Deoxynucleoside stress exacerbates the phenotype of a mouse model of mitochondrial neurogastrointestinal encephalopathy

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Balanced pools of deoxyribonucleoside triphosphate precursors are required for DNA replication, and alterations of this balance are relevant to human mitochondrial diseases including mitochondrial neurogastrointestinal encephalopathy. In this disease, autosomal recessive TYMP mutations cause severe reductions of thymidine phosphorylase activity; marked elevations of the pyrimidine nucleosides thymidine and deoxyuridine in plasma and tissues, and somatic multiple deletions, depletion and site-specific point mutations of mitochondrial DNA. Thymidine phosphorylase and uridine phosphorylase double knockout mice recapitulated several features of these patients including thymidine phosphorylase activity deficiency, elevated thymidine and deoxyuridine in tissues, mitochondrial DNA depletion, respiratory chain defects and white matter changes. However, in contrast to patients with this disease, mutant mice showed mitochondrial alterations only in the brain. To test the hypothesis that elevated levels of nucleotides cause unbalanced deoxyribonucleoside triphosphate pools and, in turn, pathogenic mitochondrial DNA instability, we have stressed double knockout mice with exogenous thymidine and deoxyuridine, and assessed clinical, neuroradiological, histological, molecular, and biochemical consequences. Mutant mice treated with exogenous thymidine and deoxyuridine showed reduced survival, body weight, and muscle strength, relative to untreated animals. Moreover, in treated mutants, leukoencephalopathy, a hallmark of the disease, was enhanced and the small intestine showed a reduction of smooth muscle cells and increased fibrosis. Levels of mitochondrial DNA were depleted not only in the brain but also in the small intestine, and deoxyribonucleoside triphosphate imbalance was observed in the brain. The relative proportion, rather than the absolute amount of deoxyribonucleoside triphosphate, was critical for mitochondrial DNA maintenance. Thus, our results demonstrate that stress of exogenous pyrimidine nucleosides enhances the mitochondrial phenotype of our knockout mice. Our mouse studies provide insights into the pathogenic role of thymidine and deoxyuridine imbalance in mitochondrial neurogastrointestinal encephalopathy and an excellent model to study new therapeutic approaches.

Keywords: MNGIE; animal model; thymidine; deoxyuridine; deoxynucleotide; mitochondrial DNA
Introduction

Balanced pools of deoxyribonucleoside triphosphates (dNTPs) are required for mitochondrial DNA replication, and alterations of this balance, caused by mutations in genes encoding proteins involved in the metabolism of deoxyribonucleoside triphosphates (RRM2B, DGUOK, TYMP and TK2) are relevant to human mitochondrial diseases (Nishino et al., 1999; Mandel et al., 2001; Saada et al., 2001; Bourdon et al., 2007).

Autosomal recessive mutations in TYMP are responsible for mitochondrial neurogastrointestinal encephalopathy (MNGIE) (Nishino et al., 1999). MNGIE is a devastating disease characterized clinically by onset between the second to fifth decades, ptosis, progressive external ophthalmoplegia, leukoencephalopathy, gastrointestinal dysmotility, and peripheral neuropathy (Hirano et al., 1994). Patients with MNGIE typically die in their late thirties, often due to gastrointestinal complications.

TYMP mutations cause severe decreases of thymidine phosphorylase activity. Thymidine phosphorylase catalyses the phosphorolysis of the pyrimidine nucleosides thymidine or deoxyuridine to the corresponding bases, thymine or uracil, and deoxyribos-1-phosphate (Desgranges et al., 1981). The lack of thymidine phosphorylase activity provokes marked elevations of thymidine and deoxyuridine in plasma and tissues, leading to pathogenic secondary instability of mitochondrial DNA manifesting as somatic multiple deletions, depletion, and site-specific point mutations (Papadimitriou et al., 1998; Nishino et al., 1999; Spinazzola et al., 2002; Marti et al., 2003; Nishigaki et al., 2003, 2004; Hirano et al., 2004; Blazquez et al., 2005; Valentino et al., 2007).

To better understand the pathogenic mechanism of MNGIE, we previously generated double knockout (Tpp−/− Upp−/−) mice, because murine uridine phosphorylase (UP) compensates for Tpp deficiency by degrading thymidine and deoxyuridine. Tpp−/−Upp−/− mice showed no abnormalities of growth, development, sexual maturation, or reproductive ability (Lopez et al., 2009). Nevertheless, Tpp−/− Upp−/− mice recapitulate several features of patients with MNGIE including thymidine phosphorylase deficiency in all tissues studied [except liver, which has 17% residual activity; likely due to Upp 2 (Johansson, 2003)], elevated levels of thymidine and deoxyuridine compared to wild-type littermates, leukoencephalopathy, dNTP pool imbalance associated with mitochondrial DNA depletion, respiratory chain defects, and white matter changes in brain (Lopez et al., 2009).

