

Title: Effects of nicotinamide riboside in mitochondrial DNA disease

Authors: Katherine R. Schon^{1,2,3*}, Alison Sleigh^{2,4*}, Zoe Golder^{1,2}, Alessandro Berghella^{5,6}, May Yung Tiet², Camilla Lyons^{1,2}, Lyuba V. Bozhilova^{1,2}, Yu Nie^{1,2}, Emma Harrison², Chloe Seikus², Patrick Yu-Wai-Man^{2,7,8,9}, Annelies Quaegebeur², Andreas Roos^{10,11,12}, Andreas Hentschel¹³, Heather Biggs², Massimo Zeviani^{14,15}, Carlo Viscomi^{16,17}, Jelle van den Ameele^{1,2}, Rita Horvath², Patrick F. Chinnery^{1,2}

Affiliations:

¹ Medical Research Council Mitochondrial Biology Unit, University of Cambridge; Cambridge, CB2 0XY, UK.

² Department of Clinical Neurosciences, University of Cambridge; Cambridge, CB2 0QQ, United Kingdom

³ Department of Genomic Medicine, University of Cambridge; Cambridge, CB2 0QQ, United Kingdom

⁴ Institute of Metabolic Science Metabolic Research Laboratories, University of Cambridge; Cambridge, CB2 0QQ, United Kingdom

⁵ Department of Biomedical and Neuromotor Sciences (DIBINEM), University of Bologna; Bologna, Italy

⁶ Sidra Medicine, Doha, Qatar

⁷ Cambridge Eye Unit, Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust; Cambridge, CB3 0QQ, United Kingdom

⁸ Moorfields Eye Hospital NHS Foundation Trust; 162 City Road, London, EC1V 2PD, United Kingdom

23 ⁹ Institute of Ophthalmology, University College London; 11-43 Bath St, London, EC1V
24 9EL, United Kingdom

25 ¹⁰ Department of Pediatric Neurology, Centre for Neuromuscular Disorders, Centre for
26 Translational Neuro- and Behavioral Sciences, University Duisburg-Essen; Essen 45147,
27 Germany.

28 ¹¹ Brain and Mind Research Institute, Children's Hospital of Eastern Ontario Research
29 Institute; Ottawa, ON K1H 8L1, Canada.

30 ¹² Department of Neurology, Medical Faculty and University Hospital Düsseldorf, Heinrich
31 Heine University; Düsseldorf 40225, Germany.

32 ¹³ Leibniz-Institut für Analytische Wissenschaften -ISAS- e.V.; 44227 Dortmund, Germany

33 ¹⁴ Professor of Neurology, University of Padova, Department of Neurosciences, Padova,
34 Italy

35 ¹⁵ Scientific Director of the IRCCS Maternal-infantile Institute “Burlo Garofolo” Trieste,
36 Italy.

37 ¹⁶ Veneto Institute of Molecular Medicine (VIMM), via Orus 2-35129 Padova, Italy

38 ¹⁷ Department of Biosciences, University of Milan; via Celoria 26-20133 Milano, Italy

39

40

41 * These authors contributed equally

42 Correspondence: pfc25@cam.ac.uk

43

44

45

46

47 **Background:**

48 Mitochondrial disorders are a common cause of inherited neurometabolic disease affecting
49 around 1 in 8000 people. There are currently no treatments for heteroplasmic mitochondrial
50 DNA (mtDNA) disorders which target the underlying disease mechanisms. Increasing
51 nicotinamide adenine dinucleotide (NAD⁺) with nicotinamide riboside (NR) has been
52 proposed as a therapeutic strategy. It was effective in several models of impaired oxidative
53 metabolism but has not been tested before in humans.

54 **Methods:**

55 We performed an experimental medicine study in eight participants affected with mtDNA
56 disorders. Three had single large-scale mtDNA deletions and five had m.3243A>G/T
57 pathogenic variants. They were treated with 4-weeks of NR (1250-2000 mg/day). Clinical
58 outcomes (timed up and go test, six-minute walk test, grip strength and quality of life
59 questionnaires), GDF15 and FGF21 blood biomarkers, ³¹P-magnetic resonance spectroscopy
60 measurement of mitochondrial function *in vivo* and quadriceps muscle biopsy were
61 performed before and after treatment. Muscle histopathology, mtDNA copy number,
62 heteroplasmy level, citrate synthase and respiratory complex activities were analysed, as well
63 as transcriptomic, proteomic and metabolomic analyses.

64 **Results:**

65 NR was bioavailable and well tolerated. Overall, NR improved the timed up and go (TUG)
66 test. In three participants with single large-scale deletions, there was a decrease in the
67 percentage of cytochrome *c* oxidase deficient and ragged-red muscle fibres, an increase in
68 respiratory chain complex I and IV activity, and reduction in mtDNA deletion levels which
69 was not seen in five participants with m.3243A>G/T. Unbiased transcriptomic and integrated

70 multi-omic analysis of all eight participants showed the signature of mitochondrial
71 biogenesis, with SIRT1/PGC-1 α activation and increased mitochondrial gene expression,
72 enhanced NAD-related metabolite levels, and coordinated changes in mitochondrial protein
73 abundance.

74 **Conclusions:**

75 We conclude that NR has potential to be the first small-molecule precision treatment for
76 heteroplasmic mtDNA disorders guided by the specific genetic diagnosis.

77 **Trial registration** The study was prospectively registered on ClinicalTrials.gov,
78 NCT03432871, Registration date 2018-02-14

79

80 **Keywords:** mitochondrial disease, mtDNA deletions, Nicotinamide Riboside, NAD⁺,
81 treatment, mitochondrial biogenesis

82

83

84

85 **Background**

86 Primary mitochondrial disorders are a common cause of inherited neurometabolic
87 disease affecting ~ 1:8000 of the population (1). Multiple tissues and organs are often
88 involved, including the brain, neuromuscular system, vision, hearing, and cardiac muscle (2).
89 Together these lead to progressive disability which impacts the quality (3) and duration of
90 life, with many patients dying prematurely despite the best supportive care (4). The principle
91 biochemical defect involves oxidative phosphorylation (OXPHOS), to maintain redox
92 (NAD⁺/NADH) balance and synthesise adenosine triphosphate (ATP), which are required
93 for all active cellular processes including protein synthesis and cell division (5). OXPHOS is
94 the final common pathway for oxidative energy metabolism and thus cannot be by-passed,
95 making it very challenging to develop new therapies for mitochondrial diseases. Although
96 there has been some progress for the tissue- specific disorder, Leber hereditary optic
97 neuropathy(6), there are no licenced treatments for the majority of patients (7). Gene therapy
98 approaches provide some hope, but genetic complexity is a major hurdle to progress (8).
99 Mitochondrial disorders can be caused by >350 different nuclear genes (9), >100 different
100 point mutations of mitochondrial DNA (mtDNA) (10), and many different mtDNA deletions
101 (11), making a comprehensive gene targeted approach highly unlikely in the foreseeable
102 future. For mtDNA mutations, there is the added complexity of heteroplasmy (12), with
103 different proportions of mutant and wild-type mtDNA in different cells and tissues. There is
104 therefore a pressing need to develop new treatments by modulating the downstream sequelae
105 of the huge range of different genetic causes.

106 Patients with mitochondrial diseases show an enhanced capacity for mitochondrial
107 proliferation and it has been proposed that inducing mitochondrial proliferation (biogenesis)
108 might be an effective treatment (13). NAD⁺ regulates mitochondrial biogenesis by acting as a
109 substrate for the enzyme Sirtuin1, an NAD⁺ dependent protein deacetylase (14,15). Increased
110 Sirtuin 1 leads to an increased amount of the deacetylated, active form of peroxisome

proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1 α) and an upregulation of genes for mitochondrial biogenesis and oxidative phosphorylation, making this an attractive target for therapy (16,17). Boosting NAD⁺ with Nicotinamide Riboside (NR), a natural NAD⁺ precursor, improved mitochondrial function in several models of impaired oxidative metabolism (14,18–23), slowing progression and delaying death, but this approach has not been systematically studied in humans. Whilst the related B3 vitamin, Niacin, has been shown to correct systemic NAD⁺ levels in five patients with a mtDNA deletion disorder (24), there were no objective clinical improvements, so the significance of these findings is unclear. NR itself has not been systematically studied in patients with mitochondrial disease to date. Here we describe an experimental medicine study of NR in eight patients with mtDNA disease. We show the treatment is well tolerated and provide preliminary evidence of both biochemical and clinical efficacy, particularly in patients with large-scale mtDNA disorders.

Methods

Study Design

We aimed to recruit ten participants to a 4-week open-label experimental medicine study to determine whether NR (10mg/kg bd) induces mitochondrial biogenesis and affects mitochondrial function. An overview of the study design is shown in **Figure 1A**. The dose of NR was chosen based on pharmacokinetic data in healthy individuals (25,26). The study was performed in the UK at the Cambridge Clinical Research Centre. Data from the SCO2 mouse model (14) suggested that effect sizes of 1.5 standard deviations or greater are possible across the outcomes of interest such as transcript expression profile of mitochondrial-related genes. On this basis, nine evaluable participants would be sufficient to

135 demonstrate superiority of Nicotinamide Riboside with 80% power and at a significance
136 level of 0.05.

137 *Eligibility Criteria*

138 *Inclusion criteria:*

- 139 • Clinical and genetic diagnosis of:
 - 140 ○ Progressive external ophthalmoplegia (PEO) caused by a single deletion of
 - 141 mitochondrial DNA
- 142 • or
 - 143 ○ Mitochondrial disease caused by the *m.3243A>G* or *m.3243A>T* mutation in
 - 144 mitochondrial DNA
- 145 • Age 18-70 years
- 146 • Women of child-bearing age only if they were not pregnant or breast feeding on
- 147 inclusion and agreed not to become pregnant during the study. Female participants had a
- 148 pregnancy test before study commencement. Participants agreed to take appropriate
- 149 contraceptive methods (intrauterine device, barrier methods).
- 150 • Signed informed patient consent

151

152 *Exclusion criteria:*

- 153 • Clinically significant liver disease (e.g., cirrhosis or a history of hepatitis)
- 154 • Presence of significant other neurological disorders (such as multiple sclerosis,
- 155 Parkinson's disease) or major co-morbidities (such as definite cognitive impairment,
- 156 psychiatric disease, heart, or lung failure, orthopaedic or rheumatological disorders)
- 157 • Females at risk of pregnancy (i.e., not using regular contraception), who were pregnant,
- 158 lactating or planning pregnancy

- Females taking the combined oral contraceptive pill (as oestrogens can induce mitochondrial biogenesis and interfere with study results)
- For MRI – medical contraindication e.g., pacemaker, deep brain stimulator, aneurysm clip or significant claustrophobia
- For biopsy – varicose veins overlying biopsy site, clinically significant lower limb oedema, active lower limb infection, use of anticoagulants or antiplatelet agents that cannot be discontinued, known bleeding diathesis
- Abnormal liver function tests (LFTs) in screening bloods (outside normal reference ranges for the local laboratory)
- Random plasma glucose greater than 20mMol/L in screening bloods (indicating poorly controlled diabetes mellitus)

Selection of Endpoints

Primary endpoints were measures of mitochondrial biogenesis from quadriceps muscle biopsy samples: citrate synthase and mitochondrial DNA copy number and phosphocreatine recovery time (kPCr) on ³¹P-MRS. Secondary endpoints were the clinical outcome measures – six-minute walk test, timed up and go test, SF-36 questionnaires.

Nicotinamide Riboside

Nicotinamide Riboside chloride was supplied free of charge by Niagen Bioscience through the ChromaDex external research programme in the form of 250mg NIAGEN ® capsules.

