THE NATURAL HISTORY OF INFANTILE MITOCHONDRIAL DNA DEPLETION SYNDROME DUE TO RRM2B DEFICIENCY

Nandaki Keshavan MBBChir MRCPCH[1,2], Jose Abdenur MD[3], Glenn Anderson FIBMS[4], Zahra Assouline PhD[5], Giulia Barcia MD PhD[5], Lamia Bouhikbar PhD[6,7], Anupam Chakrapani MBBS FRCPCH[1], Maureen Cleary MD FRCPCH [1], Marta C. Cohen MD FRCPath[8], François Feillet MD PhD[9], Carl Fratter PhD[10], Natalie Hauser MD[11], Tom Jacques PhD FRCPath[4], Amanda Lam PhD[12,13], Helen McCullagh MB ChB[14], Rahul Phadke MD FRCPath[13], Agnès Rötig PhD[15], Mark Sharrard MBBS FRCPCH[8], Mariella Simon MS PhD[3], Conrad Smith PhD[10], Ewen W. Sommerville MSc PhD[16,17], Robert W. Taylor PhD FRCPath[16], Wyatt W. Yue MA PhD[18], Shamima Rahman PhD FRCP[1,2*]

1 Metabolic Unit, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK

2 Mitochondrial Research Group, UCL Great Ormond Street Institute of Child Health, London, UK

3 Division of Metabolic Disorders Children's Hospital of Orange County, California, USA

4 Department of Histopathology, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK

5 Department of Genetics, Necker Hospital – Sick Children, Paris, France

6 GOSgene Centre for Translational Omics, UCL Great Ormond Street Institute of Child Health, London UK

7 NIHR GOSH Biomedical Research Centre, London, UK

8 Sheffield Children's NHS Foundation Trust, Sheffield, UK

9 Reference Centre for Inherited Metabolic Diseases, Nancy, France

10 Oxford Medical Genetics Laboratories, Oxford University Hospitals NHS Foundation Trust, Oxford, UK

11 Inova Translational Medicine Institute, Virginia, USA

12 Neurometabolic Unit, National Hospital of Neurology and Neurosurgery, London, UK

13 UCL Queen Square Institute of Neurology, London, UK

14 Department of Paediatric Neurology, Leeds Children's Hospital, Leeds, UK

15 Imagine Institute, Paris, France

16 Wellcome Centre for Mitochondrial Research, Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, UK

17 Division of Psychological Medicine and Clinical Neurosciences, Cardiff University, Cardiff, UK

18 Structural Genomics Consortium, Nuffield Department of Medicine, University of Oxford, Oxford, UK

***Correspondence to:**

Professor Shamima Rahman

Mitochondrial Research Group

Genetics and Genomic Medicine

UCL Great Ormond Street Institute of Child Health

London WC1N 1EH, UK.

Telephone: +44 (0)2079052608

shamima.rahman@ucl.ac.uk

ABSTRACT

Purpose

Mitochondrial DNA (mtDNA) depletion syndrome (MDDS) encompasses a group of genetic disorders of mtDNA maintenance. Mutation of *RRM2B* is an uncommon cause of infantile-onset encephalomyopathic MDDS. Here we describe the natural history of this disease.

Methods

Multinational series of new genetically confirmed cases from six paediatric centres.

Results

Nine new cases of infantile-onset RRM2B deficiency, and 22 previously published cases comprised a total cohort of 31 patients. Infants presented at a mean of 1.95 months with truncal hypotonia, generalised weakness and faltering growth. Seizures evolved in 39% at a mean of 3.1 months. Non-neurological manifestations included respiratory distress/failure (58%), renal tubulopathy (55%), sensorineural hearing loss (36%), gastrointestinal disturbance (32%), eye abnormalities (13%), and anaemia (13%). Laboratory features included elevated lactate (blood, CSF, urine, MR spectroscopy), ragged-red and cytochrome *c* oxidase-deficient fibres, lipid myopathy and multiple oxidative phosphorylation enzyme deficiencies in skeletal muscle. Eight new *RRM2B* variants were identified. Patients with biallelic truncating variants had the worst survival. Overall survival was 29% at 6 months and 16% at 1 year.

Conclusion

Infantile-onset MDDS due to RRM2B deficiency is a severe disorder with characteristic clinical features and extremely poor prognosis. Presently management is supportive as there is no effective treatment. Novel treatments are urgently needed.

[200/200 words]

Key words:

Mitochondrial disease, mtDNA depletion, ribonucleotide reductase, treatment, outcomes

INTRODUCTION

RRM2B (OMIM 604712) is a nuclear gene encoding the small p53 inducible subunit (p53R2) of the ribonucleotide reductase (RNR) complex. RNR is a heterotetramer[1-3] composed of two R1 subunits (encoded by *RRM1*), and two R2 (encoded by *RRM2*) or p53R2 (encoded by *RRM2B*) subunits. RNR localises to the cytosol[4] and reduces ribonucleotide diphosphates to deoxyribonucleotide diphosphates which is important in keeping the mitochondrial deoxyribonucleotide triphosphate (dNTP) pool replete. These dNTPs are then thought to be relocated to the nucleus and mitochondria for DNA synthesis.