However, in humans, gastrointestinal dysmotility produces the most severe clinical manifestation in MNGIE, in Tpp−/−Upp−/− mice we did not find any evidence of clinical, biochemical, or molecular abnormalities in the gastrointestinal tract. We hypothesized that the modest elevations of thymidine and deoxyuridine in mutant animals explained the mild phenotype in Tpp−/−Upp−/− mice, because in patients with MNGIE, pyrimidine nucleosides in tissues increase >100-fold, whereas in the double knockout mice the increase is <65-fold.

In the present work, we enhanced the mitochondrial phenotype in Tpp−/− Upp−/− mice by oral administration of exogenous thymidine and deoxyuridine, confirming the role of thymidine and deoxyuridine accumulation in the pathogenesis of MNGIE.

Materials and methods

Animal model and treatment

Tpp−/− Upp−/− double knockout murine model in C57BL/6j genetic background was previously characterized (Lopez et al., 2009). Fifteen animals per group (both Tpp−/− Upp−/− and Tpp+/+ Upp+/+) were treated with exogenous thymidine and deoxyuridine.

Thymidine and deoxyuridine were administered to the mice at a standard concentration of 16.6 mg/ml (deoxythymidine 68.6 mM and deoxyuridine 72.3 mM) in sterile drinking water, from weaning until sacrificed or death. For MRI studies and neuromuscular phenotyping, 15 mice with double dose were treated with 33.2 mg/ml (deoxythymidine 137.2 mM and deoxyuridine 144.6 mM) up to 1 year. All experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee of the Columbia University Medical Centre, and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed and bred according to international standard conditions, with a 12-h light, 12-h dark cycle, and sacrificed using CO2 narcosis followed by cervical dislocation. Organs (brain, liver, kidney, muscle, and small intestine) were removed and frozen in the liquid phase of isopentane, pre-cooled near its freezing point (−160°C) with dry ice. All the experiments were performed in at least three mice per group.

Thymidine and deoxyuridine measurements

Deoxythymidine and deoxyuridine levels were assessed by a gradient-elution HPLC method as described previously (Marti et al., 2004), with minor modifications. Briefly, deproteinized samples were injected into an Alliance HPLC system (Waters Corporation) with an Alltima C18 NUC reversed-phase column (Alltech) at a constant flow rate of 1.5 ml/min (except where indicated) using four buffers: eluent A (20 mM potassium phosphate, pH 5.6), eluent B (20 mM potassium phosphate–60% methanol, pH 5.6), eluent C (water) and eluent D (methanol). Samples were eluted over 60 min with the following gradient: 0–5 min, 100% eluent A; 5–25 min, 100–71% eluent A, 29% eluent B; 25–26 min, 0–100% eluent D; 26–30 min, 100% eluent D; 30–31 min, 0–100% eluent C; 31–35 min, 100% eluent C (1.5–2 ml/min); 35–45 min, 100% eluent C (2 ml/min); 45–46 min, 100% eluent C (2–1.5 ml/min); 46–47 min, 0–100% eluent D; 47–50 min, 100% eluent D; 50–51 min, 0–100% eluent A; and 51–60 min, 100% eluent A. Absorbance of the eluates was monitored at 267 nm and thymidine and deoxyuridine peaks were quantified by comparing their peak areas with a calibration curve obtained with aqueous standards. For definitive identification of thymidine and deoxyuridine peaks for each sample, we used a second aliquot treated with excess of purified E. coli thymidine phosphorylase (Sigma) to
specifically eliminate thymidine and deoxythymidine. The detection limit of this method is 0.05 mmol/l for both deoxynucleosides.

**Neuromuscular phenotype assessment**

**Rotarod**

Whole body mobility and coordination was assessed by means of an accelerating rotarod performance test (Economex Rotarod, Columbus Instruments) in thymidine-deoxythymidine-treated and untreated Tpp⁻/⁻ Upp⁻/⁻ and wild-type mice.