Safety Monitoring

Clinical observations were checked at each visit (pulse, blood pressure, temperature and weight). Pregnancy testing was also carried out if applicable. A study physician reviewed the participant's medical history including adverse events and changes to concomitant

medications. The muscle biopsy site was also reviewed at each visit. Weekly safety bloods included ALT, AST, Creatinine, and total CK. Urine was tested weekly for the presence of myoglobin. Participants were additionally followed up a week after their final visit by telephone and e-mail.

Patient and Public Involvement/Engagement

We will produce lay summaries of the results, and we will work with the Lily foundation (the foremost mitochondrial patient charity in the UK) to produce accessible content to publicise the results which will be available online.

NR/NAD⁺ measurement

Blood samples were collected in acid citric dextrose (ACD) tubes and snap frozen. Measurement of NR/NAD⁺ was completed by Keystone Bioanalytical Ltd, Pennsylvania, USA using LC-MS-MS (combining liquid chromatography with tandem mass-spectrometry). Levels of NR/NAD⁺ were measured in participant NR006 at weeks 0, 1, 2, 3 and 4 in whole blood – citric acid.

Biochemical analysis

Screening bloods included the following standard analyses: total creatinine kinase (CK), aspartate transferase (AST), creatinine, urea, electrolytes, gamma-glutamyl transferase (GGT), liver function tests (alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin, albumin) and non-fasting glucose. The fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15) levels were measured each week. These assays were performed by the NIHR Core Biochemistry Assay Laboratory, Cambridge Biomedical Research Centre. FGF21 was measured by enzyme linked immunosorbent assay (kit manufacture by R&D Systems and distributed in the UK by BioTechne, Abingdon, Oxford - product code DF2100). GDF15 was measured using an in-house chemiluminescent

207 immunoassay on the MesoScale Discovery assay platform. Monoclonal anti-GDF-15
208 antibodies were purchased from ANSH (Webster TX, USA).

209 *Six-Minute Walk Test (6MWT)*

210 Participants were assessed at weeks 0 and 4. They were asked to walk at their normal pace to
211 see how far they could walk in six minutes. A 30m track was set up in the clinical research
212 facility using cones to mark the turning points. Standardised instructions and
213 encouragements were used. The number of laps walked was recorded and the excess distance
214 was measured using a tape measure.

215 *Timed up and go (TUG)*

216 Participants were assessed at weeks 0 and 4. Participants were asked to sit in a standard chair
217 (seat height 46cm, arm height 67cm) and walk to a line 3m away and back. Walking aids
218 could be used if required. Standardised instructions were used: “*When I ask you to start, I*
219 *would like you to get up out of this chair and walk (With your stick/frame) at a comfortable*
220 *and safe pace to the mark on the floor which is 3 meters away. Cross the line, turn round*
221 *and return to the chair and sit down again*”.

222 Participants were timed from being asked to start until they returned to a seated position.
223 They were asked to wear comfortable clothing and footwear and rest for 15 minutes before
224 the test.

225 *Dynamometric measure of grip strength*

226 Grip strength was measured using a T.K.K.5401 GRIP D Grip strength dynamometer.
227 Participants were asked to squeeze the dynamometer as hard as they could while standing
228 upright with their arms down to measure grip strength. A measurement was taken for the
229 right hand and then the left hand, and then a repeat measurement for the right hand and left
230 hand.

231 *Quality of Life Scale SF-36*

232 Participants were assessed at weeks 0 and 4. They were asked to fill in a questionnaire about
233 their symptoms and quality of life. The 36-Item Short Form Health Survey (27) was
234 developed in 1992 by the RAND corporation and has been widely used in clinical studies.

235 *Adherence to study medication*

236 Adherence was assessed by participant diaries and by counting the number of tablets
237 remaining at each study visit.

238 *³¹P-magnetic resonance spectroscopy measurement of mitochondrial function in vivo*

239 Participants were asked to perform simple leg exercises by lifting and extending their leg
240 with a weight on their right ankle. Maximal voluntary contraction (MVC) was measured at
241 the screening visit using a dynamometer chair. Three attempts were done and the mean of the
242 second and third attempt was used. The ³¹P-MRS acquisition and analyses were performed
243 as described in Sleight et al (28) with the following exceptions:

244 - the coil was 11 cm (not 9cm) in diameter. It was a ³¹P-1H flex coil made by RAPID
245 Biomedical.

246 - the dynamometer used isometric (not isokinetic) exercise to determine maximum voluntary
247 contraction (MVC). MVC was recorded and the weight for exercise in situ calculated and
248 standardised in order to achieve a target phosphocreatine (PCr) depletion of 20-30% of basal
249 [PCr].

250 *Muscle biopsy*

251 Needle biopsy of the left quadriceps was performed with ethical approval and patient
252 consent. Muscle biopsy samples were split into four pieces – three pieces were snap frozen

in liquid nitrogen within 15 minutes and stored at -80 degrees. The remaining piece was taken to the tissue bank within 30 minutes.

Histological analysis

COX SDH and Gomori Trichrome staining were performed in the Cambridge University Hospitals NHS Foundation Trust human research tissue bank. Muscle biopsy sections were analysed by a consultant histopathologist (AQ) who was blinded to the participant ID and to whether the samples was before or after treatment. Density (%) of ragged red fibres, COX negative fibres and COX intermediate fibres were assessed. Density (%) of COX deficient fibres was calculated by adding the densities of COX negative and COX intermediate fibres. Density of regenerating muscle fibres was assessed from hematoxylin and eosin-stained sections.

DNA and RNA extraction

DNA and RNA were extracted from skeletal muscle biopsy samples (quadriceps) using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen 80224) and the concentration measured on the NanoDrop spectrophotometer (Thermo Scientific).

Quantification of levels of heteroplasmy/Pyrosequencing

The mitochondrial genome spanning the 3243 region was amplified from extracted DNA using the PyroMark PCR Kit (QIAGEN) and site specific primers (29). After confirming amplification, heteroplasmy was measured on the Q48 Autoprep and analysed on the PyroMark analysis software following the manufacturer's protocol.

Mitochondrial DNA Copy Number and deletion heteroplasmy/Digital PCR

mtDNA copy number and deletion levels were determined by droplet digital PCR (30) targeting the mitochondrially encoded *ND1* and *ND4*, and nuclear RNaseP genes. Droplet analysis was performed using the QX manager software (Bio-Rad).

277 *Deep sequencing and variant analysis/ NGS*

278 For high-depth mtDNA sequencing two overlapping amplicons were amplified per sample
 279 (31). Amplification was confirmed and the product bead purified, quantified, and pooled in
 280 equimolar amounts. Libraries were prepared (NEB E7805L and E7600) and sequenced using
 281 a 2 × 250-cycle MiSeq Reagent kit v3.0 (Illumina). FASTQ files generated were checked for
 282 quality using FastQC v0.11.5. After trimming, variant calling was performed with the
 283 MToolBox pipeline (32). mtDNA structural changes (deletions, mtDel; and duplications,
 284 mtDup) were determined using MitoSAlt (33). The filtering features include: cluster
 285 threshold = 5 (the minimum number of reads supporting a cluster); break threshold = 5 (the
 286 maximum deviation of split read breakpoints that have to be considered within the same
 287 cluster); hplimit = 0.01 (detected heteroplasmy threshold); deletion threshold min = 30 (the
 288 minimum size of the gap between fragments of a split read for the split read to be considered
 289 as potentially spanning a deletion); breakspan = 10 (the minimum number of bases a non-
 290 split read must span either side of a breakpoint to be considered in the heteroplasmy count);
 291 split distance threshold = 5 (the maximum length of unmapped distance between two
 292 fragments of a split read); score threshold = 30 (alignment score cut-off); eval
 293 threshold = 0.00001 (alignment eval cut-off).

294 *Respiratory chain enzyme and citrate synthase activity*

295 Respiratory chain complex I and IV activities, citrate synthase activity and protein levels
 296 were measured in muscle using the method described by Hargreaves *et al* (34). This analysis
 297 was performed by Mitochondrial Biochemistry at the Neurometabolic Unit, London NHS
 298 Highly Specialised Service for Rare Mitochondrial Disorders.

299

300 *Gene expression analysis*

RNA integrity was checked using Tapestation (Agilent). Samples were submitted to the Wellcome-MRC Cambridge Stem Cell Institute for library preparation and sequencing on a Novaseq 6000 SP flowcell with 150 bp paired end reads. Sequenced reads were quality checked with FastQC (v.0.11.8), FastQ Screen (v.0.14.1) and QualiMap (v.2.2.1). Trimming was performed with Trim_galore (v.0.6.6) and reads were aligned with STAR (v.2.7.8a) to GRCh38 genome. Quality reports were compiled using MultiQC (v.1.12). Counts were generated with STAR. Differential expression analysis was performed in R, using DESeq2 (v.1.36.0), with the experimental design used: ~patient + treatment. Pathway analysis was performed using enrichR 3.2. Genes were selected based on significance (adjusted p-value < 0.05, Benjamini-Hochberg correction) and positive logarithmic fold change. We chose three reference databases: GO_Biological_Process_2018, GO_Cellular_Component_2018 and GO_Molecular_Function_2018, to search for enriched terms. The significance threshold for enriched terms was set to 0.05 for the adjusted p-value.

Proteomic Analysis

The sample preparation, measurement and analysis was performed as previously described (35). A two-sided t-test was carried out with Microsoft Excel. P-values were adjusted for multiple hypothesis testing using the Benjamini-Hochberg correction. To control for possible changes in mitochondrial abundance, proteins with evidence of mitochondrial localisation as defined by the MitoCarta 3.0(36) list were subsetted and renormalised. The differential expression analysis was repeated for this subset only.

Targeted Metabolomic Analysis

Sample preparation, quality assurance/quality control and ultrahigh performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) were performed by UVic-Genome BC Proteomics Centre, Victoria, Canada, as well as compound identification, quantification and data curation. A total of 220 different metabolites were measured on

human muscle samples from four participants before treatment (0 weeks) and after 4 weeks of treatment. The metabolites analysed were sugars and organic acids, polar metabolites in the folate cycle, methionine cycle and transsulfuration pathways and NAD related metabolites, phosphorylated metabolites and carnitines. The assay of organic acids used a chemical derivatization liquid chromatography-multiple reaction monitoring/mass spectrometry (LC-MRM/MS) method previously described (37–39) and the assay of carnitines used a chemical derivatization LC-MRM/MS method previously described (40) with necessary modifications.

Multi-omics analyses

Multi-omics analyses were performed in R (version 4.2.2) using the mixOmics package (version 6.16.3) (41). Multi-omics integration was performed using the DIABLO method (Data Integration Analysis for Biomarker discovery using Latent cOmponents), which employs sPLS-DA (sparse Partial Least Squares Discriminant Analysis) to extract latent components that maximize correlation across omics datasets.

The model was run with two components to capture the most informative features related to the response to NR treatment. A sparse feature selection strategy was applied, retaining 10 variables per component for each omics dataset. This approach helped to reduce model complexity and focus on the most relevant features, improving interpretability of the results.

Protein–protein interaction network analysis was performed using the STRING web server (42), which provides known and predicted interactions based on multiple evidence types. Pathway enrichment analysis of metabolomics data was conducted using MetaboAnalyst (43), enabling the identification of enriched metabolic pathways associated with NAD-related metabolites. Pearson's correlation was calculated across all the three omics datasets. The p values were corrected for multiple testing using the Benjamini-Hochberg procedure. Gene set enrichment analysis was performed using the top 20 features (genes/proteins) with the highest absolute loadings on component 1, which drives sample separation in the sPLS-DA model.