Unlike nuclear DNA, which replicates during the S phase of the cell cycle before mitosis, mtDNA is synthesised throughout the non-proliferative phase (G0) and thus requires a steady pool of dNTPs. Therefore, RNR exists in two forms: that which supports *de novo* dNTP synthesis during the S phase (composed of 2R1, 2R2 subunits) and that which is responsible for maintenance of dNTP pools in G0 (composed of 2R1, 2p53R2). *p53* is a tumour suppressor gene which is activated during DNA damage. p53R2 is thought to have two roles: provision of dNTPs for DNA repair following nuclear DNA damage[2] and also a 'housekeeping role' in maintaining dNTP pools needed for mtDNA replication throughout the cell cycle in both dividing and non-dividing cells[5,6].

Mitochondrial DNA depletion syndrome (MDDS) encompasses a group of nuclear gene disorders affecting key enzymes involved in the synthesis of mtDNA and its replication. This includes several genes involved in either mtDNA replication itself or in the synthesis of nucleosides which are incorporated into mtDNA at the replication fork[7,8]. MDDS is classically categorised into different phenotypic subtypes, each with an expanding number of causative disease genes. These phenotypes are: myopathic (caused by mutations in *TK2*,

SLC25A4, MGME1), encephalomyopathic (*RRM2B, FBXL4, ABAT*), hepatocerebral (*TFAM*, *MPV17, DGUOK, SUCLA2, SUCLG1*), Alpers syndrome (*POLG*, *TWNK*) and MNGIE (*TYMP*, rarely *POLG* and *RRM2B*)[6-9]. Mutations of *RRM2B* may be associated with several different phenotypes, including infantile-onset MDDS caused by autosomal recessive inheritance of biallelic pathogenic variants[3,5,10-16]. Other phenotypes include multiple mtDNA deletions in children and adults which are caused by either autosomal recessive inheritance of biallelic pathogenic variants or autosomal dominant inheritance of a single pathogenic variant[17-21], Kearns-Sayre syndrome[19], Mitochondrial Neurogastrointestinal Encephalopathy (MNGIE) [22] and, most recently, acute liver failure[23].

We describe nine new cases of infantile-onset encephalomyopathic *RRM2B* MDDS, reporting their detailed phenotype, investigations, treatment and outcome. Taken together with a further 22 published cases, we describe the natural history of 31 patients with infantile RRM2B deficiency.

METHODS

Patient cohort

Nine new patients were recruited from three British centres, two centres in USA and one French centre. Patients were included if they had confirmed biallelic *RRM2B* variants and an infantile-onset clinical phenotype, and were not previously published. Data were collected retrospectively by clinicians in each centre using a standardised questionnaire. This study was approved by Great Ormond Street Hospital (GOSH) as a clinical audit.

Molecular studies in new patients

MtDNA copy number relative to nuclear DNA was estimated and compared with age-matched normal controls by real-time quantitative PCR[24]. For the UK cases, this was performed as described previously except that the nuclear DNA probe was labelled with Vic and the assays were performed simultaneously using a PE7500 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). All exons and exon-intron boundaries of *RRM2B* (GenBank accession number NM_015713.4, exons 1-9 numbered sequentially) were Sanger sequenced using capillary electrophoresis in a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). For probands in whom Sanger sequencing only identified a single heterozygous pathogenic variant, *RRM2B* exon copy number was assessed by multiplex ligation-dependent probe amplification (SALSA MLPA P089-B1, MRC-Holland, Amsterdam, The Netherlands). For two cases, genetic diagnoses were obtained by genome sequencing in a rapid genome sequencing project for critically ill infants[25]. For a further case, the causal *RRM2B* variants were identified by exome sequencing as previously described[26].

Literature Search

We collected data from all published cases of infantile-onset *RRM2B*-related MDDS by searching PubMed for relevant publications. Search terms were 'RRM2B' OR 'ribonucleotide reductase' OR 'p53R2' with filters for English language publications and human species (search completed 21 October 2018). This yielded 1948 publications including 10 papers containing relevant cases.

Statistical Analyses

Stata software version 15 was used for all statistical analyses (https://www.stata.com/). A P value <0.05 was considered statistically significant.

Modelling

FoldX server was used to model the new missense variants[27].

RESULTS

Patient cohort

Nine previously unreported cases are included in our multinational cohort (patients A-I). They presented with symptoms at an average of 2.3 months (range 0 – 4 months). Twenty-two further cases were identified from the literature (patients 1-22), making a total of 31 cases of infantile-onset RRM2B-deficient MDDS[3,5,10-16].

Molecular analyses

Mitochondrial DNA studies

Residual mtDNA levels in skeletal muscle in the new cohort of 9 previously unreported infants with biallelic *RRM2B* variants ranged from 2 to 20% (median 4.5%) which is far below the accepted diagnostic limit of 30% for MDDS[28].