The Rotarod test was performed with a 3.5-cm diameter rod. Mice were positioned on the rotating rod, four mice were tested simultaneously, with each mouse separated by a 3 mm wide x 60 cm high opaque Plexiglas wall. The Rotarod was started at 4 rpm and accelerated after 15 s at a constant rate of 1 rpm/s. The latency to fall was measured within 60 s; when a mouse ran for 60 s without falling from the rod, the test session was ended. After a training phase of three trials, three motor performances of each mouse were averaged and analysed. Data of each animal were expressed as percentages of controls (untreated double knockout or untreated wild-type animals depending on the test).

**Grip strength**

Grip strength was measured using a Grip Strength Meter (Columbus Instruments) on thymidine-deoxythymidine-treated and untreated Tpp⁻/⁻ Upp⁻/⁻ mice. Mice were allowed to grip a rectangular grid with all four limbs, followed by pulling the mice until they released; three force measurements were recorded in each separate trial.

**Magnetic resonance imaging**

FLAIR images were used to screen for leukencephalopathy. The animals were studied in an Achieva 3 T whole body magnetic resonance scanner (Philips Healthcare) using a research mouse coil (volume receive coil with inner diameter of 40 mm placed perpendicular to the main field). The imaging sequences consisted of coronal T₁-weighted gradient echo (repetition time/echo time = 157/4.6 ms, flip angle = 70°, field of view = 25 x 25 x 10 mm³, voxel size = 0.25 x 0.26 x 0.75 mm³, reconstruction matrix = 256 x 256, averages = 3, total scan duration = 46 s), coronal FLAIR (repetition time/echo time/inversion time = 5500/125/1800 ms, turbo spin echo factor = 15, field of view = 25 x 25 x 22 mm³, voxel size = 0.15 x 0.15 x 0.75 mm³, thickness = 1.5 mm, reconstruction matrix = 176 x 176, averages = 1, total scan duration = 13:48 min), and coronal T₂-weighted turbo spin echo (repetition time/echo time = 6000/109 ms, turbo spin echo factor = 15, field of view = 25 x 25 mm², thickness = 19.5 mm, voxel size = 0.15 x 0.15 x 0.75 mm³, thickness = 0.75 mm, reconstruction matrix = 176 x 176, averages = 6, total scan duration = 13:48 min).

FLAIR brain MRI images were transformed into heatmaps with Tuxpi Photo Editor®. FLAIR intensity signals were quantitated using NIH ImageJ 1.37 V software. Average grey values were calculated within selected areas as the sum of the grey values of all the pixels within selected areas divided by the number of pixels. Four different brain sections of each animal were quantified, and data were expressed in terms of per cent relative to wild-type mice.

**Mitochondrial DNA quantification**

Mouse mitochondrial DNA was quantitated by real-time PCR using an ABI PRISM 7000 sequence detection system using Taqman® MGB primers and probes for murine mt-Co1 gene (mitochondrial DNA) and mouse Gapdh (nuclear DNA) (Spinazzola et al., 2006). Mitochondrial DNA levels were normalized by nuclear DNA, and the data were expressed in terms of per cent relative to wild-type mice.

**Mitochondrial DNA deletions**

Mitochondrial DNA deletions were assessed by long PCR as described previously (Tynismaa et al., 2004). Briefly, mouse mitochondrial DNA was amplified from 25 ng of total DNA with the primers at nucleotides 1953–1924 (reverse) and 2473–2505 (forward) of mouse mitochondrial DNA and using Takara LA Taq™ (Takara Bio Inc.) with buffer 2 and the following PCR conditions: 98°C for 10 s and 68°C for 15 min, x 30 cycles.

**Activities of mitochondrial respiratory chain complexes**

To measure the complex activities, 40 mg tissue was homogenized in CPT medium up to 10% concentration, sonicated for 10 s and centrifuged at 2500 g for 20 min at 4°C. The supernatant was used for protein determination and mitochondrial respiratory chain enzymes analysis as previously described (DiMauro et al., 1987; Quinzi et al., 2013).

**Mitochondrial respiratory chain protein levels**

Thirty micrograms of whole brain tissue extracts were electrophoresed in a SDS 12% PAGE gel, transferred to ImmunoBlot™ PVDF membranes (Bio-Rad) and probed with MitoProfile®. Total OXPHOS Rodent WB Antibody Cocktail of antibodies (MitoSciences). Protein–antibody interaction was detected with peroxidase-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich), using Amersham™ ECL Plus western blotting detection system (GE Healthcare Life Sciences). Quantification of proteins was carried out using NIH ImageJ 1.37 V software. Average grey value was calculated within selected areas as the sum of the grey values of all the pixels in the selection divided by the number of pixels.