Statistical analysis

Statistical analyses were performed in R version 4.2.2. The Wilcoxon signed-rank test (two sided) was used for paired data. A standard threshold for significance of $p < 0.05$ was used for clinical outcome measures. For the differential expression analysis, the Wald test was used to compare the expression of each gene before and after 4 weeks of NR, and p-value correction was done using Benjamini-Hochberg.

Approvals, funding, sponsorship and consent

The study was prospectively registered ClinicalTrials.gov (NCT03432871, registration date 2018-02-14) and the study was approved by the Health Research Authority Committee East of England Cambridge South (REC Ref 17/EE/0120). The sponsor was the University of Cambridge and Cambridge University Hospitals NHS Foundation Trust. Informed consent was obtained from all participants. The research conformed to the principles of the Helsinki Declaration. The study was funded through a UK MRC Confidence in Concept award to the University of Cambridge. The funder did not have a role in the design, conduct, analysis and reporting of the study.

Timeline

The first participant enrolled on 20th September 2018. The last patient enrolled on 10th May 2022 and completed the study on 17th June 2022. All samples were analysed after the final participant completed the study. Respiratory chain enzyme and citrate synthase results were received on 25th November 2022. Next generation sequencing of mitochondrial DNA was completed on 30th November 2022. Mitochondrial DNA copy number analysis was completed on 10th January 2023. Clinical data was collated on 11th January 2023. GDF-15 and FGF-21 results were received on 24th January 2023. Analysis of ³¹P-MRS data was completed on 27th January 2023. RNA sequencing results were received for analysis on 11th April 2023. Analysis of the histology was completed on 11th August 2023. Metabolomic

results were received on 01st May 2024 and proteomic results were received on 10th May 2024.

Results

Safety, tolerability, and target engagement

Eight participants were recruited from the Cambridge and London mitochondrial clinics with a genetically confirmed heteroplasmic mitochondrial disorder: four with m.3243A>G, one with m.3243A>T and three with single large-scale mtDNA deletions (mt-Del). Overall, five were male and three were female with a median age of 53.5 years (range 21-68 years) (**Table 1**). On meeting screening criteria, participants underwent baseline clinical testing (**Figure 1A, Sup Table S1**) before receiving NR 10mg/kg twice daily for four weeks (2000mg/day n=3; 1250mg/day n = 5). Tablet counts and patient diaries found 7 out of 8 participants adhered fully to the regimen. One participant missed two evening doses. A total of 446 doses were taken out of 448 expected, meaning a 99.5% adherence to the dosing regimen. All eight participants completed the study.

New onset ‘heartburn’ (dyspepsia) was noted by one participant on commencing NR. Other reported adverse events included constipation, diarrhoea, cough, mouth ulcers, palpitations, abnormal sensation on the left leg and urinary frequency: each was reported once, were described as mild and not considered likely to be due to the NR (**Sup Table S2**). The dyspepsia was treated by the participant with over-the-counter Gaviscon. There were no other changes to concomitant care or medications. No new abnormalities were observed in the weekly safety blood tests and no myoglobinuria was detected. There was no significant change in the participants’ weight, body mass index (BMI) or blood pressure during the

study. Blood NAD⁺ and NR were measured in one participant (NR006) at baseline and each week confirming bioavailability and target engagement (**Figure 1B and 1C**).

Muscle histopathology, OXPHOS biochemistry and molecular genetics

Baseline muscle histopathology fell into two groups reflecting the underlying genetic diagnosis (**Figure 2**): participants with m.3243A>G/T had a median of 3.60% cytochrome c oxidase (COX) deficient muscle fibres and a median of 0.451% ragged-red fibres (RRF), whereas participants with single large-scale mtDNA deletions (mt-Del) had a median of 12.3% COX-deficient fibres and a median of 0.256 % RRF at baseline. This difference was also reflected in the muscle OXPHOS biochemistry (**Fig.3A-C**). Given that the potential for improvement in these parameters was strikingly different between the two main genetic groups, we highlight the difference in the subsequent data presentation.

After NR treatment, the number of COX-deficient fibres was lower in all three mt-Del patients. The RRF fell from 6.47% to 2.29% in one mt-Del patient and remained at a low level (<1%) in the other two. There was a corresponding increase in median complex I and IV activity normalised to CS as a marker of mitochondrial mass, and substantial increase in citrate synthase activity in one mt-Del patient (**Fig.3A-C, Sup Figure S1**). This was accompanied by an increase in the total amount of skeletal muscle mtDNA in two out of three (**Fig.3D**) and a decrease in the proportion of mutant mtDNA (**Fig. 3E-G, Sup Figure A1, Sup Table S3**). The absolute amount of wild type mtDNA increased in two deletion patients where this could be calculated (mean zero = 1087, mean week 4 = 2001). The same trends were not seen for m.3243A>G/T (**Sup Figure 2**), and overall, the results did not reach statistical significance (**Sup Table S4**).

In vivo biomarkers of mitochondrial function

In vivo mitochondrial function was assessed by ³¹P-magnetic resonance spectroscopy (³¹P-MRS) to determine the phosphocreatine (PCr) recovery rate constant, kPCr, in

quadriceps femoris (opposite side the biopsy). The median kPCr increased in the mt-Del group, but not in m.3243A>G/T participants (**Fig. 3H, Sup Fig E**), mirroring the muscle histopathology, OXPHOS biochemistry, and molecular genetic data.

Two proposed serum biomarkers of mitochondrial disease severity, fibroblast growth factor 21 (FGF-21)(44,45), and growth and differentiation factor 15 (GDF-15)(46), showed a trend towards decreasing in both mt-Del and m.3243 A>G/T participants, although results did not reach statistical significance. (For the whole cohort of eight participants, FGF21: median baseline = 329 pg/ml, IQR 107 pg/ml; median week 4 = 267 pg/ml, IQR 115 pg/ml; $P = 0.11$. GDF15: median baseline = 2016 pg/ml, IQR 1235 pg/ml; median week 4 = 1994 pg/ml, IQR 1221 pg/ml; $P = 0.38$; **Fig 3I, 3J, Sup Fig 1F, 1G**).

Clinical outcomes and quality-of-life assessments

Clinical outcomes and quality-of-life assessments are reported for the whole cohort of eight participants. The timed up and go improved after NR treatment (TUG: median time zero = 11.9s, IQR 7.51s; median week 4 = 9.96s, IQR 7.39; $P = 0.04$, **Fig. 3K, Sup Fig 1H**). However, there was no similar trend for the six-minute walk test (6MWT: median time zero = 360m, IQR 158m; median week 4 = 352m, IQR 172m; $P = 0.58$, **Fig 3L, Sup Fig 1I**), or dynamometric measure of grip strength (maximum grip strength: median strength zero = 24.2kg, IQR 12.3kg, median week 4=24.3kg, IQR 18.6kg, $P=0.20$). The SF-36 quality of life score also did not change (SF-36: median time zero = 101, IQR 3; median week 4 = 94.5, IQR 9.5; $P = 0.16$). There were no obvious genotype correlations for the clinical outcomes.

Skeletal muscle transcriptomic, proteomic and metabolomic analysis

We first performed individual statistical analyses on the transcriptomics, proteomics, and metabolomics datasets to identify differentially regulated molecules. Transcriptomic and proteomic analysis was performed in all eight individuals. Transcriptomic analysis identified

sixty-two differentially expressed genes in skeletal muscle after 4-weeks of NR treatment (adjusted p -value <0.05). Genes encoding mitochondrial proteins (MitoCarta 3.0 (36)) were 4.3-fold enriched in the upregulated group (55 genes, **Sup Fig S3, Sup Table S5**), including: *UQCRC1*, *SLC25A3*, *ATP5F1D*, *MT-CO3*, *NDUFB5*, *GOT2*, *GATM*, *SDHD*, *NIPSNAP3B*, *COX5A*, *CHCHD10*, *VDAC3*, *MT-ND3*, *MT-CO1*, *FTH1* and *GLRX5*. Up-regulated non-mitochondrial genes included *CKM* (creatine kinase-M type). None of the 7 down-regulated genes were predicted to code for mitochondrial proteins (36) (**Sup Table S6**). Pathway analysis using GO cellular components showed that the most enriched terms corresponded to the mitochondrial (MT) inner membrane, MT respiratory chain complex IV, MT respiratory chain complex I, apical dendrite and mitochondrion. The most enriched GO biological processes were respiratory electron transport chain, MT ATP synthesis coupled electron transport, muscle contraction, MT electron transport- NADH to ubiquinone, and skeletal muscle tissue development (**Sup Fig S4**).

In contrast, proteomic analysis of skeletal muscle tissue revealed no significantly regulated proteins after 4-weeks of NR treatment after false discovery rate correction for multiple hypothesis testing. 367 proteins in the dataset matched the MitoCarta 3.0 list(36). The differential expression analysis on this subset revealed increased abundance of three nominally significant proteins ($p < 0.05$) — MRPS36, PCCA, and NAXD — and decreased abundance of one, ISOC2, following NR treatment (**Sup Fig. S5**).

Targeted metabolomic analysis of skeletal muscle tissue was performed in four individuals. It was not performed in the other three because they had no sample remaining after the other analyses. There was a significant increase in key metabolites, including 2,3-Diphosphoglyceric acid, Nicotinamide N-oxide, 4PY (N^1 -methyl-4-pyridone-3-carboxamide), and 2PY (N^1 -methyl-2-pyridone-5-carboxamide), which were upregulated following NR treatment (**Sup Fig S6, Sup Fig S7A, Sup Table S7**).

Integrated multi-omics analysis

Building on these individual analyses, we proceeded to perform an integrated multi-omics analysis combining transcriptomic, proteomic, and metabolomic data using the DIABLO framework. This integration revealed a strong correlation (0.86) between the transcriptomic and proteomic datasets (**Fig. 4A**), suggesting a coordinated biological effect across these two omics layers driven by NR treatment. To further support this, we visualized

the individual omics datasets along the two principal components (**Fig. 4B**), which showed an alignment between the transcriptomic and proteomic data.

Most of the proteins involved in the separation linked to the myogenesis pathway and are associated with the Z-disc in striated muscle cells, alongside two mitochondrial proteins, FAM162A and Mitochondrial Antiviral Signalling Protein (MAVS), which were downregulated and upregulated, respectively. However, the top-ranked protein driving the separation was Nexilin (NEXN), which was downregulated following NR treatment (**Fig. 4C**). Notably, protein interaction network analysis showed there is strong evidence for a functional relationship between NEXN and MIGA1, a mitochondrial protein involved in mitochondrial fusion and energy metabolism (STRING score 0.716). In the transcriptomic data, the largest contribution came from the downregulation of Long Intergenic Non-Protein Coding RNA 664 (**Fig. 4D**), however its specific function is not yet fully understood. The top ten features contributing to component 1 of the diablo model are shown in **Sup Fig S7B-D** and the Pearson correlations across the transcriptomic, proteomic and metabolomic datasets are shown in **Sup Fig 8**.

Given previous observations, we speculated that omics correlation scores might be influenced by the underlying genetic diagnosis, also reflected in the histopathological differences between m.3243A>G/T and mtDNA deletions patients. Sub-group analysis revealed a stronger correlation between the omics datasets analysed separately (0.99 for SNV and 0.97 for Deletion) (**Sup Fig S9A-B**), compared to the group analysis (0.86). While these results suggest potentially distinct molecular responses between genotypes, this could also reflect the limited sample size. Gene set enrichment analysis was performed based on the top 20 features (genes/proteins) with the highest absolute loadings on component 1, which drives sample separation in the sPLS-DA model (**Sup Fig S10**).

