columbia University (led by Dr. Hirano) and investigators in Spain (including Dr. Martí and Dr. Paradas) to develop a clinical trial to assess dT/dC therapy. Modis planned to strive towards full FDA and EMA approvals for the use of dT/dC for TK2d, which would eliminate many of the barriers to treatment, previously noted by Mr Nogués.

Following with the topic of designing a clinical trial assessing dT/dC treatment for TK2d, the next speaker, Dr. Thompson, gave details on the methodology from both clinical and statistical points of view with a few examples of clinical trials for rare diseases. In order to increase statistical power, clinical researchers design clinical trials to recruit as many patients as possible. However, this is not always the best strategy, and TK2d is an example of this. TK2d is a rare disease and the number of new patients not yet under treatment is limited. Trying to increase statistical power by increasing the number of participants and then mixing different sub-phenotypes (infant-, childhood- and adulthood-onset) may result in confusing results. Dr. Thompson suggested setting up different trials for each sub-group of patients.

Dr. Thompson reviewed the case of KANUMA, a treatment for Lysosomal Acid Lipase (LAL) deficiency, also a rare disease with two forms: the first type (known as Wolman disease) is often fatal in the first year of life; the second form (known as cholesteryl ester storage disease, CESD) is milder, with an onset in early childhood or later and variable life expectancy. The approval of KANUMA was based on two different clinical trials, each of them targeting a specific group of patients. This led to a better assessment of the different outcome measurements. For instance, in patients with Wolman disease, the focus was the survival rate at age 12 mo among treated patients as compared to the untreated historical cohort. In contrast, the CESD trial focused on the levels of low-density lipoprotein (LDL) cholesterol and other disease-related measures, as compared to placebo after 20 weeks of treatment. Having different clinical trials and different outcome measures, according to each group of patients, facilitated the detection of significant differences and overall improved the power of each clinical trial to demonstrate therapeutic effects. Notably, the suggestions made by Dr. Thompson, and the contributions and conclusions derived from this ENMC workshop, culminated in the establishment of a phase 2 clinical trial for assessing dT/dC clinical efficacy and safety in TK2d patients (NCT03845712).

7. Therapy for MNGIE

The next session focused on the treatment of another specific mtDNA replication disorder, mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). In contrast to most MDDSs, MNGIE has available treatments, as well as active research for new therapeutic strategies. For these reasons the workshop dedicated a session to discuss this specific topic.

MNGIE is a rare autosomal recessive disease caused by loss-of-function mutations in *TYMP*, which encodes the cytosolic enzyme thymidine phosphorylase (TP) [11]. TP deficiency leads to accumulations of dT and dU in plasma and in cells, which impair mtDNA maintenance and integrity as described above. Clinically, the disease manifests as PEO, gastrointestinal dysmotility, cachexia, peripheral neuropathy, and leukoencephalopathy with onset typically in adolescence and with a mean age-at-death of 37 years. Therapeutic approaches have been targeted at lowering levels of the toxic nucleosides, dT and dU, by (1) peritoneal dialysis [43], (2) temporary enzyme replacement, e.g. blood cell encapsulated TP infusions [44], or (3) permanent restoration of TP activity through allogeneic hematopoietic stem cell transplantation (AHSCT) [45] or orthotopic liver transplantation (OLT) [46]. Since severity of symptoms appears to be inversely correlated with success of HSCT and OLT, early intervention is crucial [45,46]. Potential future options include gene therapy, which is still under pre-clinical development [47].

At least 30 patients have undergone AHSCT treatment for MNGIE making this the most frequent disease modifying treatment for this disease. AHSCT successfully restores buffy coat TP activity and markedly lowers plasma dT and dU with delayed stabilization or partial improvements of clinical manifestations after recovery from the conditioning regimen and donor cell engraftment. However, AHSCT has been associated with a high mortality (17/30, 56%) due to variable combinations of late-stage of the disease at time of AHSCT, transplant related complications, and disease progression. Thus, AHSCT should be considered only in an early stage of the disease when there is a HLA 10/10 matched donor.