Novel pathogenic RRM2B variants

We identified eight novel pathogenic *RRM2B* variants not present in the Gnomad database (https://gnomad.broadinstitute.org/gene/ENSG00000048392). These are c.599G>A p.Gly200Glu, c.313G>A p.Glu105Lys, c.181G>C p.Ala61Pro, c.165G>A p.Met55Ile, c.(321+1_322-1)_(684+1_685-1)del (exon 4-6 deletion), c.635_636insAAG p.Gly212_Leu213insSer, c.400C>G p.His134Asp and c.59C>G p.Ser20*. The six missense and insertion variants are found in amino acid positions with a high degree of evolutionary conservation across species (Clustal Omega, [http://www.clustal.org/], see Supplementary Figure 1 for alignments, Figure 1a and Supplementary Figure 2 for the biallelic variants identified in the cohort); at these positions, the amino acids of the human sequence are also shared by 65-96% of RRM2B orthologues (n=150 orthologues; data from Consurf).

The six non-truncation variants are distributed across the N-terminal two-thirds of the RRM2B protein, a 351-residue polypeptide (Figure 1b). The crystal structure of human

RRM2B reveals a homodimer with an active site di-iron cofactor from each monomer[1]. Two variants, p.Ala61Pro and p.Gly200Glu, result in marked changes to the amino-acid side-chains, and are predicted by the FoldX server to reduce protein stability (ΔΔG 3.97 and 11.60 kcal/mol respectively). His134 is one of the iron ligating residues in the structure, hence the p.His134Asp variant likely interferes with metal binding, and is also predicted by FoldX to reduce protein stability (ΔΔG 6.50 kcal/mol). Glu105 is located at the dimer interface, forming salt bridges with Arg40 and Arg121 from the opposite dimer subunit. The p.Glu105Lys variant could therefore interfere with structural integrity of the homodimer. The p.Met55Ile and p.Gly212_Leu213insSer variants are located at the protein exterior, away from the core active centres; the effects of these substitutions are less clear from the structure.

Disease course of nine new cases

The disease phenotype was multisystemic, with an initial myopathic presentation followed by evolution of respiratory distress and failure leading to assisted ventilation in all cases. Multisystem involvement included the neurological, renal, cardiac, respiratory, gastrointestinal and haematological systems, the eyes, hearing and growth. Table 1 and Figure 2 detail the biochemical and histological findings. Supplementary Figure 3 illustrates the neurological, respiratory, renal and gastrointestinal features and growth of the 9 new cases. Supplementary Figure 4A-C illustrate the treatments administered to the new cases, including respiratory support, supportive feeding and cofactor supplementation. Supplementary Figure 4D illustrates the survival outcomes for the new cases. By 6 months 8/9 patients had already developed respiratory failure and 6/9 had died. All nine patients died by 22 months. The causes of death are illustrated in Supplementary Figure 4E.

Natural history of cohort of 31 patients

Considering the entire cohort of 31 infants with RRM2B deficiency, median age of symptom onset was 2 months (mean 1.95 months, range 0-6 months). The most frequent presenting features were muscular hypotonia, feeding difficulty, failure to thrive, hearing loss and respiratory distress. The clinical phenotype was multisystemic. Neurological involvement occurred in all cases: encephalopathy in 15/31 (48%), seizures in 12/31 (39%) presenting at an average age of 3.1 months (range 20 days-7 months), respiratory involvement in 18/31 (58%), renal involvement in 17/31 (55%), growth abnormalities in 16/31 (51%), hearing loss in 11/31 (36%), GI involvement in 10/31 (32%), eye abnormalities in 4/31 (13%), and anaemia in 4/31 (13%) of cases. The most consistent biochemical abnormality was elevated lactate seen in blood, CSF and urine, followed by elevated alanine and mildly elevated creatine kinase. The most frequent skeletal muscle histopathological features were COX-deficient fibres, abnormal lipid deposition and ragged-red fibres. The most frequent OXPHOS biochemical abnormalities in muscle were decreased activities of complexes I, III and IV with compensatory increases in both complex II and citrate synthase activities. Neuroimaging most frequently showed a structurally normal brain, and less frequently evidence of demyelination, white matter abnormality or generalised atrophy (Table 2). Elevated lactate peak was frequently observed on MR spectroscopy. Detailed clinical, biochemical and radiological features of all 31 cases are summarised in Table 2.

Survival outcomes for the cohort were poor. A Kaplan-Meier survival curve of all 31 cases of *RRM2B*-associated infantile MDDS (Figure 3) shows that survival at 3 months was 64.5% (20/31), at 6 months 29% (9/31), at 1 year 16% (5/31) and at 2 years 6% (2/31).