**Mitochondria isolation and mitochondrial dNTP pool determination**

We measured the mitochondrial dNTP pool by DNA polymerase extension assay as previously described (Marti et al., 2012). Briefly, homogenates of liver and brain were centrifuged at 10000 g for 5 min at 4°C and supernatants were centrifuged at 13 000 g for 2 min at 4°C (twice). The mitochondrial pellets were suspended in 1 ml cold MTSE buffer (210 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl pH 7.5, 0.2 mM EGTA) and an aliquot of 10 μl was taken to measure proteins. The dNTPs were extracted with 60% methanol and after evaporation, the dry residue was suspended in 65 μl water.

To minimize ribonucleotide interference, total dNTP pools were determined as reported (Ferraro et al., 2010). Briefly, 20 μl volume reactions was generated by mixing 5 μl of sample or standard with 15 μl of reaction buffer [0.025 U/ml Thermo Sequenase™ DNA polymerase (GE Healthcare), 0.75 μM H-dTTP or H-dATP (Moravek Biochemicals), 0.25 μM specific oligonucleotide, 40 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 5 mM DTT]. After 60 min at 48°C, 18 μl of reaction were spotted on Whatman DE81 filters, air dried and washed...
three times for 10 min with 5% Na₂HPO₄, once in distilled water and once in absolute ethanol. The retained radioactivity was determined by scintillation counting.

Morphological studies
To visualize histological features and mitochondrial abnormalities, 8-μm thick frozen sections of brain (cerebellum), quadriceps muscle and small intestine were cut in a cryostat, and haematoxylin and eosin, DAPI (DNA-intercalating dye 4,6-diamidino-2-phenylindole), Masson trichrome, Gomori trichrome, cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) stains were performed (Quinzii et al., 2013). Sections were examined using an Olympus BX51 microscope with a computer-assisted image analysis system, and the images were scanned under uniform light conditions with QCapture software (QImaging).

Nuclei density was quantified in DAPI stained serial transverse sections. Images were acquired at ×200 magnification and processed by NIH ImageJ 1.37 V software. The number of nuclei in muscularis propria was calculated from analyses of 10 different areas per animal of at least three animals per group. The number of nuclei was normalized to the delimited area of the section.

Statistical methods
Data were expressed as the mean ± SD of at least three experiments per group. Log rank test was used to compare the survival proportion of each group of mice. Unpaired t-test with Welch’s correction and Mann Whitney test were used to compare wild-type versus thymidine and deoxyuridine treated Tpp⁻/⁻ Upp⁻/⁻ mice, for molecular and biochemical studies. A P-value of <0.05 was considered to be statistically significant.

Results
Exogenous thymidine and deoxyuridine administration increases levels of thymidine and deoxyuridine
To test the hypothesis that elevated levels of nucleotides cause pathogenic mitochondrial DNA instability in MNGIE, we treated the double knockout (Tpp⁻/⁻ Upp⁻/⁻) mice with exogenous thymidine and deoxyuridine for 2 years. We noted ~3–2000-fold increases of thymidine levels over baseline in treated Tpp⁻/⁻ Upp⁻/⁻ mice in all the organs studied (small intestine, 2000-fold; liver, 800-fold; brain, 78-fold; kidney, 20-fold; and muscle, 3-fold) after 6 months of treatment, compared with untreated wild-type (Table 1).

| Table 1 Analysis of thymidine and deoxyuridine levels |
|---------------------------------|----------|----------|----------|----------|----------|
|                               | Liver    | Kidney   | Brain    | Muscle   | Small intestine |
| Thymidine (pmol/mg protein)    |          |          |          |          |              |
| Untreated wild-type            | ND       | 67       | 3.7      | 50       | ND              |
| Treated wild-type              | 89       | 133      | 19       | ND       | 50              |
| Untreated Tpp⁻/⁻ Upp⁻/⁻         | 138 ± 48 | 196 ± 98 | 17 ± 9   | 27 ± 9   | 431 ± 124       |
| Treated Tpp⁻/⁻ Upp⁻/⁻           | 797 ± 41 | 1419 ± 555 | 236 ± 79 | 144 ± 73 | 2975 ± 1748     |
| Deoxyuridine (pmol/mg protein) |          |          |          |          |              |
| Untreated wild-type            | ND       | 15       | 11       | 2        | ND              |
| Treated wild-type              | ND       | 83       | 57       | 48       | ND              |
| Untreated Tpp⁻/⁻ Upp⁻/⁻         | 23 ± 15  | 83 ± 36  | 22 ± 6   | 16 ± 2   | 1139 ± 1204     |
| Treated Tpp⁻/⁻ Upp⁻/⁻           | 192 ± 63 | 1547 ± 442 | 590 ± 295 | 183 ± 69 | 97 ± 106        |

Thymidine and deoxyuridine levels were increased in all analysed tissues of Tpp⁻/⁻ Upp⁻/⁻ mice treated with exogenous thymidine and deoxyuridine compared with wild-type and untreated double knockout mice. Animals were sacrificed at age 6 months. Data are expressed as mean ± standard deviation of three animals in Tpp⁻/⁻ Upp⁻/⁻ groups and single wild-type animals. ND = not detectable.