The session continued with Dr. Carelli presenting the outcomes of liver transplantation in cases of MNGIE. This organ is rich in TP activity. Therefore, the liver from the donor would work as an enzyme replacement therapy, as the hepatocytes would catabolize high levels of dU and dT from blood. Since these are the toxic metabolites that interfere with mtDNA replication at a systemic level, liver would systemically remove these deoxyribonucleosides, thereby indirectly cleaning other tissues [48]. Nevertheless, Dr. Carelli stressed that liver is also affected in MNGIE patients, since routine histology often shows COX-deficiency, steatosis and fibrosis. Therefore, liver transplantation is expected to solve simultaneously two problems: mitochondrial hepatopathy and lack of TP enzyme activity in the patient. Dr. Carelli presented two MNGIE patients who were transplanted. The first patient was a 25 years-old man misdiagnosed with Crohn's disease. When he was later diagnosed with MNGIE, he was already in an advanced stage of the disease. After liver transplant, he had to undergo temporary decompressive gastrostomy and ileostomy and required parenteral nutrition. Nevertheless, his overall health status improved over time after transplantation, with increased leg strength and recovery of the ability to walk. Karnofsky score improved from 30 to 60. Plasma deoxyribonucleoside levels were dramatically reduced within two days after liver transplant, from 16.5 μM dT and 16.2 μM dU to undetectable levels. The second patient was a 21 yo woman with typical manifestations of MNGIE (ptosis, fatigue, ataxia, distal limb paresthesia). She was in an early stage of disease when she underwent liver transplant. Buffy coat TP activity was undetectable with elevated levels of dT (8.0 μM) and dU (17.0 μM). After liver transplant, SF36 quality of life scale score improved from 46 to 60, and plasma levels of dT and dU were undetectable. In both cases, TP activity in plasma remained undetectable and no changes in weight were observed. These two patients have been reported together with a third MNGIE patient

[49,50], who underwent liver transplant in an advanced stage of disease. After liver transplantation, the patient was diagnosed with bacterial pneumonia and suffered pulmonary failure. Mechanical ventilation and parenteral nutrition were temporarily necessary. The patient was recovering from these complications and showed reductions in dT (18.1 μM) and dU levels (25.3 μM). Thus, the preliminary liver transplants have shown safer profiles compared to HSCT, even in advanced stages of disease, and the ability to detoxify plasma from high dT and dU levels.

However, because both AHSCT and liver transplantation are dependent on availability of compatible tissue donors and pose risks for MNGIE patients, gene therapy may be a potential safer alternative. Dr. Martí summarized all preclinical results obtained for the use of adenoassociated virus (AAV) vectors for MNGIE. The first work demonstrating that this is a feasible approach was reported in 2014 [51]. An AAV vector (serotype 8) was generated containing the coding sequence of the human *TYMP* gene, under the control of the hepatic promoter of the thyroxine binding globulin. The vector was injected intravenously at different doses to the murine model of the disease, the double knockout Tymp$^{-/-}$/Upp1$^{-/-}$, which resulted in restoration of TP activity in the liver of the animals and dose-dependent systemic clearance of the accumulated substrates dT and dU [51]. Additional studies indicated that these results were maintained long-term for intermediate and high doses of the vector, as well as improved efficacy with an alternative hepatic promoter (alpha-1-antitrypsin), relative to the results previously observed with the thyroxine binding globulin promoter [47,52,53]. Dr. Martí also presented the international consortium, comprised of several clinical and academic centers in Europe and USA, built to serve as a platform for the future implementation of an AAV-based gene therapy clinical trial for MNGIE, for which an orphan drug designation was obtained from the EMA and FDA in 2014, as well Scientific Advice from the EMA in 2015. However, the implementation of this program is dependent on funding opportunities given the elevated costs of good manufacturing practices (GMP)-compliant production of clinical grade AAV vectors, and the validation of its safety and efficacy in both preclinical and clinical phases.