Although the single-omics analysis did not identify any significantly regulated proteins after multiple testing correction, the integrated analysis highlighted biologically relevant proteins including MAOA, NDUFAF2, and SCAF1 (**Sup Fig S9C-9D**), which were differentially modulated between groups and are associated with mitochondrial structure and NADH metabolism. In addition, differential modulation of Ras superfamily members, including RalA and R-Ras, was observed. Although not definitive due to the small number of participants, these findings suggest genotype-specific effects in response to NR treatment.

Finally, the inclusion of metabolomics data revealed a significant increase in NAD-related metabolites after NR treatment. Pathway enrichment analysis identified the upregulation of *Nicotinate and Nicotinamide Metabolism* and *NAD⁺ Biosynthesis* pathways (**Fig. 5A and 5B**), confirming bioavailability and on-target effects of the NR treatment regime used in this study which stimulates the NADH pathway, supporting transcriptomic and proteomic evidence that NR promotes mitochondrial bioenergetic activity (**Fig. 5C**). Altogether, this multi-omics exploratory approach supports a treatment effect of NR which is genotype-dependent and warrants further investigation.

Discussion

Here we report an experimental medicine study of the effects of NR 10mg/kg twice daily for one month in eight patients with mtDNA disease. NR treatment was well tolerated, and blood NAD⁺ and NR levels were in keeping with pharmacokinetic data in healthy individuals (25,26), confirming that our dosing regime was adequate as reflected in the metabolomic analysis. Considering all eight patients together, only one measure showed marginal evidence of a clinical treatment effect: the timed up and go (TUG) test. The TUG has gained favour in recent years because of strong inter- and intra-rater performance in a

wide range of clinical contexts. Our findings provide preliminary evidence that NR may be beneficial in mtDNA diseases. The main limitations of our study were the short duration and limited number of participants, which prevented robust statistical data. A larger randomised controlled trial is required to validate these findings before NR is used routinely to treat mitochondrial disorders.

Although the results of any *post hoc* analysis should be interpreted with caution, the striking difference in the severity of the muscle pathology at baseline pathology prompted us to highlight differences between mt-Del and m.3243A>G/T patients. After treatment with NR, several parameters improved in the mt-Del patients, including the percentage of COX deficient skeletal muscle fibres, the percentage of RRF, OXPHOS complex I and IV activity, and the percentage of mutant mtDNA in biopsy samples from the same skeletal muscle. Although these trends were only seen in three people, the fact that all of these measures change in the same direction is reassuring. When linked to the increase in CS activity and mtDNA content in skeletal muscle, these findings are consistent with the anticipated treatment effect with NR inducing mitochondrial biogenesis that has been seen in animal models of mitochondrial disease (14,18), including the mtDNA-deletor mouse (18). However, the decrease in mutation load implies additional mechanisms are activated in our human study.

NAD⁺ levels regulate the activity of the class III histone deacetylase SIRT1 which deacetylates FOXO1 to upregulate PGC1 α , the master regulator of mitochondrial biogenesis. In keeping with this, we saw upregulation of CS and mtDNA levels, indicating an increased mitochondrial mass skeletal muscle. SIRT1 also preferentially targets the acetylated lysine 16 of histone H4 (H4K16ac), which activates muscle satellite cell proliferation in part by upregulating the muscle specific transcription factor MyoD, promoting satellite cell fusion with existing muscle fibres. Satellite cells in patients with mtDNA mutations typically have a much lower mutation level than juxtaposed mature skeletal muscle fibres, particularly in

patients with single mtDNA deletions (47). This observation underpinned several experimental medicine studies aimed at activating satellite cells by damaging muscle with myotoxins (48) or eccentric exercise (49), leading to a reduction in the number of COX-deficient muscle fibers. Building on these combined observations, it is plausible that NR also ‘delivers’ wild-type mtDNAs to mature skeletal muscle through the activation and fusion of endogenous satellite cells, explaining why the mutation load decreased in the mt-Del patients. In keeping with this we observed slightly more regenerating muscle fibres after treatment, although this failed to reach formal statistical significance on group analysis, so a larger study is required to validate this finding and dissect out the underlying mechanism.

We recognise that the effects of NR may not be specific to mt-Del patients. One of the mouse models which responded to NR was the *Sco2* knock in/knock out mouse (14), a model for autosomal recessive complex IV deficiency due to biallelic pathogenic variants in the *SCO2* gene. It would be interesting to study the effect of NR in individuals with *SCO2* variants and other mitochondrial disorders caused by nuclear gene defects but these studies would be extremely challenging because each individual genetic cause is very rare, restricting opportunities to build cohorts. As noted above, the histological and biochemical abnormalities in the m.3243A>G/T muscle were mild, leaving little room for improvement. It is therefore possible that similar effects of NR may be seen in more severely affected individuals with a range of different mtDNA disorders. These observations are relevant for the design of future therapeutic studies in mitochondrial disorders: inclusion criteria will need to ensure participants have significant pathology (and disability) to maximise the possibility of detecting any treatment effect using these measures. This will be challenging for a rare disease, particularly when the most common genetic group (m.3243A>G) often have very mild symptoms.

Conclusions

This was an open-labelled study not designed to provide definitive evidence of a treatment effect of NR. However, our findings are encouraging, particularly in the more severe mt-Del subgroup, where consistent pattern of improvement was seen across several laboratory measures. In addition, the results of an unbiased transcriptomic and integrated multi-omic analysis showed evidence that NR upregulates the synthesis of mitochondrial in skeletal muscle, as anticipated from the expected mode of action. NR was well tolerated by all eight participants, endorsing a larger prospective randomised controlled trial to determine whether this approach should be used as a treatment for mtDNA disorders.

List of abbreviations

6MWT	Six minute walk test
ALT	Alanine Transaminase
ALP	Alkaline Phosphatase
ATP	Adenosine triphosphate
CK	creatine kinase
COX	cytochrome c oxidase
CPEO	chronic progressive external ophthalmoplegia
CS	Citrate synthase
DIABLO	Data Integration Analysis for Biomarker discovery using Latent cOmponents
FGF-21	Fibroblast Growth Factor 21
GDF-15	Growth and Differentiation Factor 15
GGT	Gamma-glutamyl Transferase

603	kPCr	phosphocreatine recovery rate constant
604	LC-MRM/MS	Liquid Chromatography-Multiple Reaction Monitoring/Mass Spectrometry
605	MVC	Maximum voluntary contraction
606	MRS	Magnetic resonance spectroscopy
607	Mt-Del	mitochondrial DNA deletion
608	Mt-Dup	mitochondrial DNA duplication
609	mtDNA	mitochondrial DNA
610	NAD ⁺	nicotinamide adenine dinucleotide
611	NR	Nicotinamide Riboside
612	OXPHOS	Oxidative phosphorylation
613	PCr	Phosphocreatine
614	PCR	Polymerase Chain Reaction
615	PGC-1 α	peroxisome proliferator-activated receptor-gamma coactivator 1-alpha
616	RRF	Ragged red fibres
617	SF36	36-Item Short Form Health Survey
618	SNV	Single nucleotide variant
619	sPLS-DA	sparse Partial Least Squares Discriminant Analysis
620	TUG	Timed up and go
621	UPLC-MS/MS	Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy
622	List of Supplementary Materials	

623 Tables S1 to S6

624 Figures S1 to S10

625 **Declarations**

626 **Ethics approval and consent to participate**

627 The study was approved by the Health Research Authority Committee East of England
628 Cambridge South (REC Ref 17/EE/0120). The sponsor was the University of Cambridge
629 and Cambridge University Hospitals NHS Foundation Trust. Informed consent was obtained
630 from all participants. The research conformed to the principles of the Helsinki Declaration.

631 **Consent for publication**

632 The participants' informed consent included consent for publication of the results.

633 **Availability of data and materials:**

634 RNA sequencing data has been deposited at the European Genome-phenome Archive
635 (EGA), which is hosted by the European Bioinformatics Institute and the Centre for
636 Genomic Regulation, under accession number EGAS50000001947. The mass spectrometry
637 proteomics data have been deposited to the ProteomeXchange Consortium via the
638 PRIDE(50) partner repository with the dataset identifier PXD083307.

639

640 **Competing interests:**

641 The authors have no relevant conflicts of interest. PFC is Executive Chair of the Medical
642 Research Council. He also has roles in the Royal College of Physicians, Royal College of
643 Pathologists, Academy of Medical Sciences and Royal Society. RH is an Associate Editor of
644 Brain. No other authors report competing interests.

645 **Funding:**

646 University of Cambridge's institutional MRC Confidence in Concept grant (PFC, MZ)

647 NIHR Clinical Lectureship (KS)

648 NIHR Cambridge Clinical Research Facility Grant number NIHR (203312) (AS)

649 Advanced Fellowship Award from the UK National Institute of Health Research (NIHR

650 301696 (PYWM)

651 Rosetrees Trust PGL23/100048 (PYWM, RH, PFC)

652 Fight for Sight UK (PYWM)

653 Isaac Newton Trust UK (PWYM)

654 Moorfields Eye Charity GR001376 (PYWM)

655 Addenbrooke's Charitable Trust (PYWM, KRS)

656 National Eye Research Centre UK (PYWM)

657 International Foundation for Optic Nerve Disease (PWYM)

658 Rare Diseases Translational Research Collaboration, the NIHR Cambridge Biomedical

659 Research Centre NIHR203312 (PYWM)

660 NIHR Biomedical Research Centre based at Moorfields Eye Hospital NHS Foundation Trust

661 and UCL Institute of Ophthalmology NIHR203322 (PYWM).

662 Deutsche Gesellschaft für Muskelkranke DGM (AR)

663 European Regional Development Fund ERDF; projects NME-GPS (AR, AH) and B2B-

664 RARE (AR)

665 Ministerium für Kultur und Wissenschaft des Landes Nordrhein-Westfalen (AH)

666 Der Regierende Bürgermeister von Berlin, Senatskanzlei Wissenschaft und Forschung (AH)

667 Telethon Foundation GGP20013 and GSP24003 (CV),

668 AFM-Téléthon 23706 (CV)

669 Associazione Luigi Comini Onlus (MitoFight2) (CV).

670 Wellcome Clinical Research Career Development Fellowship 219615/Z/19/Z (JvdA)

671 Wellcome Discovery Award 226653/Z/22/Z (JvdA, RH, PFC)

672 UKRI BBSRC Responsive Mode Research Grant BB/X00256X/1 (JvdA)

673 Core funding from the Medical Research Council (MRC) to the MRC Mitochondrial
 674 Biology Unit MC_UU_00028/8 (JvdA).
 675 Medical Research Council UK MR/V009346/1 (RH)
 676 Hereditary Neuropathy Foundation (RH)
 677 AFM-Telethon (RH)
 678 Ataxia UK (RH)
 679 Action for AT (RH)
 680 Muscular Dystrophy UK (RH)
 681 Horizon Europe Guarantee MSCA Doctoral Network Programme Project 101120256: MMM
 682 (RH)
 683 NIHR Cambridge Biomedical Research Centre BRC- BRC-1215-20014 (RH)
 684 Wellcome Collaborative Award 224486/Z/21/Z (PFC)
 685 Medical Research Council Mitochondrial Biology Unit MC_UU_00028/7 (PFC)
 686 Biological and Biotechnology Research Council BB/Y003209/1 (PFC)
 687 LifeArc Centre to Treat Mitochondrial Diseases (LAC-TreatMito) under grant no. 10748.
 688 LifeArc is a charity registered in England and Wales under no. 1015243 and in Scotland
 689 under no. SC037861 (KRS, PYWM, JvdA, RH, PFC)
 690 NIHR Cambridge Biomedical Research Centre (BRC-1215-20014) (PFC)

691

692 **Authors' contributions:**

693 MZ, CV, RH and PFC designed the study. AS designed the ³¹P-MRS measurement of
 694 mitochondrial function in vivo and analysed the data. KRS, MYT, CS, EH, HB, JvdA, RH
 695 and PFC carried out the clinical assessments. ZG performed DNA and RNA extraction,
 696 quantified levels of heteroplasmy and measured mtDNA copy number and deletion
 697 heteroplasmy. CL analysed the RNAseq results. YN analysed the m.3243A>G/T next

generation sequencing results and performed the MitoSAlt analysis of mtDNA deletions. AR and AH performed the proteomic analysis. AQ performed the histopathology analysis. LB performed statistical analysis and visualisation of the data. AB performed the integrated transcriptomic, proteomic and metabolomic analysis. PYWM, PFC, RH, JvdA and KRS recruited patients to the study. HB, EH and CS were responsible for project administration. MZ and PGC acquired funding for the study. PFC and RH supervised the study. PFC and KRS wrote the original draft. All authors read and approved the final manuscript.