Exogenous thymidine and deoxyuridine administration reduces survival, body mass and motor functions in double knockout mice
Untreated and treated wild-type animals survived up to 24 months. In contrast, 16% of untreated Tpp⁻/⁻ Upp⁻/⁻ mice, and 74% of thymidine-deoxyuridine treated Tpp⁻/⁻ Upp⁻/⁻ mice (P < 0.001) died before 24 months of age (Fig. 1A).

Thymidine-deoxyuridine administration reduced body weight in Tpp⁻/⁻ Upp⁻/⁻ animals compared with untreated Tpp⁻/⁻ Upp⁻/⁻, wild-type treated, and untreated animals after 1 year of age.

The drop in body mass was particularly evident at 17 months of age (27.5 ± 0.7 g treated Tpp⁻/⁻ Upp⁻/⁻ versus 32 ± 3.5 g untreated Tpp⁻/⁻ Upp⁻/⁻ versus 38.5 ± 2 g untreated wild-type; P < 0.05; Fig. 1B). This difference was less significant in aged animals (Fig. 1C), probably because only a limited number of treated mutant animals survived.

We evaluated the effects of the treatment on motor skills every month between ages 3 and 12 months. Six to 9 month-old treated Tpp⁻/⁻ Upp⁻/⁻ mice showed exercise intolerance, compared with untreated knockouts. Conversely, untreated wild-type and Tpp⁻/⁻ Upp⁻/⁻ mice showed similar performance in motor tests at these ages (Fig. 2).

Motor coordination and exercise tolerance evaluated by Rotarod showed that thymidine-deoxyuridine treated Tpp⁻/⁻ Upp⁻/⁻ mice between the ages of 6 and 9 months were unable to perform as untreated Tpp⁻/⁻ Upp⁻/⁻ (81.4 ± 26.4% treated Tpp⁻/⁻ Upp⁻/⁻ versus 100 ± 15% untreated Tpp⁻/⁻ Upp⁻/⁻; P < 0.01); whereas treated and untreated wild-type animals had similar latency times to fall (97.9 ± 15% untreated Tpp⁻/⁻ Upp⁻/⁻ versus 100 ± 11% untreated wild-type) (Fig. 2A).
Limb strength evaluated by the grip test showed weakness in treated Tpp⁻/⁻ Upp⁻/⁻ compared with untreated Tpp⁻/⁻ Upp⁻/⁻ mice (88.4 ± 9.8% treated Tpp⁻/⁻ Upp⁻/⁻ versus 100 ± 9.6% untreated Tpp⁻/⁻ Upp⁻/⁻; P < 0.05). Wild-type treated and untreated mice at the same ages (6–9 months) did not show any differences (98.5 ± 9.6% untreated Tpp⁻/⁻ Upp⁻/⁻ versus 100 ± 12% untreated wild-type; Fig. 2B).

Similar to the decrease in body weight, the deficit in motor function was less evident in aged animals, probably because only a limited number of treated knockout animals survived and because the age-related increase of weight affects Rotarod performance.

**Muscle histology in thymidine–deoxyuridine-treated Tpp⁻/⁻ Upp⁻/⁻ mice**

Because of the motor function impairment in treated Tpp⁻/⁻ Upp⁻/⁻ mice, muscle histology was studied in the animals. No morphological changes were observed with haematoxylin and eosin, COX, modified Gomori trichrome and succinate dehydrogenase stains in muscle of the thymidine–deoxyuridine-treated Tpp⁻/⁻ Upp⁻/⁻ mice (Supplementary Fig. 1).

