Combinatorial glucose, nicotinic acid, and N-acetylcysteine therapy has synergistic effect in preclinical C. elegans and zebrafish models of mitochondrial complex I disease

Sujay Guha¹#, Neal D. Mathew¹#, Chigoziri Konkwo¹, Julian Ostrovsky¹, Young Joon Kwon¹, Erzsebet Polyak¹, Christoph Seiler², Michael Bennett³, Rui Xiao⁴, Zhe Zhang⁵, Eiko Nakamaru-Ogiso¹, Marni J. Falk¹.⁶*¹

¹Mitochondrial Medicine Frontier Program, Division of Human Genetics, Department of Pediatrics, The Children’s Hospital of Philadelphia, Philadelphia, PA;

²Aquatics Core Facility, The Children’s Hospital of Philadelphia, Philadelphia, PA;

³Department of Pathology and Laboratory Medicine, The Children’s Hospital of Philadelphia and University of Pennsylvania Perelman School of Medicine, Philadelphia, PA;

⁴Department of Biostatistics, Epidemiology and Informatics, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA;

⁵Center for Biomedical Informatics, The Children’s Hospital of Philadelphia, Philadelphia, PA;

⁶Department of Pediatrics, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA.

#, equal contribution

*Corresponding Author

Marni J. Falk, MD
ARC 1002c
3615 Civic Center Blvd
Philadelphia, PA 19104
Office 215-590-4564; Fax 267-426-2876
Email falkm@email.chop.edu
**Supplemental Figures Legends.**

**Supplemental Figure 1:** *List of 37 compounds that were previously evaluated individually in our research group to identify effects on wild-type development and gas-1(fc21) lifespan in C. elegans.* N2 Bristol larvae were used to test the concentration range for each compound listed to determine the maximal dissolvable, non-toxic molar dosing that did not impair wild-type animal development. The maximal obtainable dose that was non-toxic was then assessed on gas-1(fc21) lifespan relative to buffer-only treated gas-1(fc21) and N2 Bristol worm populations. Individual lifespan data was not studied in this paper, but top hits used to identify combinatorial treatments that were studied here. Buffer used is indicated for each compound, where water buffer was preferred when possible. All compounds were obtained from Sigma, unless otherwise indicated.

**Supplemental Figure 2:** *Combinatorial therapy regimens experimental details in C. elegans gas-1(fc21) complex I mutant worms.* Table of all compound names, concentrations, and 11 randomly selected combinations of one drug each across lead compounds previously identified in three treatment groups (metabolic modifiers, signaling modifiers, antioxidants) that were studied here relative to buffer-control in *C. elegans* complex I mutant gas-1(fc21) worms. AICAR, 5-Aminimidazole-4-carboxamide ribonucleotide (an AMP analogue). Carn, L-carnitine. Cyst, cysteamine bitartrate (provided by Raptor Therapeutics). CoQ, coenzyme Q10 (obtained from Novasol). DCA, dichloroacetate (provided by Dr. Peter Stacpool at University of Florida). Epi, (+) epicatechin (provided by Cardero Therapeutics). FA, folinic acid. Glu, glucose. LpA, lipoic acid. NA, nicotinic acid. NAC, N-acetylcysteine. Rap, rapamycin. Resv, resveratrol. Ribo, riboflavin or vitamin B2. Thi, thiamine or vitamin B1. Vit E, vitamin E. All compounds were obtained from Sigma, unless otherwise indicated.

**Supplemental Figure 3:** *Lifespan effects of 11 triple combinatorial therapy regimens in C. elegans gas-1(fc21) complex I mutant worms.* Table details median lifespan results in gas-1 (fc21) worms of 11 triple combination therapies (per File S2) initiated from L1 early larval development. All lifespan analyses were performed by
manual perturbation with microscopy analysis at 20°C compared to buffer-only treated gas-1(fc21) and N2 (WT) worms, divided between two experienced research technicians. * indicates analyses performed with 100 µM FUdR to prevent offspring development. At least 2 biological replicate experiments were performed per condition. Statistical analysis were performed in Graphpad Prism 7.04 by using a log-rank (Mantel-Cox) test compared to buffer-only treated gas-1(fc21).

Supplemental Figure 4: Lifespan effects of two lead combinatorial regimens single, pair-wise, and triple treatments in C. elegans gas-1(fc21) complex I mutant worms. Table details pair-wise therapies median lifespan effects for the two lead triple combination therapy regimens that were found on initial screen to significantly improve gas-1(fc21) lifespan (Figure 2 and Supplemental Figure 3). Individual and pair-wise component combinations of FA+Cyst+Resv (folinic acid, cysteamine bitartrate, and resveratrol) therapy found resveratrol effects drove treatment response, without synergy observed upon triple compared to pair-wise therapy. All combinations were given at L1 early larval development at 20°C as compared to concurrent buffer-only treated gas-1(fc21) and N2 (WT) worms. Two biological replicate experiments were performed per condition. Statistical analyses were performed by log-rank (Mantel-Cox) test compared to buffer-only treated gas-1(fc21).

Supplemental Figure 5: Gene-level heat map of RNAseq-based transcriptome changes in C. elegans gas-1(fc21) complex I mutant worms relative to wild-type worms or upon Glc+NA+NAC therapy. (A) The gene-level heat maps list individual genes (names on right vertical axis) that were down-regulated (n = 260) or up-regulated (n = 90) in gas-1(fc21) relative to N2 (WT) C. elegans worms, and reversed by at least one drug treatment (single or multiple drug combination) having false discovery rate (FDR) < 0.05 and p value < 0.05. Each color block represents the mean of samples for each gene within the same treatment group. Data of each gene were subtracted by mean of all groups and then divided by their standard deviation. Red and yellow colors convey high and low expression, respectively. (B) Parametric analysis of gene set enrichment (PAGE) scores are shown for selected
KEGG pathways that were significantly dysregulated in gas-1(fc21) worms compared to N2 (wild-type) worms or upon treatment with Glu+NA+NAC and its single or double components at the young adult stage (YA) for 24 hours. Red and blue indicate upregulated and downregulated expression, respectively, in the second group relative to the first group named in each two-way comparison. Color intensity indicates significance of effect.