Another gene therapy approach is targeted to the hematopoietic stem cells of the patients to enable production of functional TP protein, followed by transplantation of the genetically corrected autologous hematopoietic stem cells. Dr. De Coo presented pre-clinical results using this procedure. They used a self-inactivating lentiviral vector containing the human phosphoglycerate kinase promoter and human *TYMP*. Bone marrow from male *Tymp*$^{-/-}$ *Upp1*$^{-/-}$ mice were isolated, transduced with this vector and transplanted into female *Tymp*$^{-/-}$ *Upp1*$^{-/-}$ mice. Blood TP activity in the treated mice was above normal, and levels of dT and dU in blood and urine were undetectable. White matter edema and vacuolization were absent in treated mice, and astrocyte processes thickness surrounding blood vessels were reduced to normal levels. Importantly, mice were followed for 11 months to evaluate the safety of this approach. Pre-end point euthanized mice due to high discomfort scores did not show vector-positive hematological clonal expansion. Overall, over-expression of TP did not disturb hematopoietic cells homeostasis, no genotoxicity was observed and the safety profile was similar to that of other lentiviral vectors already under assessment in clinical trials [54].

Finally, Dr. Hirano gave a summary of the main recommendations for treatment of MNGIE. Based upon the limited experience with disease-modifying treatments, the consensus of MNGIE experts concluded that early therapeutic intervention, before severe permanent organ damage occurs, is essential. [55] Bridge therapy with peritoneal dialysis or erythrocyte encapsulated TP may be considered in three situations: in symptomatic patients who are reluctant to undergo a risky permanent treatment; in severely affected patients who are unlikely to tolerate a transplant; and if no permanent therapy is available (e.g. no suitable transplant donors). Response to therapy appears to depend upon extent of liver and gastrointestinal damage; therefore, a comprehensive assessment of disease status may predict treatment efficacy.

8. Discussion and conclusions

During the last session of this workshop, participants discussed several topics that emerged from the previous sessions and achieved several agreements.

First, the participants agreed on the importance of greater attention to the mtDNA maintenance disorders (Table 1) in the scientific and biomedical communities (healthcare agents, patient associations, and biomedical companies). Increased awareness of these diseases will enhance early and correct diagnosis and treatment of patients.

To enhance visibility and to develop differential diagnoses of these disorders, the participants summarized actual and potential misdiagnoses (**Table 3**).

The discussion then turned to a focus on the two currently treatable diseases: MNGIE and TK2d. Regarding therapeutic recommendation, participants agreed to follow recommendation for treatment of MNGIE, as summarized in the Section 7 above, recommendations that have been recently reported in a position article [55].

Regarding TK2d, participants agreed to make an effort to include *TK2* in all clinical diagnostic genetic panels for patients whose clinical presentation and/or mtDNA molecular results (depletion or presence of multiple deletions) suggest possible TK2d. This will maximize the detection of pathogenic mutations in this gene and will facilitate early treatment of patients.

There was further debate about parenteral (intravenous) treatment of TK2d with deoxyribonucleosides, and participants agreed that this therapy may be recommended only if future studies demonstrate very significant advantage(s) over oral therapy. Another discussion regarding the need for muscle biopsy took place. Muscle biopsy can be very informative, since it can show significant findings like dystrophic features, COX-deficiency or ragged-red fibbers. Nevertheless, it is a very invasive technique for infants and children, therefore participants agreed that it may be necessary only in patients with adult onset.

Regarding other diseases (mutations in *DGUOK, RRM2B, POLG, POLG2*, and *TWNK*), there was an agreement on the potential plausibility to treat them with deoxyribonucleosides, based on available results obtained *in vitro* [31,32,34,39,40,56], but it was deemed reasonable to wait for additional *in vivo* data confirming the *in vitro* information before recommending treatment with deoxyribonucleosides to patients with mutations in those genes. The participants agreed that some exceptions to this general rule may be acceptable, but only in patients with life-threatening courses or in potentially terminal phases of the disease. In such cases, compassionate use of desoxyribonucleosides can be considered given the relative safety profile of this therapy, with only mild secondary effects, as had been discussed in the workshop and later reported [42].