**Brain magnetic resonance imaging**

As leukoencephalopathy is a hallmark of MNGIE, we performed MRI in Tpp⁻/⁻ Upp⁻/⁻ mice treated with the two doses of thymidine and deoxyuridine for up to 2 years. Animals analysed after 1 year of treatment showed slightly stronger FLAIR signal in the brains of untreated double knockout mice (Fig. 3B). In contrast, in treated Tpp⁻/⁻ Upp⁻/⁻ mice, FLAIR MRI showed distinct diffuse cerebral hyperintensity, which was most prominent around the third ventricle and the lateral ventral cortex (Fig. 3C and D). When quantified, cerebral FLAIR hyperintensity in treated mutant mice showed clear trends towards increases (132 ± 16.1% untreated Tpp⁻/⁻ Upp⁻/⁻ versus 163 ± 39.7% treated Tpp⁻/⁻ Upp⁻/⁻), even higher in animals treated for 1 year with a double dose of thymidine and deoxyuridine (204 ± 17.5%). Thus, a dose-related effect was identified in
the brains of mice treated with the standard doses versus those treated with the double dose, increasing the hyperintensity (Fig. 3C, D and I). The duration of the treatment also worsened the leukoencephalopathy evident as further increased FLAIR signal intensity (Fig. 3H). After 2 years, Tpp<sup>−/−</sup>Upp<sup>−/−</sup> animals treated with the lower doses of nucleosides showed more intense FLAIR signal than wild-type mice (151 ± 25.5% untreated Tpp<sup>−/−</sup>Upp<sup>−/−</sup> versus 166 ± 34.3% treated Tpp<sup>−/−</sup>Upp<sup>−/−</sup>; Fig. 3G, H and J). Consistently, the brightest cerebral FLAIR signals were observed in double knockout after 2 years of nucleoside administration, relative to age-matched untreated Tpp<sup>−/−</sup>Upp<sup>−/−</sup> (Fig. 3H), and untreated wild-type animals.

Figure 3 FLAIR MRI signal in the cerebral hemispheres of 12-month-old (A–D) and 24-month-old (E–H) mice showed increased signal, which was more intense in nucleoside treated Tpp<sup>−/−</sup>Upp<sup>−/−</sup> animals (C, D and H) compared with age-matched untreated Tpp<sup>−/−</sup>Upp<sup>−/−</sup> (B and G), which showed slightly increased signal relative to wild-type (WT) animals (A and E). Brain MRI FLAIR signal in mutant animals increased with dose and duration of treatment (D, G, H, I, and J). At least three animals per group were analysed by MRI. Dd = double dose.
Exogenous thymidine and deoxyuridine administration causes mitochondrial DNA depletion in the brain and small intestine of stressed Tpp<sup>−/−</sup> Upp<sup>−/−</sup> mice

Treated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> mice showed mitochondrial DNA depletion in brain (37%) and small intestine (27%), compared to wild-type mice (relative mitochondrial DNA: 62.9 ± 11.6% treated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> versus 85.8 ± 22.1% untreated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> versus untreated wild-type in brain; 72.7 ± 10.2% treated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> versus 121.8 ± 31.4% untreated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> versus untreated wild-type in small intestine) (P < 0.05) (Fig. 4A and B).

Mitochondrial DNA amount in liver (relative mitochondrial DNA: 82.6 ± 25.3% treated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> versus 91.7 ± 19.4% untreated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> versus untreated wild-type) and muscle (relative mitochondrial DNA: 83.3 ± 27.3% treated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> versus 84.4 ± 23.2% untreated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> versus untreated wild-type) showed trends toward reduction compared with untreated wild-type (Fig. 4C and D).

Long PCR to amplify nearly full-length mouse mitochondrial DNA in brain, small intestine and muscle was performed to assess mitochondrial DNA deletions. We did not find any deletions in small intestine of treated and untreated Tpp<sup>−/−</sup> Upp<sup>−/−</sup>, and wild-type mice (Supplementary Fig. 2). Similar levels of deleted mitochondrial DNA PCR products were observed only in muscle, and brain of treated and untreated knockout, and wild-type mice, likely because of the normal ageing process.

Exogenous thymidine and deoxyuridine administration causes mitochondrial respiratory chain deficiency in the brain and reduction of smooth muscle cells in small intestine of thymidine-deoxyuridine stressed Tpp<sup>−/−</sup> Upp<sup>−/−</sup> mice

In the brain of 24-month-old thymidine-deoxyuridine treated mutants, COX (complex IV) biochemical activity normalized to succinate dehydrogenase was significantly decreased, relative to wild-type animals (68.3 ± 8.2% treated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> versus untreated wild-type) (P < 0.05) whereas NADH-dehydrogenase (complex I) activity showed a trend toward reduction (Fig. 5A and B) (Supplementary Table 1) consistent with the observed mitochondrial DNA depletion. In haematoxilin and eosin and COX stained cerebellar sections, no histological changes were detected in treated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> mice (Supplementary Fig. 3A–H). Succinate dehydrogenase activity, an indicator of mitochondria mass, was normal in brain of treated and untreated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> mice (Fig. 5C), indicating absence of mitochondrial proliferation.