Phenotypic correlations

Mitochondrial DNA content

Patients with lower residual mtDNA levels in skeletal muscle tended to die earlier, however this observation did not meet statistical significance (Spearman rank test p=0.06). There was no statistically significant correlation between residual mtDNA content and age of onset (Spearman rank test p = 0.44) or number of organ systems affected (Spearman rank test p =0.14). We compared patients who developed seizures (12/31) as a subgroup to patients who did not develop seizures (19/31) and noted no difference in average mtDNA levels between these two groups (Mann Whitney U test p = 0.85). Similarly, we noted no significant difference in average residual mtDNA levels in patients who developed renal disease (n=17) compared to those who did not develop renal disease (n=14) (Mann Whitney U test p = 0.29).

Age of onset of symptoms

Infants presenting earlier appeared to die earlier, however, this did not reach statistical significance (Spearman rank test, p= 0.07). There was no correlation between age of onset and number of organs affected (Spearman rank test, p=0.20, nor between age of onset and development of seizures (Mann Whitney U test p= 0.88). Interestingly patients who developed renal disease presented earlier (median age of onset for patients who developed renal disease was 2 months vs 3 months for those without renal disease, Mann Whitney U test p=0.01*).

Age of death

There was no correlation between age of death and number of organs affected (Spearman rank test p=0.42), nor between age of death and occurrence of seizures (Mann Whitney U test p=0.56). Patients with renal disease tended to die earlier, however this did not reach statistical significance (Mann Whitney U test p= 0.07).

Genotype-phenotype correlations

Patients were divided into two groups based on genotype. Group 1 included 7 patients whose pathogenic variants are expected to result in the formation of only truncated mRNA which is likely to be subject to nonsense mediated decay (5 with biallelic nonsense variants [patients 1-3, 21, 22], one with biallelic frameshift variants [patient 19], and one compound heterozygote with a nonsense variant and a splice site variant [patient I]). Group 2 patients included the remaining 24 patients whose variants are not expected to result in the formation of solely truncated mRNA (15 patients with biallelic missense variants [patients A,B,D,G,H, 6,8,11,13-18,20], 3 compound heterozygotes with a missense variant and a splice site variant [patients 4,5,12], 3 compound heterozygotes with a missense variant and a truncating variant [patients C, 9,10], 2 patients with biallelic in-frame insertions [patients E,F], and one compound heterozygote for a missense variant and an in-frame deletion [patient 7]).

Group 1 patients had a worse prognosis than group 2. Median age of death was 2.5 months for group 1 compared to 5 months for group 2 (Mann Whitney U test p=0.002**). There was no statistically significant difference between age of onset or number of organs affected between the two groups (Mann Whitney U test p=0.33 and 0.16 respectively), nor any correlation between genotypic group and development of seizures (Chi squared test, p=0.53) or renal disease (Chi squared test, p=0.31).

DISCUSSION

Here we have shown that infantile MDDS due to biallelic *RRM2B* variants presents in the very first months of life with hypotonia, poor feeding, and faltering growth leading to hospitalisation. Elevated blood and CSF lactate may be the clue to an underlying mitochondrial disease, prompting multi-system assessment revealing involvement of other organs, frequently including SNHL and renal tubulopathy/nephrocalcinosis. Infants may also develop GI symptoms such as recurrent vomiting/reflux. All deteriorate with respiratory insufficiency/failure leading to assisted ventilation. Generalised seizures may develop later in the disease course. This infantile clinical phenotype is distinct from paediatric and adult-onset disease associated with multiple mtDNA deletions due to recessive (biallelic) and dominant (heterozygous) *RRM2B* variants, where PEO, ptosis and proximal muscle weakness are the most frequent features and encephalopathy, gastrointestinal and renal involvement are uncommon.[21] Interestingly, SNHL loss is present to the same degree (36% of infantile-onset MDDS and 36% of cases with paediatric/adult-onset disease).

Although we have shown that the phenotype of infantile MDDS due to RRM2B deficiency is relatively homogeneous, we did observe some phenotypic heterogeneity within the cohort. Notably, although MDDS due to RRM2B deficiency is classically described as encephalomyopathic[6], encephalopathy was seen in 15/31 cases (49%) and seizures were observed in only 12 infants (39%). It is possible that some patients died due to severe myopathy before the development of encephalopathy or indeed that they would never have developed encephalopathy. Incomplete penetrance of neurological features is seen in other genetic causes of MDDS e.g. infantile-onset DGUOK deficiency where individuals may develop liver disease which can be fatal, or go on to develop multisystemic disease with additional brain involvement including encephalopathy. Conversely in our cohort, renal tubulopathy was seen in 17/31 (55%) of cases, and SNHL in 12/31 (39%). The high

incidence of renal tubulopathy in infants with MDDS is reminiscent of the phenotype of the *rrm2b*$^{-/-}$ exon 3 and 4 deletion mouse model in which renal failure is the most prominent feature and cause of death[29].

Mutational analysis of the new patients in our cohort revealed that several variants seen in patients with infantile MDDS have also been seen in children and adults with multiple mtDNA deletions presenting with PEO and myopathy. This confirms previous reports suggesting that missense variants may behave recessively (homozygous or compound heterozygous) as well as in a heterozygous dominant negative manner in different individuals. The presence of normal p53R2 protein in cases of heterozygosity may not always rescue the presence of mutated p53R2, owing to a dominant negative effect which may be mediated by disruption of normal allosteric interactions between the heterotetrameric subunits of the RNR complex[21].