**Supplemental Figure 6:** Combinatorial treatment effects on HPLC-based whole worm free amino acid profiles in gas-1(fc21) mutant worms. Whole worm population free amino acid absolute concentrations were measured by HPLC and normalized to overall protein concentration (nmol/mL per mcg protein). Symbol color denotes experimental condition as defined in key, with separate symbols shown to indicate results of three independent replicate experiments performed. Random effects ANOVA statistical analysis was used to account for experimental batch effects. *, p < 0.05 when compared to buffer (water) only exposed gas-1(fc21) worms.

**Supplemental Figure 7:** Combinatorial treatment effects on GC/MS-based whole worm stable isotopic enrichment profiles in glycine (GLY), serine (SER), aspartate (ASP), amino acids in gas-1(fc21) mutant worms. Absolute isotopic enrichment in all molecular species of each metabolite (indicating the number of carbon atoms in each molecular species having enrichment) was quantified by GC/MS in synchronous adult worm populations following U-13C6-glucose feeding to N2 (WT) or gas-1(fc21) worms for 24 hours on the first day of adulthood. Symbol color denotes experimental condition as defined in key, with separate symbols shown to indicate results of three independent replicate experiments performed. Random effects ANOVA statistical analysis was used to account for experimental batch effects. APE, atoms percent excess. *, p < 0.05 when compared to buffer (water) only exposed gas-1(fc21) worms.

**Supplemental Figure 8:** Combinatorial treatment effects on GC/MS-based whole worm stable isotopic enrichment profiles in citrate, succinate, malate, and lactate organic acids in gas-1(fc21) mutant worms.
Absolute isotopic enrichment all molecular species of each metabolite (indicating the number of carbon atoms in each molecular species having enrichment following U-13C6-glucose fed to the worms) was quantified by GC/MS in synchronous adult worm populations following U-13C6-glucose feeding to N2 (WT) or gas-1(fc21) worms for 24 hours on the first day of adulthood. Separate symbols indicate results of independent replicate experiments. Random effects ANOVA statistical analysis was used to account for experimental batch effects. Three biological replicate experiments were performed. *, p < 0.05 and **, p < 0.01 when compared to buffer (water) only exposed gas-1(fc21) worms.

Supplemental Figure 9: Combinatorial treatment effects on swimming activity and additional biochemical analytes in rotenone-treated zebrafish larvae (A) Swimming activity raw data is shown, as summarized in Figure 7. Glu+NA+NAC triple combination therapy begun at 5 dpf prevented neuromuscular defects that result from rotenone-based complex I inhibition in zebrafish at 7 dpf. The line graph represents the average activity for each minute measured using the ZebraLab Software (Viewpoint Life Sciences). The light cycle is a 20-minute light on at (60% light intensity) 20-minute light off (0% light intensity) which is repeated for 600 minutes. Data shown is from one biological replicate experiment, with n = 8 zebrafish per condition. (B) Pyruvate levels were not significantly changed with rotenone or Glu+NA+NAC therapy in zebrafish. Pyruvate concentration in zebrafish (20 larvae per replicate condition) was 75.5 pmol per larvae in wild-type (AB) zebrafish (n = 4 biological replicates), 78.6 pmol per larvae in 150 nM rotenone x4 hours exposed wild-type (AB) zebrafish (n = 4 biological replicates), and 99.4 pmol per larvae in Glu+NA+NAC pre-treated larvae from 5 dpf before rotenone exposure for 4 hours on 7 dpf. (C) NAD+ levels. NAD+ concentration in zebrafish (20 larvae per replicate condition) was 53.2 pmol per larvae in wild-type (AB) zebrafish (n = 4 biological replicates), 34.7 pmol per larvae in 150 nM rotenone treated wild-type (AB) zebrafish (n = 4 biological replicates), and 35.8 pmol per larvae in Glu+NA+NAC pre-treated larvae from 5 dpf before rotenone exposure for 4 hours on 7 dpf (n= 6 biological replicates). (D) NADH levels. NADH concentration in zebrafish (20 larvae per replicate condition) was 5.1 pmol per larvae in wild-type (AB) zebrafish (n = 4 biological replicates), 5.2 pmol per larvae in 150 nM rotenone
treated wild-type (AB) zebrafish (n = 6 biological replicates), and 6.9 pmol per larvae in Glu+NA+NAC pre-treated larvae from 5 dpf before rotenone exposure for 4 hours on 7 dpf (n= 6 biological replicates).  

(E) **NADH:NAD\(^+\) ratio.** No significant differences were seen between wild-type (AB) larvae in buffer-only control or 150 nM rotenone exposure for 4 hours alone or following Glu+NA+NAC pre-treatment from 5 dpf.  

(F) **GSSG Level.** Oxidized glutathione GSSG) concentration in zebrafish (20 larvae per replicate condition) was 6.4 pmol per larvae in wild-type (AB) zebrafish (n = 6 biological replicates), 6.2 pmol per larvae in 150 nM rotenone treated wild-type (AB) zebrafish (n = 7 biological replicates), and 3.9 pmol per larvae in Glu+NA+NAC pre-treated larvae from 5 dpf before rotenone exposure for 4 hours on 7 dpf (n= 7 biological replicates). Error bars represent mean and standard error of mean *, p < 0.05.