Although 24-month-old thymidine–deoxyuridine-treated mutant mice showed depletion of mitochondrial DNA in the small intestine, this tissue had normal biochemical activities of respiratory chain enzymes activities (Fig. 5D and E, and Supplementary Table 1) and COX histochemistry (Fig. 6D and H).

However, histology of the small intestine muscularis propria of thymidine–deoxyuridine-treated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> showed fewer smooth muscle cells in the external layer (Figs 6D and 7D), and increased fibrous tissue relative to untreated mice (Fig. 6L). Relative density of nuclei in the muscularis propria was significantly reduced in treated mutants when compared with untreated wild-type mice (Fig. 7D). Although the treatment did not alter the number of smooth muscle cells of normal animals, untreated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> mice showed a mild reduction in the smooth cell density of muscularis propria (100% ± 9.1% untreated wild-type versus 88.8% ± 17.5% treated wild-type versus 78.3 ± 23.1% untreated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> versus 59.3 ± 9.2% treated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> P < 0.01; Fig. 7E).

By western blot of brain extracts, only mitochondrial DNA-encoded protein COX subunit I (COXI) and the nuclear DNA-encoded protein NDUF8, of complex I, showed non-significant reduction (COXI: 93.8 ± 42.2% treated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> versus untreated wild-type; NDUF8: 91.5 ± 18.4% treated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> versus untreated wild-type) (Supplementary Fig. 4A and C). In contrast, levels of other nuclear-encoded oxidative phosphorylation (OXPHOS) components, ATP5A (complex V), UQRC2 (complex III), and SDHB (complex II) were similar in treated and untreated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> and wild-type mice (Supplementary Fig. 4A and C). The normal to slightly decreased levels of the mitochondrial OXPHOS proteins were consistent with the mild reduction observed in the activity of the respiratory chain complexes. In contrast, and in line with the respiratory chain activity data, no differences in mitochondrial OXPHOS proteins were observed in the small intestine of treated double mutants relative to untreated wild-type mice by immunoblotting (Supplementary Fig. 4B and D).

Unbalanced mitochondrial dNTP in liver and brain of Tpp<sup>−/−</sup> Upp<sup>−/−</sup> mice treated with thymidine–deoxyuridine

Because imbalance of the dNTP precursors of mitochondrial DNA is thought to be the cause of mitochondrial DNA instability in post-mitotic cells in MNGIE, we measured levels of mitochondrial dNTPs in 24-month-old treated double knockout and wild-type animals. Two different tissues were chosen: brain, which harbours a large population of non-dividing neurons and manifests mitochondrial DNA depletion in the double mutant mice, and liver, an organ with replicating cells and no reduction in mitochondrial DNA.

In both tissues, the absolute values of dTTP in mitochondria were significantly increased compared to wild-type and untreated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> littersmates. However, the absolute values of the other three deoxynucleoside triphosphates were not significantly altered in liver, while dGTP was increased in brain (Supplementary Fig 5 and Supplementary Table 2). Notably, levels of dCTP were not significantly decreased in any of these tissues in the treated Tpp<sup>−/−</sup> Upp<sup>−/−</sup> mice, except in the brain of untreated knockouts,
which did not show depletion of mitochondrial DNA (Supplementary Fig. 5 and Supplementary Table 2).

The relative percentages of mitochondrial dNTPs were also assessed. In liver of treated Tpp^{−/−}Upp^{−/−} compared with untreated normal and mutant mice, the proportion of dTTP (dTTP/dNTP) was higher, whereas dCTP/dNTP showed no significant reduction (Fig. 8A).

In treated double knockout brain, the organ with the lowest mitochondrial DNA amount, dTTP/dNTP was higher whereas dCTP/dNTP was less than half of the normal untreated mice (3.5 ± 1.1% treated Tpp^{−/−}Upp^{−/−} versus 11.6 ± 7.2% untreated wild-type) indicating that the mitochondrial dNTP pool imbalance is more severe in brain than in liver (Fig. 8B).