In this study we sought to determine whether there are any phenotypic and genotype-phenotype correlations in *RRM2B*-associated MDDS. This is important for prognostication for affected infants and in future may enable assessment of suitability for candidate therapies. As MDDS is defined as residual mtDNA <30% of age-matched healthy controls, all of the patients met this criterion[28]. In this natural history study, we investigated whether mtDNA quantification can be used not just in diagnosis but also in predicting severity of disease. As it was not possible to apply a specific scoring system, for example the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS), in entirety to each patient, the markers of severity of disease that we analysed in this study were age of onset, presence of seizures, number of organs affected and survival. Taken pairwise, no statistically significant correlation was seen between these markers of disease severity and mtDNA quantification. Nevertheless, important trends were revealed: earlier disease onset tended to be associated with presence of

renal disease later in the disease course and an earlier mortality. While there was no statistical correlation between the presence of seizures and age of death, for the 12 patients in the cohort who did develop seizures, death followed after an average of 2.9 months (median 1.2 months, range 0-15 months) after the appearance of seizures, defining a point of significant clinical deterioration in the disease course. Therefore, we suggest that the presence of seizures indicates significant brain involvement and therefore severe disease.

When we grouped patients into two variant subgroups we found that patients with biallelic variants resulting in truncated transcripts had a relatively shorter survival compared to patients with missense/in-frame insertion/deletions resulting in formation of mRNA transcripts that are aberrant but not subject to degradation by cellular nucleases. This finding was statistically significant despite a small cohort and is consistent with our findings for other natural history studies in mitochondrial disease[30].

It should be noted that there is a limitation of using age of death as a marker of disease severity since in some cases death followed redirection of care after recognising futility of treatment. Severe disease may paradoxically be associated with a longer than expected lifespan, owing to prolonged artificial ventilation. Another consideration is that meaningful correlation between percentage mtDNA levels and genotype based on protein truncation could not be determined due to small numbers. Finally, it is assumed that the formation of truncated mRNA will result in mRNA degradation and therefore little enzyme activity. However, an exception to this has been demonstrated in myocytes of adult patients with exon 9 deletions where mRNAs escaped nonsense mediated decay and western blots showed persistent presence of truncated protein[17].

We have demonstrated that patients with biallelic variants resulting in truncated mRNA transcripts have the poorest prognosis overall. This is an important finding in terms of prognostication and appears to be the most robust genotype-phenotype correlation. The prognosis overall however is extremely poor, with a 6-month survival of 29% and a one-year survival of 16%, underscoring the need to find new effective treatments.

In RRM2B deficiency, inability to reduce ribonucleotide diphosphates to deoxyribonucleotide diphosphates (dNDPs) results in mtDNA depletion. For a different form of myopathic MDDS, thymidine kinase 2 (TK2) deficiency, it has been proposed that bypassing the defective step of nucleoside salvage by supplementation with deoxyribonucleotides may represent a therapeutic strategy for MDDS. This has been trialled in tk2-deficient mice with some success. A combination of orally supplemented deoxycytidine monophosphate (dCMP) and deoxythymidine monophosphate (dTMP) delayed disease onset and prolonged survival but did not prevent death in a knock-in model of tk2 deficiency[31]. Deoxyribonucleotide monophosphates appeared able to cross the immature blood-brain barrier before P13 but further development of the blood-brain barrier after P13 was likely to account for CNS disease at P29 and ultimate death despite therapy. Similar results were seen with oral supplementation with deoxycytidine (dC) and deoxythymidine (dT) in mice with tk2 deficiency, which is thought to be due to compensatory upregulation of the cytosolic enzymes tk1 and dck[32]. For RRM2B deficiency, *in vivo* studies using a suitable animal knockout model are yet to be undertaken, since the existing animal model does not effectively recapitulate human disease[29].

For RRM2B deficiency, *in vitro* studies of supplementation with deoxynucleosides in cultured human cells yielded contradictory results. RRM2B deficient patient fibroblasts do not express mtDNA depletion in culture unless they are exposed to ethidium bromide or

prolonged serum starvation[33]. Deoxynucleoside supplementation increased intracellular dNTP pools and normalized mtDNA synthesis in ethidium bromide treated quiescent patient fibroblasts harbouring a lethal homozygous missense *RRM2B* variant[34]. However, in a second study, supplementation with deoxynucleotide monophosphates (dNMPs) in cultured myotubules/myoblasts from another patient was ineffective in restoring mtDNA copy number[35]. *In-vivo* delivery of all four deoxyribonucleosides via any route has not been investigated in any model of MDDS. Furthermore, the CNS penetrance of systemically delivered deoxyribonucleosides in humans is unknown. The half-life of adenosine is extremely brief when delivered intravenously and there is a risk of toxicity since adenosine induces atrioventricular node blockade. It is clear that in the presence of many unknowns, detailed pharmacodynamic, pharmacokinetic and efficacy studies in a knock-out animal model of RRM2B deficiency which effectively recapitulates neurological disease seen in humans will be essential to answer these questions.