Acknowledgements:

The authors wish to acknowledge the UVic-Genome BC Proteomics Centre, Victoria, Canada for targeted metabolomic studies, Niagen Bioscience for supplying Niagen®, nicotinamide riboside chloride (NR) for the study through the ChromaDex external research program, the NIHR Core Biochemistry Assay Laboratory, Cambridge Biomedical Research Centre for performing the FGF21 and GDF15 assays and the Mitochondrial Biochemistry, Neurometabolic Unit, London NHS Highly Specialised Service for Rare Mitochondrial Disorders for performing the respiratory enzyme analysis. The authors also thank Zoe MacIntyre for working on the study set up, Thiloka Ratnaike and Dominic O'Donovan for help with study visits, Malwina Prater for initial work on the transcriptomic analysis and Yuelin Yao for depositing the RNA sequencing data. The views expressed are those of the author(s) and not necessarily those of the NIHR or the Department of Health and Social Care.

References

1. Gorman GS, Schaefer AM, Ng Y, Gomez N, Blakely EL, Alston CL, et al. Prevalence of nuclear and mitochondrial DNA mutations related to adult mitochondrial disease. Ann

- 723 Neurol. 2015 May;77(5):753–9. doi:10.1002/ana.24362 PubMed PMID: 25652200;
724 PubMed Central PMCID: PMC4737121.
- 725 2. Chinnery PF, Hudson G. Mitochondrial genetics. *Br Med Bull.* 2013 Jun;106(1):135–59.
726 doi:10.1093/bmb/ldt017 PubMed PMID: 23704099; PubMed Central PMCID:
727 PMC3675899.
- 728 3. van de Loo KFE, van Zeijl NT, Custers JAE, Janssen MCH, Verhaak CM. A conceptual
729 disease model for quality of life in mitochondrial disease. *Orphanet J Rare Dis.* 2022 Jul
730 15;17(1):263. doi:10.1186/s13023-022-02411-9 PubMed PMID: 35841006; PubMed
731 Central PMCID: PMC9287990.
- 732 4. Kaufmann P, Engelstad K, Wei Y, Kulikova R, Oskoui M, Sproule DM, et al. Natural
733 history of MELAS associated with mitochondrial DNA m.3243A>G genotype.
734 *Neurology.* 2011 Nov 29;77(22):1965–71. doi:10.1212/WNL.0b013e31823a0c7f
735 PubMed PMID: 22094475; PubMed Central PMCID: PMC3235358.
- 736 5. Vafai SB, Mootha VK. A Common Pathway for a Rare Disease? *Science.* 2013 Dec
737 20;342(6165):1453–4. doi:10.1126/science.1248449 PubMed PMID: 24357304.
- 738 6. Newman NJ, Yu-Wai-Man P, Biouesse V, Carelli V. Understanding the molecular basis
739 and pathogenesis of hereditary optic neuropathies: towards improved diagnosis and
740 management. *The Lancet Neurology.* 2022 Sep 22;0(0). doi:10.1016/S1474-
741 4422(22)00174-0 PubMed PMID: 36155660.
- 742 7. Pitceathly RDS, Keshavan N, Rahman J, Rahman S. Moving towards clinical trials for
743 mitochondrial diseases. *J Inherit Metab Dis.* 2021 Jan;44(1):22–41.
744 doi:10.1002/jimd.12281 PubMed PMID: 32618366; PubMed Central PMCID:
745 PMC8432143.
- 746 8. Falabella M, Minczuk M, Hanna MG, Viscomi C, Pitceathly RDS. Gene therapy for
747 primary mitochondrial diseases: experimental advances and clinical challenges. *Nat Rev*
748 *Neurol.* 2022 Nov;18(11):11. doi:10.1038/s41582-022-00715-9
- 749 9. Stenton SL, Prokisch H. Genetics of mitochondrial diseases: Identifying mutations to
750 help diagnosis. *EBioMedicine.* 2020 Jun;56:102784. doi:10.1016/j.ebiom.2020.102784
751 PubMed PMID: 32454403; PubMed Central PMCID: PMC7248429.
- 752 10. Ratnaike TE, Greene D, Wei W, Sanchis-Juan A, Schon KR, van den Aamee J, et al.
753 MitoPhen database: a human phenotype ontology-based approach to identify
754 mitochondrial DNA diseases. *Nucleic Acids Res.* 2021 Aug 24;gkab726.
755 doi:10.1093/nar/gkab726 PubMed PMID: 34428295.
- 756 11. Grady JP, Campbell G, Ratnaike T, Blakely EL, Falkous G, Nesbitt V, et al. Disease
757 progression in patients with single, large-scale mitochondrial DNA deletions. *Brain.*
758 2014 Feb;137(Pt 2):323–34. doi:10.1093/brain/awt321 PubMed PMID: 24277717;
759 PubMed Central PMCID: PMC3914470.
- 760 12. Stewart JB, Chinnery PF. The dynamics of mitochondrial DNA heteroplasmy:
761 implications for human health and disease. *Nat Rev Genet.* 2015;16(9):530–42.
762 doi:10.1038/nrg3966 PubMed PMID: 26281784.

- 763 13. Viscomi C, Bottani E, Civiletto G, Cerutti R, Moggio M, Fagiolari G, et al. In vivo
764 correction of COX deficiency by activation of the AMPK/PGC-1 α axis. *Cell Metab.*
765 2011 Jul 6;14(1):1. doi:10.1016/j.cmet.2011.04.011 PubMed PMID: 21723506; PubMed
766 Central PMCID: PMC3130927.
- 767 14. Cerutti R, Pirinen E, Lamperti C, Marchet S, Sauve AA, Li W, et al. NAD(+)-dependent
768 activation of Sirt1 corrects the phenotype in a mouse model of mitochondrial disease.
769 *Cell Metab.* 2014 Jun 3;19(6):1042–9. doi:10.1016/j.cmet.2014.04.001 PubMed PMID:
770 24814483; PubMed Central PMCID: PMC4051987.
- 771 15. Lapatto HAK, Kuusela M, Heikkinen A, Muniandy M, Van Der Kolk BW,
772 Gopalakrishnan S, et al. Nicotinamide riboside improves muscle mitochondrial
773 biogenesis, satellite cell differentiation, and gut microbiota in a twin study. *Sci Adv.*
774 2023 Jan 13;9(2):eadd5163. doi:10.1126/sciadv.add5163
- 775 16. Damgaard MV, Treebak JT. What is really known about the effects of nicotinamide
776 riboside supplementation in humans. *Science Advances.* 2023 Jul 21;9(29):eadi4862.
777 doi:10.1126/sciadv.adi4862
- 778 17. Cantó C, Houtkooper RH, Pirinen E, Youn DY, Oosterveer MH, Cen Y, et al. The
779 NAD(+) precursor nicotinamide riboside enhances oxidative metabolism and protects
780 against high-fat diet-induced obesity. *Cell Metab.* 2012 Jun 6;15(6):6.
781 doi:10.1016/j.cmet.2012.04.022 PubMed PMID: 22682224; PubMed Central PMCID:
782 PMC3616313.
- 783 18. Khan NA, Auranen M, Paetau I, Pirinen E, Euro L, Forsström S, et al. Effective
784 treatment of mitochondrial myopathy by nicotinamide riboside, a vitamin B3. *EMBO*
785 *Mol Med.* 2014 Jun;6(6):721–31. doi:10.1002/emmm.201403943 PubMed PMID:
786 24711540; PubMed Central PMCID: PMC4203351.
- 787 19. Schöndorf DC, Ivanyuk D, Baden P, Sanchez-Martinez A, De Cicco S, Yu C, et al. The
788 NAD⁺ Precursor Nicotinamide Riboside Rescues Mitochondrial Defects and Neuronal
789 Loss in iPSC and Fly Models of Parkinson's Disease. *Cell Rep.* 2018 Jun
790 5;23(10):2976–88. doi:10.1016/j.celrep.2018.05.009 PubMed PMID: 29874584.
- 791 20. Hong Y, Zhang Z, Yangzom T, Chen A, Lundberg BC, Fang EF, et al. The NAD⁺
792 Precursor Nicotinamide Riboside Rescues Mitochondrial Defects and Neuronal Loss in
793 iPSC derived Cortical Organoid of Alpers' Disease. *Int J Biol Sci.* 2024;20(4):1194–217.
794 doi:10.7150/ijbs.91624 PubMed PMID: 38385069; PubMed Central PMCID:
795 PMC10878163.
- 796 21. Walker MA, Chen H, Yadav A, Ritterhoff J, Villet O, McMillen T, et al. Raising NAD⁺
797 Level Stimulates Short-Chain Dehydrogenase/Reductase Proteins to Alleviate Heart
798 Failure Independent of Mitochondrial Protein Deacetylation. *Circulation.* 2023 Dec
799 19;148(25):2038–57. doi:10.1161/CIRCULATIONAHA.123.066039 PubMed PMID:
800 37965787; PubMed Central PMCID: PMC10842390.
- 801 22. Yoon JY, Daneshgar N, Chu Y, Chen B, Hefti M, Vikram A, et al. Metabolic rescue
802 ameliorates mitochondrial encephalo-cardiomyopathy in murine and human iPSC
803 models of Leigh syndrome. *Clin Transl Med.* 2022 Jul;12(7):e954.
804 doi:10.1002/ctm2.954 PubMed PMID: 35872650; PubMed Central PMCID:
805 PMC9309541.