Nevertheless, during these discussions, participants highlighted the need for pre-clinical studies using animal models presented during the workshop (IOSCA mouse [26], *Dguok* mouse [24] and zebrafish models [33], *Rrm2b* mouse [57], *Mpv17* mouse [28,30]) to obtain data that would support treatment in humans. Participants also emphasized the need for more natural history studies to increase understanding of these disorders and to improve assessment of potential therapies in clinical trials. Additionally, it was commented the interest in validating molecules, such as GDF-

Table 3
Misdiagnoses for MNGIE and TK2d.

MNGIE
– Anorexia nervosa
– Inflammatory bowel disease
– Superior mesenteric artery syndrome
– Coeliac disease
– Esophagitis
– Gastritis
– Irritable bowel disease
– Whipple disease
– Chronic intestinal pseudo-obstruction
– Intestinal obstruction
– Charcot Marie Tooth disease
– Chronic inflammatory demyelinating neuropathy
– Other mitochondrial diseases (chronic progressive external ophthalmoplegia, Kearns-Sayre syndrome)
TK2d
– Pompe disease
– Limb-girdle muscular dystrophy
– Fascioscapulohumeral dystrophy
– Spinal muscular atrophy
– Inflammatory myopathy

15 and FGF-21, as mitochondrial disease biomarkers, which may be included eventually in future clinical trials as surrogate biomarkers. Finally, new results confirming the plausibility of this proposal have been recently reported [19].

The last discussion of the meeting focused on design of clinical trials in general, and the design of a clinical trial to assess therapeutic efficacy of dT/dC therapy in TK2d patients in particular. Participants agreed that, since it is a rapidly progressive and fatal disease, placebo groups can be avoided and open-label designs are indicated.

9. Recent developments in the field related with the topic of this workshop

As mentioned in multiple sections of this report, research advances after the workshop have reinforced interest in treatment options for MDDS. Several concepts discussed at that time have been supported by new evidence, such as the publication of the first report indicating efficacy of deoxyribonucleoside treatment in TK2d patients [42], followed by current ongoing clinical trials for this therapy (NCT03845712 and NCT04581733). Additional reports have recently expanded our knowledge about the biochemical mechanisms and limitations of this therapy [37,38]. It is noteworthy that the cytokine GDF-15 has been shown to be a reliable biomarker of the therapeutic response of TK2d [19], an observation that is particularly relevant to the ongoing clinical trials of this disease and perhaps other mitochondrial disorders. Of utmost importance for these clinical trials is the publication of a detailed retrospective natural history study of TK2d [18]. Importantly, new publications have documented characterization of a *Dguok* knockout mouse that was initially described at the workshop [24] as well as preclinical evidence that *POLG* mutant MDDS is also a candidate for deoxynucleoside therapy [40]. In parallel, significant advances supporting the feasibility of gene therapy for two MDDS forms, MNGIE [47] and TK2d, have been reported and for TK2d, a combination of gene therapy and deoxyribonucleosides has been shown to synergistically ameliorate Tk2 deficient mice [58]. Thus, the study of MDDS is a dynamic topic in mitochondrial medicine with rapidly advancing knowledge and therapies, which are already under assessment in clinical trial programs.

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Declaration of Competing Interest

RM, MH, and YC report grants, non-financial support, personal fees and other suppot from Zogenix/UCB. In addition, RM and MH have a patent “Deoxynucleoside therapy for diseases caused by unbalanced nucleotide pools including mitochondrial DNA depletion syndromes” (PCT/US16/038110) with royalties paid by Zogenix/UCB; MH reports grants and other support from Entrada Therapeutics. RM and YC have a patent “Treatment of mitochondrial diseases” (PCT/EP2016/062636) with royalties paid by Zogenix/UCB.These relationships for MH are de minimus for Columbia University Irving Medical Center and for RM and YC are de minimus for Vall d’Hebron Research Institute and CIBERER.

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