Prior to designing a clinical trial for an experimental treatment for *RRM2B*-related infantile MDDS in humans, it will be necessary to determine at which time point CNS involvement is considered irreversible. This should consider both the natural history of disease in humans and the optimal timepoint for effective drug delivery in preclinical drug trials using an appropriate animal model. Difficult decisions will need to be made in the advent of experimental therapies as to whether a specific patient is deemed to have reversible disease, especially since conventional neuroimaging is often normal early on in infants who have quite severe functional neurodisability, and seizures are not universally seen. However, in cases for which there are structural abnormalities on neuroimaging or prolonged/refractory electrical or clinical seizures, we suggest that CNS disease has progressed beyond the point of reversibility.

Whether or not irreversible disease should be treated with an experimental treatment ought to be considered on a case-by-case basis utilising existing ethical frameworks. This is predicated on determining whether the disease modifying treatment is likely to improve the quality of life rather than just prolonging life. The Royal College of Paediatrics and Child Health have provided an ethical framework which states that the best interests of the child need to be considered to 'determine whether or not there is an overall benefit in prolonging life because of the adverse impact entailed'[36]. The ethical implications of prolonging life with long term ventilation in the context of commencement of an experimental therapy need to be weighed carefully against those of redirection of supportive care. Indeed, long term ventilation may be requested as a bridge to emerging or experimental therapies and its use in this setting is difficult to evaluate[37]. An ethics committee should be called upon to meet to assess the individual clinical case considering the opinion of the infant's parents and available second opinions provided by other clinical teams.

It is clear that the natural history of infantile-onset RRM2B MDDS is characterised by rapid neurological disease progression. Prompt diagnosis is essential for optimal management of these infants, particularly avoidance of prolonged invasive ventilation as currently there are no effective disease-modifying therapies for this and most other mitochondrial diseases[38]. In future, if a clinical trial of a disease-modifying therapy is to be undertaken, clinical and ethical discussions regarding suitability for trial recruitment of the affected infant need to commence soon after a genetic diagnosis is made.

To facilitate more rapid diagnosis, we suggest that where there is a high index of clinical suspicion indicated by a combination of several of the following six features: hypotonia developing insidiously after birth, renal tubulopathy, sensorineural hearing loss, feeding difficulty, muscle weakness and respiratory failure, targeted single gene sequencing for

RRM2B should be prioritised over muscle biopsy and exome sequencing. Where the index of clinical suspicion is lower, that is when only a few of these features are present, the differential diagnosis includes potentially treatable disorders such as spinal muscular atrophy[12], benign reversible infantile respiratory chain deficiency disorders[39], congenital myopathies and other causes of MDDS e.g. TK2 deficiency. In this scenario a rapid exome sequencing approach with consideration of early muscle biopsy is more appropriate. Of note, creatine kinase levels are normal or only mildly elevated in RRM2B deficiency effectively distinguishing this disorder from myopathic MDDS due to TK2 deficiency[40].

CONCLUSIONS

Infantile-onset *RRM2B*-related MDDS should be suspected in cases where mtDNA depletion has been demonstrated in an infant with a combination of encephalomyopathy, respiratory failure, and renal or hearing impairment. The majority of infants are already symptomatic with hypotonia and faltering growth well before presentation with respiratory failure to emergency medical services. The disease evolves relentlessly with a one-year survival of 16%. Biallelic truncating variants predict the worst survival outcome. Whilst awaiting a genetic diagnosis there is evolving brain injury, implying a narrow therapeutic window for treatment with disease-modifying therapies, should these become available. The usefulness of deoxyribonucleoside supplementation remains to be elucidated in an animal model that adequately recapitulates human neurological disease before considering clinical trials in humans.

[3978/4000 words]