- 806 23. Romani M, Sorrentino V, Oh CM, Li H, de Lima TI, Zhang H, et al. NAD⁺ boosting
807 reduces age-associated amyloidosis and restores mitochondrial homeostasis in muscle.
808 Cell Rep. 2021 Jan 19;34(3):108660. doi:10.1016/j.celrep.2020.108660 PubMed PMID:
809 33472069; PubMed Central PMCID: PMC7816122.
- 810 24. Pirinen E, Auranen M, Khan NA, Brilhante V, Urho N, Pessia A, et al. Niacin Cures
811 Systemic NAD⁺ Deficiency and Improves Muscle Performance in Adult-Onset
812 Mitochondrial Myopathy. Cell Metab. 2020 Jun 2;31(6):1078-1090.e5.
813 doi:10.1016/j.cmet.2020.04.008 PubMed PMID: 32386566.
- 814 25. Trammell SAJ, Schmidt MS, Weidemann BJ, Redpath P, Jaksch F, Dellinger RW, et al.
815 Nicotinamide riboside is uniquely and orally bioavailable in mice and humans. Nat
816 Commun. 2016 Oct 10;7:12948. doi:10.1038/ncomms12948 PubMed PMID: 27721479;
817 PubMed Central PMCID: PMC5062546.
- 818 26. Airhart SE, Shireman LM, Risler LJ, Anderson GD, Nagana Gowda GA, Raftery D, et
819 al. An open-label, non-randomized study of the pharmacokinetics of the nutritional
820 supplement nicotinamide riboside (NR) and its effects on blood NAD⁺ levels in healthy
821 volunteers. PLoS One. 2017;12(12):e0186459. doi:10.1371/journal.pone.0186459
822 PubMed PMID: 29211728; PubMed Central PMCID: PMC5718430.
- 823 27. Monica 1776 Main Street Santa, California 90401-3208. 36-Item Short Form Survey
824 from the RAND Medical Outcomes Study [Internet]. [cited 2023 Mar 8]. Available
825 from: https://www.rand.org/health-care/surveys_tools/mos/36-item-short-form.html
- 826 28. Sleight A, Lupson V, Thankamony A, Dunger DB, Savage DB, Carpenter TA, et al.
827 Simple and effective exercise design for assessing in vivo mitochondrial function in
828 clinical applications using ³¹P magnetic resonance spectroscopy. Sci Rep. 2016 Jan
829 11;6(1):1. doi:10.1038/srep19057
- 830 29. White HE, Durston VJ, Seller A, Fratter C, Harvey JF, Cross NCP. Accurate detection
831 and quantitation of heteroplasmic mitochondrial point mutations by pyrosequencing.
832 Genet Test. 2005;9(3):190–9. doi:10.1089/gte.2005.9.190 PubMed PMID: 16225398.
- 833 30. Burr SP, Chinnery PF. Measuring Single-Cell Mitochondrial DNA Copy Number and
834 Heteroplasmy using Digital Droplet Polymerase Chain Reaction. J Vis Exp. 2022 Jul
835 12;(185). doi:10.3791/63870 PubMed PMID: 35913155.
- 836 31. Nie Y, Murley A, Golder Z, Rowe JB, Allinson K, Chinnery PF. Heteroplasmic
837 mitochondrial DNA mutations in frontotemporal lobar degeneration. Acta Neuropathol.
838 2022 Jun;143(6):687–95. doi:10.1007/s00401-022-02423-6 PubMed PMID: 35488929;
839 PubMed Central PMCID: PMC9107417.
- 840 32. Calabrese C, Simone D, Diroma MA, Santorsola M, Guttà C, Gasparre G, et al.
841 MToolBox: a highly automated pipeline for heteroplasmy annotation and prioritization
842 analysis of human mitochondrial variants in high-throughput sequencing.
843 Bioinformatics. 2014 Nov 1;30(21):3115–7. doi:10.1093/bioinformatics/btu483 PubMed
844 PMID: 25028726; PubMed Central PMCID: PMC4201154.
- 845 33. Basu S, Xie X, Uhler JP, Hedberg-Oldfors C, Milenkovic D, Baris OR, et al. Accurate
846 mapping of mitochondrial DNA deletions and duplications using deep sequencing. PLoS
847 Genet. 2020 Dec;16(12):e1009242. doi:10.1371/journal.pgen.1009242 PubMed PMID:
848 33315859; PubMed Central PMCID: PMC7769605.

- 849 34. Hargreaves P, Rahman S, Guthrie P, Taanman JW, Leonard JV, Land JM, et al.
 850 Diagnostic value of succinate ubiquinone reductase activity in the identification of
 851 patients with mitochondrial DNA depletion. *J Inherit Metab Dis*. 2002 Feb;25(1):7–16.
 852 doi:10.1023/a:1015104910239 PubMed PMID: 12004863.
- 853 35. Gangfuß A, Hentschel A, Heil L, Gonzalez M, Schönecker A, Depienne C, et al.
 854 Proteomic and morphological insights and clinical presentation of two young patients
 855 with novel mutations of BVES (POPDC1). *Molecular Genetics and Metabolism*. 2022
 856 Jul;136(3):226–37. doi:10.1016/j.ymgme.2022.05.005
- 857 36. Rath S, Sharma R, Gupta R, Ast T, Chan C, Durham TJ, et al. MitoCarta3.0: an updated
 858 mitochondrial proteome now with sub-organelle localization and pathway annotations.
 859 *Nucleic Acids Res*. 2021 Jan 8;49(D1):D1541–7. doi:10.1093/nar/gkaa1011 PubMed
 860 PMID: 33174596; PubMed Central PMCID: PMC7778944.
- 861 37. Han J, Lin K, Sequeira C, Yang J, Borchers CH. Quantitation of low molecular weight
 862 sugars by chemical derivatization-liquid chromatography/multiple reaction
 863 monitoring/mass spectrometry. *Electrophoresis*. 2016 Jul;37(13):1851–60.
 864 doi:10.1002/elps.201600150
- 865 38. Han J, Gagnon S, Eckle T, Borchers CH. Metabolomic analysis of key central carbon
 866 metabolism carboxylic acids as their 3-nitrophenylhydrazones by UPLC / ESI - MS.
 867 *Electrophoresis*. 2013 Oct;34(19):2891–900. doi:10.1002/elps.201200601
- 868 39. Han J, Lin K, Sequeira C, Borchers CH. An isotope-labeled chemical derivatization
 869 method for the quantitation of short-chain fatty acids in human feces by liquid
 870 chromatography–tandem mass spectrometry. *Analytica Chimica Acta*. 2015 Jan;854:86–
 871 94. doi:10.1016/j.aca.2014.11.015
- 872 40. Han J, Higgins R, Lim MD, Atkinson K, Yang J, Lin K, et al. Isotope-labeling
 873 derivatization with 3-nitrophenylhydrazine for LC/multiple-reaction monitoring-mass-
 874 spectrometry-based quantitation of carnitines in dried blood spots. *Analytica Chimica*
 875 *Acta*. 2018 Dec;1037:177–87. doi:10.1016/j.aca.2018.01.045
- 876 41. Rohart F, Gautier B, Singh A, Lê Cao KA. mixOmics: An R package for 'omics feature
 877 selection and multiple data integration. *PLoS Comput Biol*. 2017 Nov;13(11):e1005752.
 878 doi:10.1371/journal.pcbi.1005752 PubMed PMID: 29099853; PubMed Central PMCID:
 879 PMC5687754.
- 880 42. Szklarczyk D, Kirsch R, Koutrouli M, Nastou K, Mehryary F, Hachilif R, et al. The
 881 STRING database in 2023: protein-protein association networks and functional
 882 enrichment analyses for any sequenced genome of interest. *Nucleic Acids Res*. 2023 Jan
 883 6;51(D1):D638–46. doi:10.1093/nar/gkac1000 PubMed PMID: 36370105; PubMed
 884 Central PMCID: PMC9825434.
- 885 43. Chong J, Xia J. MetaboAnalystR: an R package for flexible and reproducible analysis of
 886 metabolomics data. *Bioinformatics*. 2018 Dec 15;34(24):4313–4.
 887 doi:10.1093/bioinformatics/bty528 PubMed PMID: 29955821; PubMed Central PMCID:
 888 PMC6289126.
- 889 44. Lehtonen JM, Forsström S, Bottani E, Viscomi C, Baris OR, Isoniemi H, et al. FGF21 is
 890 a biomarker for mitochondrial translation and mtDNA maintenance disorders.

- 891 Neurology. 2016 Nov 29;87(22):2290–9. doi:10.1212/WNL.0000000000003374
892 PubMed PMID: 27794108; PubMed Central PMCID: PMC5270510.
- 893 45. Suomalainen A, Elo JM, Pietiläinen KH, Hakonen AH, Sevastianova K, Korpela M, et
894 al. FGF-21 as a biomarker for muscle-manifesting mitochondrial respiratory chain
895 deficiencies: a diagnostic study. *Lancet Neurol*. 2011 Sep;10(9):806–18.
896 doi:10.1016/S1474-4422(11)70155-7 PubMed PMID: 21820356; PubMed Central
897 PMCID: PMC7568343.
- 898 46. Yatsuga S, Fujita Y, Ishii A, Fukumoto Y, Arahata H, Kakuma T, et al. Growth
899 differentiation factor 15 as a useful biomarker for mitochondrial disorders. *Ann Neurol*.
900 2015 Nov;78(5):814–23. doi:10.1002/ana.24506 PubMed PMID: 26463265; PubMed
901 Central PMCID: PMC5057301.
- 902 47. Spendiff S, Reza M, Murphy JL, Gorman G, Blakely EL, Taylor RW, et al.
903 Mitochondrial DNA deletions in muscle satellite cells: implications for therapies. *Hum*
904 *Mol Genet*. 2013 Dec 1;22(23):4739–47. doi:10.1093/hmg/ddt327 PubMed PMID:
905 23847047; PubMed Central PMCID: PMC3820134.
- 906 48. Clark KM, Bindoff LA, Lightowlers RN, Andrews RM, Griffiths PG, Johnson MA, et al.
907 Reversal of a mitochondrial DNA defect in human skeletal muscle. *Nat Genet*. 1997
908 Jul;16(3):222–4. doi:10.1038/ng0797-222
- 909 49. Murphy JL, Blakely EL, Schaefer AM, He L, Wyrick P, Haller RG, et al. Resistance
910 training in patients with single, large-scale deletions of mitochondrial DNA. *Brain*. 2008
911 Jun 21;131(11):2832–40. doi:10.1093/brain/awn252
- 912 50. Perez-Riverol Y, Bandla C, Kundu DJ, Kamatchinathan S, Bai J, Hewapathirana S, et al.
913 The PRIDE database at 20 years: 2025 update. *Nucleic Acids Res*. 2025 Jan
914 6;53(D1):D543–53. doi:10.1093/nar/gkae1011 PubMed PMID: 39494541; PubMed
915 Central PMCID: PMC11701690.

916

917

918 **Figures**

919 **Figure 1. Study overview and Pharmacokinetic studies.** (A) Participants attended for a
920 screening visit to check eligibility and gain informed consent. Muscle biopsies were
921 performed at the baseline and end of treatment visits. Concentration of (B) Nicotinamide
922 Adenine Dinucleotide+ (NAD⁺), and (C) Nicotinamide Riboside (NR) in participant NR006
923 at baseline (v1) and after 1 (v2), 2 (v3), 3 (v4) and 4 weeks of treatment with NR 10mg/kg
924 BD.

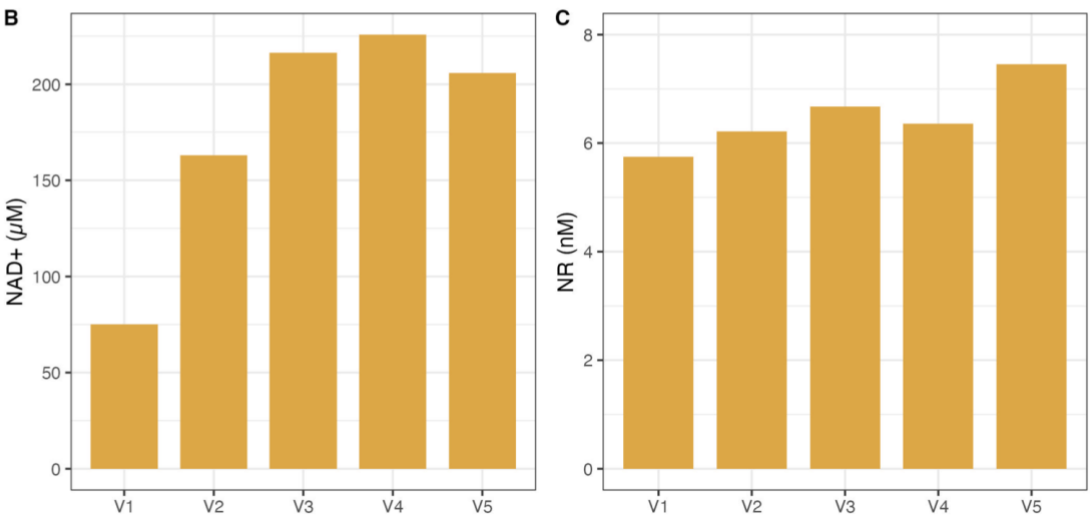
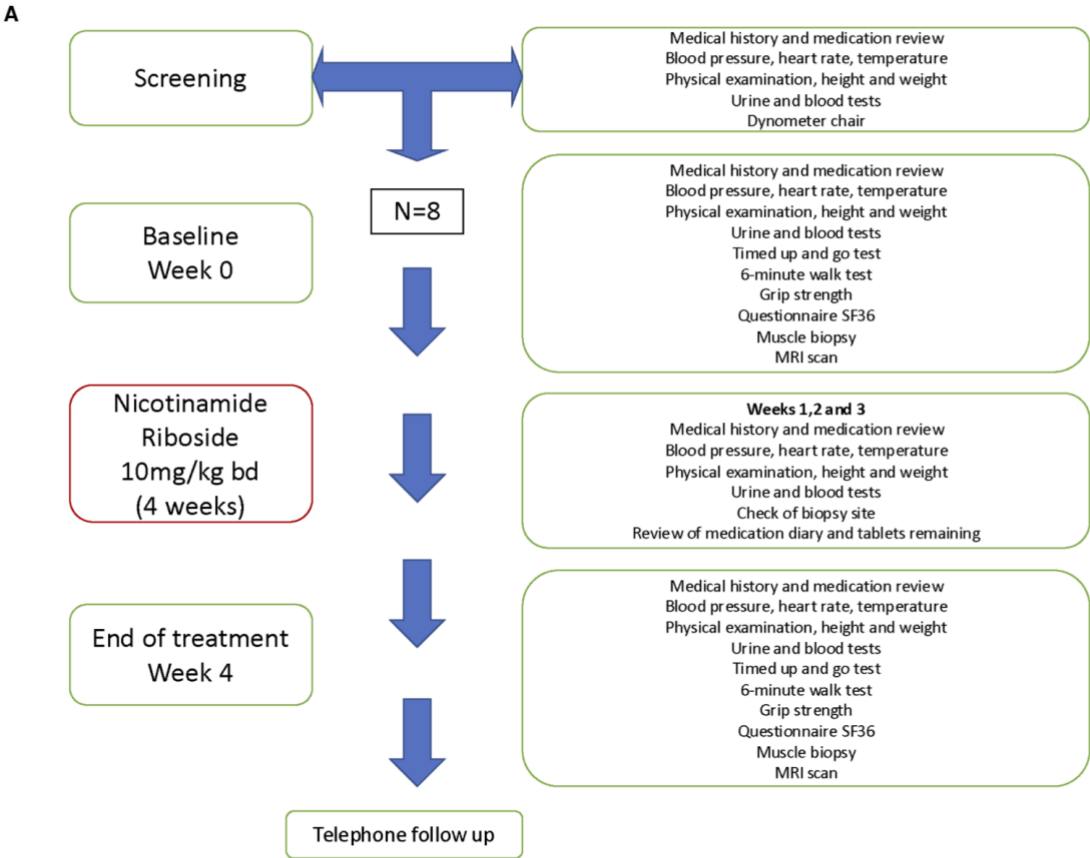


Figure 2. Skeletal muscle histochemistry before and after treatment with NR. (A) Dual cytochrome c oxidase (COX)-Succinate Dehydrogenase (SDH) staining showing COX negative fibres (blue) and COX intermediate fibres (intermediate brown / blue) in the pre-treatment quadriceps muscle biopsy from a participant with a large scale mtDNA deletion (NR011). Magnification 20X. Scale bar 20 um. (B) Percentage of COX deficient fibres in pre-treatment quadriceps muscle biopsy (v1) and after 4 weeks of NR treatment (v5). (C) Gomori trichrome staining showing ragged red fibres in the pre-treatment quadriceps muscle biopsy from a participant with a large scale mtDNA deletion (NR011). Magnification 40X. Scale bar 20um. (D) Percentage ragged red fibres in pre-treatment quadriceps muscle biopsy (v1) and after 4 weeks of NR treatment (v5) in participants with deletions and SNVs. Deletion = large scale single deletion of mtDNA. SNV = single nucleotide variant of mtDNA.

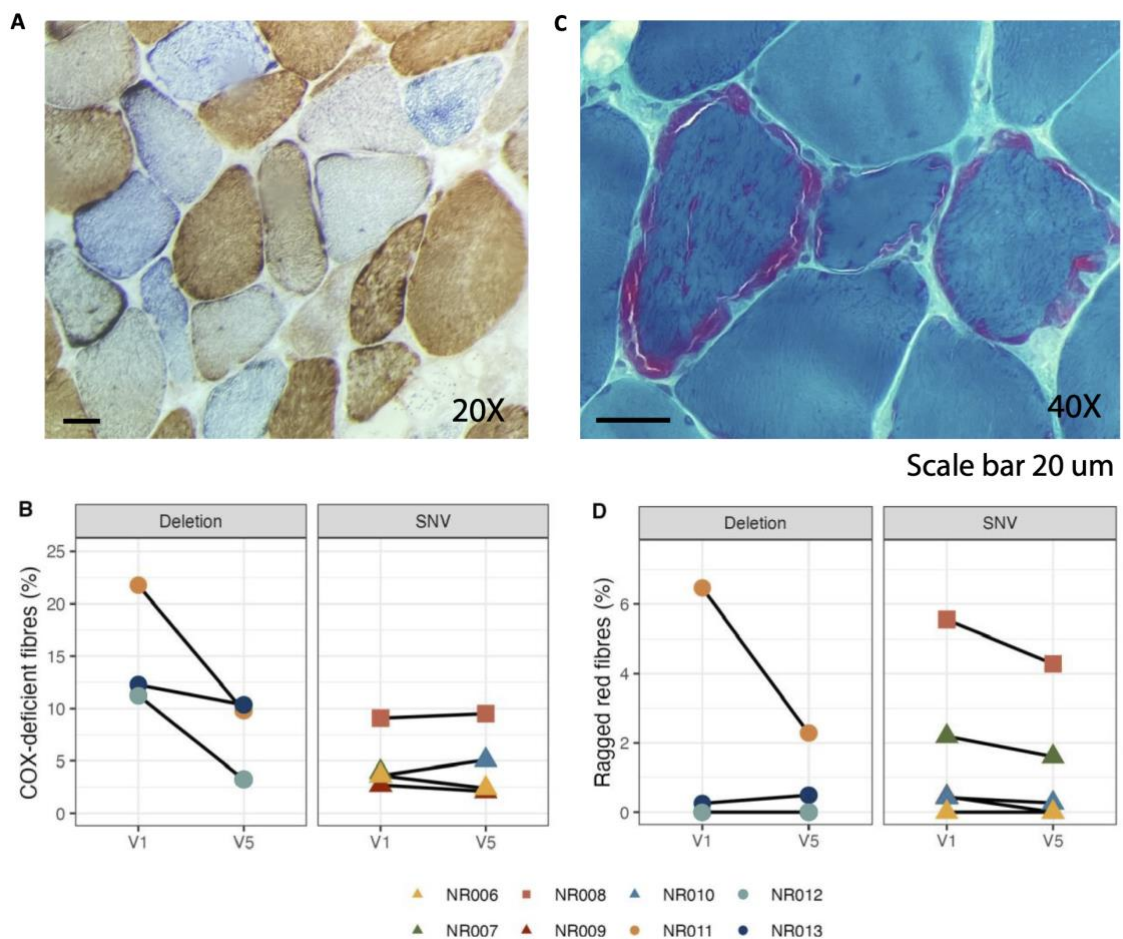


Figure 3. Respiratory chain enzymes, mitochondrial DNA copy number, mitochondrial DNA deletion analysis, biomarkers and clinical outcomes before and after treatment with NR. Muscle biopsy analysis, biomarkers and clinical outcomes pre-treatment (v1) and after 4 weeks of NR treatment (v5). **(A)** Complex I activity normalised to citrate synthase; **(B)** Complex IV activity normalised to citrate synthetase. **(C)** Citrate synthase activity. **(D)** Bulk mitochondrial DNA copy number measured by droplet digital PCR. **(E and F)** Mitochondrial DNA deletions visualised by MitoSAlt. The outer circle represents the mitochondrial genome. The lines represent different deletions detected. The colour of the line represents how many of each deletion were detected (darker colour meaning a higher number). Participants NR010, NR011 and NR012 had different single deletions (positions reported under bar plot in G). F shows the deletions identified in participant NR012 before and after treatment. We note that the heteroplasmy levels calculated by MitoSAlt are higher than those measured by droplet digital PCR. **(G)** Bar plot showing the deletion heteroplasmy in muscle before and after treatment, calculated by the MitoSAlt pipeline. **(H)** Agarose gel showing PCR products for the three participants with large-scale mtDNA deletions. The ladder on the left-hand side shows markers in thousands of base pairs. The wild type PCR product is around 9000 bp long (top arrow). Shorter PCR products (bottom two arrows) indicate mtDNA deletions. **(I)** Phosphocreatine recovery rate constant (kPCr). **(J)** Serum FGF21 level. **(K)** Serum GDF15 level. **(L)** Timed up and go (TUG) test. **(M)** 6-minute walk test distance. Deletion = large scale single deletion of mtDNA. SNV = single nucleotide variant of mtDNA.