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Patient	Highest serum Lactate	Lactate: pyruvate ratio	CSF lactate	Creatine Kinase	Free carnitine	Acylcarnitines	Plasma amino acids	Urinary Organic Acids	Complex I*	Complex II*	Complex III*	Complex II+III*	Complex IV*	Citrate Synthase†	Mt DNA‡
A	7	ND	ND	1209 (<170)	ND	ND	Normal	Increased lactate, 4-OH-phenyllactate, 4-OH-phenylpyruvate, Kreb cycle intermediates, 3OHB	13%	16%	16%	ND	16%	234%	ND
B	4.6	38	3.5	330 (<145)	93 (14-53)	Global mildly increased acylcarnitines,hydroxyacyl-carnitines	Raised alanine and glycine. Decreased serine, valine, cysteine	Increased lactate, 4-OH-phenyllactate, succinate, benzoate, mildly increased 3OHB, adipate, suberate	4 (12-29) 33%	112 (23-49) 228% †	43 (223-454) 19%	ND	10 (114-250) 8%	377 (82-150) 550%	6%
C	3.2	ND	ND	361(<400)	37 (13-80)	Normal	Normal	Strong lactate, moderate pyruvate, fumarate, citrate, malate, mild 2OH valerate, 3OHB, 2OHB	0.087 (0.104- 0.268) 83%	ND	ND	0.061 (0.04-0.204) 100%	0.005 (0.014-0.034) 35%	ND	5%
D	5	ND	ND	91 (<240)	ND	Normal	Normal	Variably raised fumarate	0.004 (0.104-0.268) 3.8%	ND	ND	0.005 (0.04-0.204) 12.5%	0.001 (0.014-0.034) 7%	ND	20%
E	5.7	ND	5.6	546 (<204)	ND	Elevated C2	Normal	Slightly increased lactate, fumarate, malate	ND	ND	ND	ND	ND	ND	ND
F	6.1	ND	ND	340 <374)	31.6 (21-53)	Normal	Raised alanine	Markedly increased 3OHB, moderate lactate, pyruvate, fumarate, malate, aconitate, citrate, Mild 2- ethyl hydracrylate.	ND	ND	ND	ND	ND	ND	4%
G	8.2	54	3.3	220 (<400)	35 (13-80)	Mildly raised malonyl-carnitine	Raised alanine	Grossly raised lactate, pyruvate, 2OHB, fumarate, citrate, 4OHphenyllactate, 4OHphenylpyruvate, 3OHB, 3OHISV	0.018 (0.104-0.268) 17.3%	ND	ND	undetectable	undetectable	ND	6%
H	7.4	7.4	4.7	292 (<200)	Normal	Normal	Raised alanine	Normal	0.006 (0.068-0.14) 8.8%	0.08 (0.098-0.192) 81.6%	0.046 (0.209-0.899) 22%	ND	0.031 (0.613-1.635) 5%	ND	3%
I	41.7	ND	2	223 (<400)	17 (13-80)	Normal	Raised alanine	Grossly elevated lactate, pyruvate, moderated 2OHB, mildly 2OH valerate, 2 oxoisocaproate, 3MGA, 3OH adipate, 4OH phenyllactate	0.127 (0.104-0.268) 100%	ND	ND	0.025 (0.04-0.204) 62.5%	0.002 (0.014-0.034) 14%	ND	2%

Table 1: Biochemical parameters observed in 9 new cases of RRM2B deficiency.

Abbreviations: MGA: methylglutaconate, OH hydroxy, OHB hydroxybutyrate, ND Not done. Units: lactate and CSF lactate: mmol/L, CK: U/L, free carnitine: umol/L. Normal range for serum lactate <2mmol/L and for CSF lactate <2.2mmol/L. Other reference ranges are included in parentheses. * % of the lower reference range value, † % of the higher reference range value, ‡ % of age matched control values.

Clinical features	Number of patients during clinical course
Neuromuscular system	31/31(100%)
Truncal hypotonia	30/31 (96.7%)
Encephalopathy	15/31 (48.4%)
Gross motor delay	15/31 (48.4%)
Feeding difficulty	14/31 (45.2%)
Seizures	12/31 (38.7%)
Lack of head control	12/31 (38.7%)
Generalised weakness	10/31 (32.2%)
Loss of reflexes	9/31 (29%)
Discoordinated suck	9/31 (29%)
Ptosis	6/31 (19.3%)
Progressive external ophthalmoplegia	6/31 (19.3%)
Discoordinated Swallow	6/31 (19.3%)
Peripheral neuropathy	2/31 (6.5%)
Nystagmus	1/31 (3.2%)
Dystonia	1/31 (3.2%)
Respiratory system	18/31 (58%)
Respiratory distress	17/31 (54.8%)
Respiratory failure	15/31 (48.4%)
Pneumonia	5/31 (16.1%)
Renal system	17/31 (54.8%)
Tubulopathy	16/31 (51.6%)
Aminoaciduria	7/31 (22.5%)
Glycosuria	5/31 (16.1%)
Microalbuminuria	3/31 (9.7%)
Elevated RBP/Cr	3/31 (9.7%)
Calciuria	2/31 (6.5%)
Elevated NAG/Cr	3/31 (6.5%)
Phosphaturia	1/31 (3.2%)
Nephrocalcinosis	6/31 (19.3%)
Auditory system	11/31 (36%)
Sensorineural hearing loss	11/31 (36%)
Gastrointestinal system	10/31 (32%)
Recurrent vomiting	7/31 (22.5%)
Feed intolerance	3/31 (9.7%)
Chronic diarrhoea	2/31 (6.5%)
Ophthalmological system	4/31 (12.9%)
Pigmentary retinopathy	2/31 (6.5%)
Cataract	1/31 (3.2%)
Megacornea	1/31 (3.2%)
Haematological system	4/31(12.9%)
Anaemia	4/31 (12.9%)
Cardiovascular system	4/31 (12.9%)
Left ventricular hypertrophy	2/31 (6.5%)
Cardiomyopathy	1/31 (3.2%)
Ventricular septal defect	1/31 (3.2%)
Growth abnormalities	16/31 (51.6%)
Failure to thrive	16/31 (51.6%)
Microcephaly	5/31 (16.1%)

Biochemical Features	Number of patients during clinical course
General Biochemistry	
Hyperlactataemia	31/31(100%)
Elevated lactate/pyruvate ratio	4/5 (80%)
Elevated CSF lactate	8/10 (80%)
Elevated creatine kinase	8/19 (42%)
Low free carnitine	3/11 (27%)
Plasma amino acids	
Elevated alanine	10/16 (63%)
Elevated branched chain amino acids	4/16 (25%) 3/16 (19%)
Multiple low species	4/16 (25%)
Normal	
Urine organic acids	
Elevated lactate	12/16 (75%)
Elevated pyruvate	5/16 (31%)
Elevated ketones	7/16 (43%)
Elevated Krebs cycle intermediates	6/16 (38%)
Normal	3/16 (19%)
Hypocalcaemia	2/31 (6%)

Skeletal muscle spectrophotometry	Low	Normal	High
Complex I	17/18	1/18	0/18
Complex II	3/13	2/13	8/13
Complex III	7/7	0/7	0/7
Complex IV	20/20	0/20	0/20
Complex V	3/6	1/6	2/6
Complex I+III	4/4	0/4	0/4
Complex II+III	9/11	2/11	1/11
Citrate synthase	1/13	4/13	8/13

Muscle Histopathology	
Ragged-red fibres	12/19
Increased SDH	7/19
COX deficient fibres	16/19
Lipid storage	14/19
Atrophic fibres	2/19
Radiological Features	
MRI Head	
Generalised hypo/ demyelination	1/14
White matter abnormalities	2/14
Generalised atrophy	2/14
Normal	9/14
MR Spectroscopy	
Elevated lactate	8/10
Localisation:	
Basal ganglia	5/8
White matter	3/8
CSF	1/8
Not stated	3/8
Reduced choline/NAA	1/10
Normal	2/10

Table 2 Clinical, biochemical and radiological features of 31 patients with RRM2B deficiency. Abbreviations: COX cytochrome *c* oxidase, NAA N-Acetyl aspartate, NAG N-acetyl beta-D glucosaminidase, RBP retinol binding protein, RRF ragged-red fibre, SDH succinate dehydrogenase.

FIGURE LEGENDS

Figure 1a depicting the 9 exons (E1-9) of RRM2B mRNA showing the location of each of the variants identified in the 9 new patients (blue for new variants and green for previously reported variants). p53R2 comprises 351 amino acid residues. Each exon is labelled with the segment of the amino acid chain it encodes. Variants in the 22 previously reported patients are indicated in black. fs frameshift; * stop codon

Figure 1b 3D structure of a RRM2B dimer depicting the location of new non-truncating variants observed in the new cases.

The two subunits of the RRM2B dimer, taken from the reported structure (PDB code 3HF1), are shown in ribbon representation. The wild-type amino acids bearing the novel variants from this study are shown as sticks, coloured yellow (for missense changes) and cyan (for indel). The di-iron metal centre for one RRM2B subunit is represented by the two Fe atoms shown as spheres.

Figure 2 Histopathological findings associated with RRM2B deficiency.

The images show the histochemical and ultrastructural features of patients C, D, G and I. On haematoxylin and eosin staining (A, B, C, D), there were myopathic features, increased variation in fibre size with excess small fibres, and vacuoles. On Gömöri trichrome staining (E, F, G, H), there was accumulation of mitochondria in the vacuolated fibres and there were some ragged-red fibres. Lipid staining with oil red O (I, J, K, L) showed marked excess lipid in the vacuolated fibres. SDH histochemistry showed fibres with increased staining (M, N, O, P). Cytochrome oxidase showed frequent negative fibres in all four patients (Q, R, S, T). Electron microscopy was available in patients D, G and I and showed clusters of enlarged atypical mitochondria (U, V, W). Nemaline rods were also present (not illustrated) in patients

G and I. Patient D had two muscle biopsies 4 months apart, the second taken after a low-fat diet. The two biopsies were similar except for reduced lipid following the low-fat diet. All the images of this patient show the first biopsy except for panel N, which is from the second. The table summarises muscle biopsy findings for 8/9 new patients for whom this was available. Abbreviations: CoQ Coenzyme Q, COX cytochrome c oxidase, EM electron microscopy, NADH reduced nicotinamide adenine dinucleotide, ND Not done, RRF ragged-red fibre, RBF ragged-blue fibres, SDH succinate dehydrogenase.

Figure 3 Kaplan-Meier Curve of survival of the 31-patient cohort of RRM2B-associated MDDS.

The figure shows survival of 31 cases including 9 new cases from this cohort and 22 previously published cases. 3-month survival was 64.5%, 6-month survival was 29%, 1-year survival was 16%, 2-year survival was 9.6%.