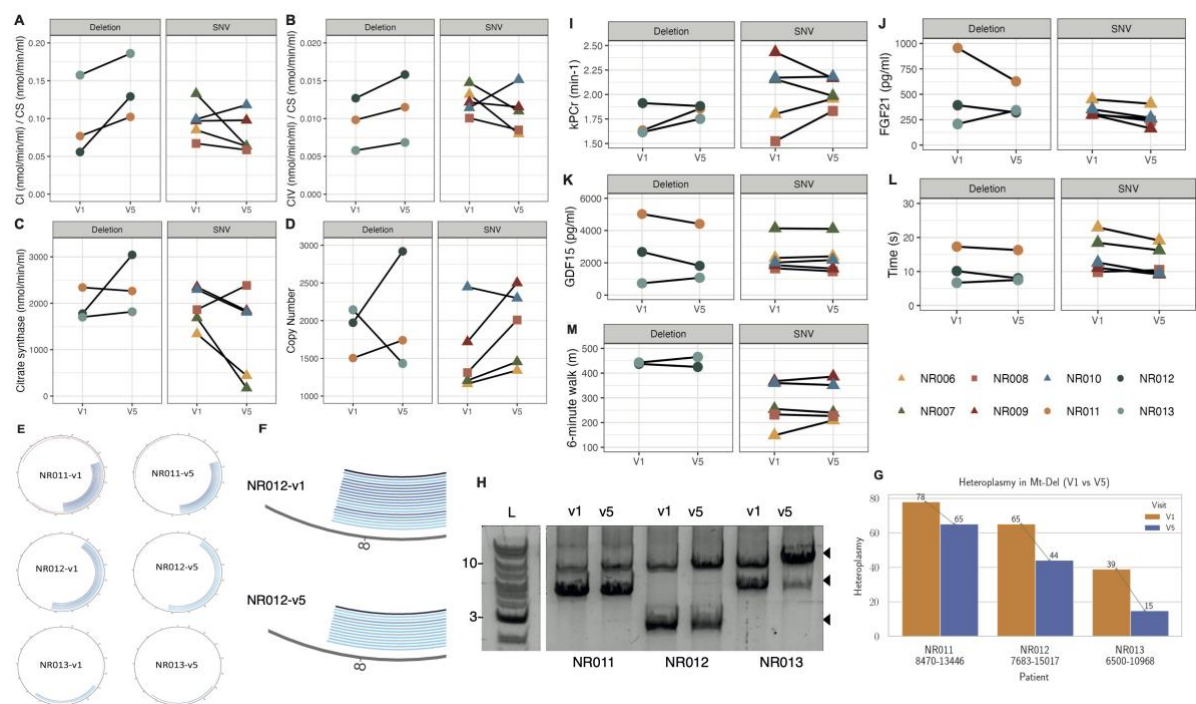


Figure 4. Integrated transcriptomic and proteomic analysis in muscle before and after treatment with NR. (A) Relationship between transcriptomics and proteomics datasets based on the first two components of the DIABLO analysis showing the strength of the correlation (0.86) between these two omics layers. (B) Distribution of individual samples along the first two principal components for both proteomics (left) and transcriptomics (right) data. Samples coloured according to treatment groups (V1 and V5). (C) Feature Loadings Plot for proteomics showing the top proteins driving the separation in the first component of the DIABLO analysis showing the contribution of the top 10 proteins to the variance captured by Component 1. (D) Feature Loadings Plot for transcriptomics showing the top genes driving the separation in the first component of the DIABLO analysis showing the contribution of the top 10 transcripts to the variance captured by Component 1.

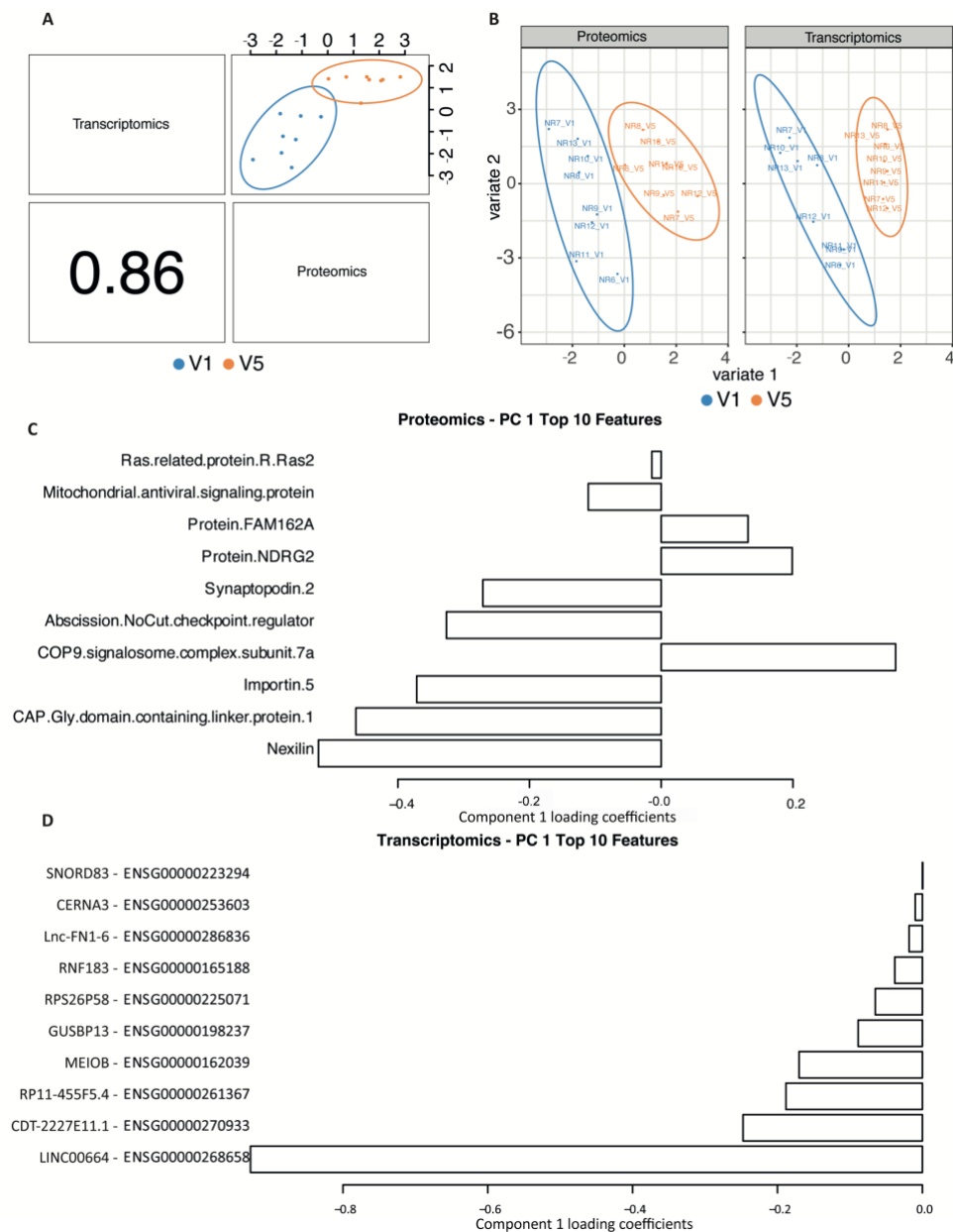
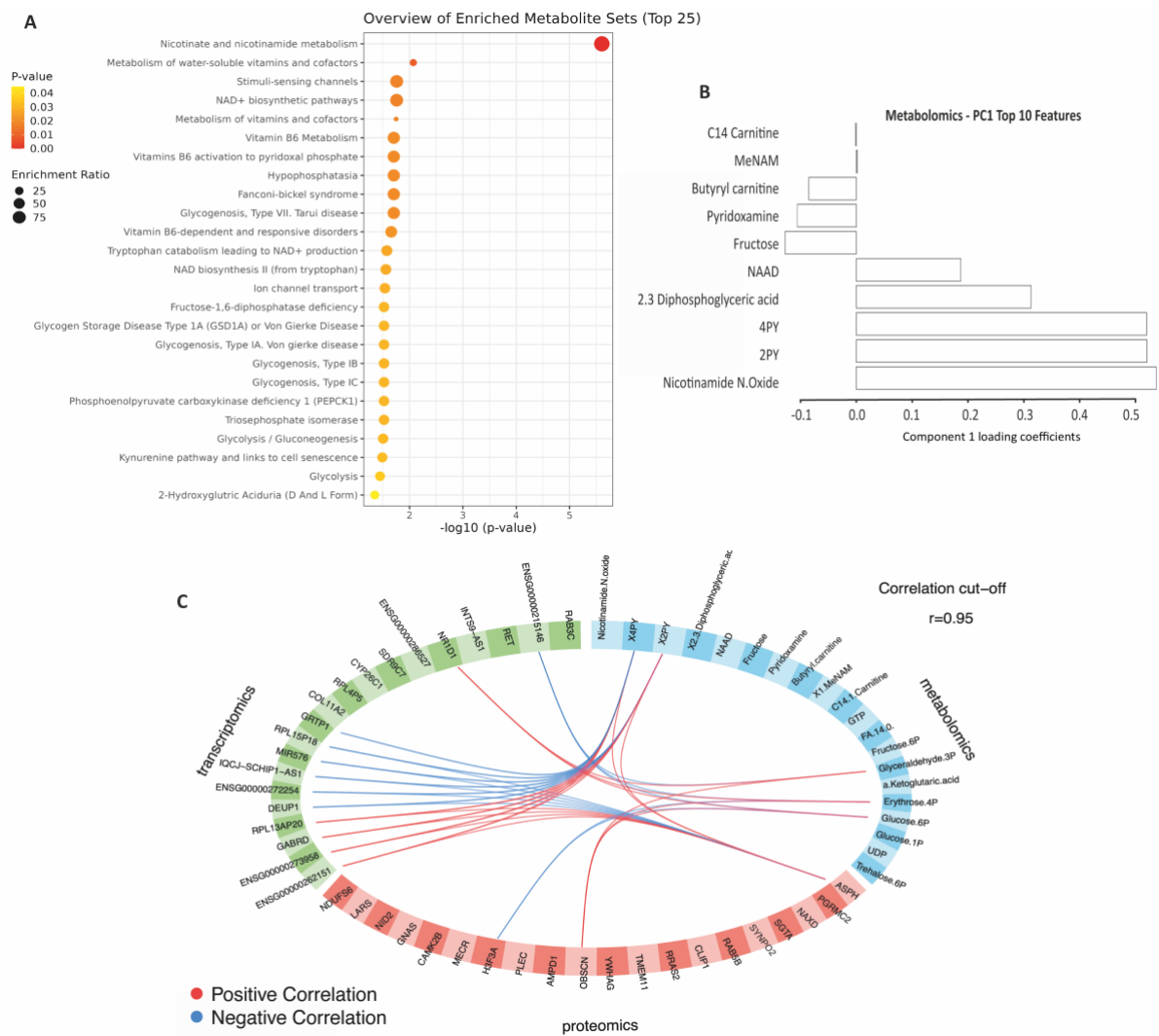


Figure 5. Metabolomics Contribution, Pathway Enrichment, and Multi-Omics

Correlation in muscle before and after treatment with NR. (A) Pathway enrichment analysis of discriminant metabolites identified significant involvement of Nicotinate and Nicotinamide Metabolism and NAD⁺ Biosynthetic Pathways, among others. Dot size represents enrichment ratio, and color intensity reflects p-value significance. **(B)** Feature Loadings Plot for metabolomics data, showing the top metabolites contributing to Component 1 of the DIABLO analysis. Notably, several NAD-related metabolites, including Nicotinamide N-oxide, 2PY, 4PY, and NAAD, show strong positive loadings. **(C)** Omics correlation plot (circo plot) depicting interconnections across transcriptomics, proteomics, and metabolomics layers. Lines represent pairwise correlations (positive in red, negative in blue) among the most strongly associated features, filtered at a correlation cut-off of $r = 0.95$.



991 **Table 1 Clinical characteristics of the study participants.**

Participant	Sex	Age (1 st visit)	Genetics	Main clinical features	Age at symptom onset
NR006	M	53	m.3243A>G	Type II diabetes, painful peripheral neuropathy, hearing impairment, seizures, ataxia	51
NR007	M	68	m.3243A>G	Type II diabetes, hearing impairment, proximal myopathy, ptosis, CPEO	54
NR008	F	21	m.3243A>T	Ptosis, fatigue, raised CK, myalgia, migraine, neuropathic pain, long GI transit time, myopathy	5
NR009	M	34	m.3243A>G	Type I diabetes, vertigo, hearing impairment, left ventricular hypertrophy	26
NR010	F	57	m.3243A>G	Mild macular dystrophy, type II diabetes, hearing loss	51
NR011	M	56	Single mtDNA deletion	CPEO, proximal myopathy	28
NR012	M	54	Single mtDNA deletion	CPEO, retinitis pigmentosa, proptosis right eye, episode of symptomatic bradycardia, proximal myopathy, fatigue, headaches, type II diabetes, fatty liver, hypertension	35
NR013	F	38	Single mtDNA deletion	CPEO	38

992 CPEO = chronic progressive external ophthalmoplegia, CK = serum creatine kinase.

993