**Discussion**

MNGIE is a rare inherited fatal disease with mean age-at-onset of 19 years and a progressive degenerative course characterized by...
ptosis, progressive external ophthalmoplegia peripheral neuropathy, leukoencephalopathy, gastrointestinal dysmotility and cachexia leading to death at a mean age of 35 years (Garone et al., 2011), caused by TYMP mutations (Nishino et al., 1999). Patients with TYMP mutations have 4–100-fold elevations of thymidine and deoxyuridine that has been shown to cause mitochondrial DNA instability in vitro (Song et al., 2003; Ferraro et al., 2005; Pontarin et al., 2006). Therefore, an early therapeutic strategy was removal of the excess thymidine and deoxyuridine from plasma by haemodialysis or peritoneal dialysis (Spinazzola et al., 2002; la Marca et al., 2006; Yavuz et al., 2007). However, thymidine and deoxyuridine plasma levels did not decrease by peritoneal dialysis and only transiently decreased by haemodialysis, most likely due to the continuous cellular production of nucleosides.

Enzyme replacement by platelet infusions transiently restored thymidine phosphorylase activity and decreased plasma deoxythymidine and deoxyuridine levels (Lara et al., 2007). Other approaches to replace the enzyme by erythrocyte-encapsulated thymidine phosphorylase (Levene et al., 2013), and by polymeric nanometre-sized nanoreactors (De Vocht et al., 2009), which are still in early developmental phases.

Therapeutic benefit has been achieved using allogeneic haematopoietic stem cell transplantation in patients (Hirano et al., 2006; Schüpbach et al., 2009). Nevertheless, despite biochemical and clinical benefit from successful allogeneic haematopoietic stem cell transplantation for patients, the treatment has demonstrated an unacceptably high mortality rate due to the unique clinical features of this disease including: physical frailty of late-stage patients and risk of bowel diverticular perforation (Halter et al., 2011). Thus, a good animal model for MNGIE disease would be very useful to assess efficacy and risks of novel therapies as well as new approaches to overcome the therapeutic risks. Furthermore, unresolved questions remain regarding the role of thymidine and deoxyuridine imbalance in the pathogenesis of the disease such as the tissue-specific vulnerability to thymidine phosphorylase deficiency and slow clinical progression of this inborn error of metabolism. Such issues may best be investigated in vivo to better understand the disease mechanism and to develop new and more rationale therapeutic approaches.

We previously generated and characterized Tpp–/–Upp–/– double knockout mice that manifest 4–65-fold increases of thymidine and deoxyuridine levels in plasma and tissues, compared with levels in wild-type animals (Lopez et al., 2009); however, these...
Figure 7. Nuclei of muscularis propria of small intestine of 24 month-old wild-type and Tpp−/−Upp−/− mice. DAPI staining showed reduced numbers of smooth cells in muscularis propria of thymidine and deoxyuridine-treated Tpp−/−Upp−/− mice (D) compared with the other groups (A–C). (E) Quantification of the relative nuclei density (number of DAPI-stained nuclei/high power field) in muscularis propria of wild type and double knockout mice untreated and treated with thymidine and deoxyuridine shows deoxynucleoside supplementation reduces significantly the number of smooth cells in treated mutant. Bar represents 100 μm. Data are expressed as percent relative to untreated wild-type mice, and expressed as mean ± standard deviation of at least three animals per group (**P < 0.01).

Figure 8. Thymidine and deoxyuridine treated Tpp−/−Upp−/− mice showed altered mitochondrial dNTPs pools. Proportions of dTTP relative to total dNTPs in both liver (A) and brain (B) were increased in treated Tpp−/−Upp−/− compared to untreated and wild-type mice. In brain of mutant mice, the proportion of dCTP relative to total dNTP was ~50% relative to brain of wild-type animals (*P < 0.05).
elevations of nucleosides are less than those observed in patients with MNGIE. The lower levels of thymidine and deoxyuridine in mutant animals compared to patients with MNGIE or the shorter mouse lifespan may account for the mild phenotype of the double mutant animal model (Lopez et al., 2009). Our new results demonstrate that exogenous thymidine and deoxyuridine enhances the phenotype of this animal model.

Exogenous administration of nucleosides in Tpp
−/−Upp
−/− animals increased thymidine and deoxyuridine tissue concentrations >100-fold above those in wild-type animals; levels that are comparable to those observed in plasma and tissues of patients with MNGIE. Consistent with the hypothesis that accumulation of pyrimidine nucleosides is pathogenic in MNGIE, thymidine and deoxyuridine-treated Tpp
−/−Upp
−/− mice showed reduced body weight, weakness, incoordination, and decreased survival that are analogous to the cachexia, neuromuscular weakness, and short lifespan observed in the human disease (Garone et al., 2011). Furthermore, in the treated mutant mice, we observed molecular and biochemical abnormalities in brain and small intestine, which are unaffected in untreated Tpp
−/−Upp
−/− mice.

Although patients with MNGIE typically manifest gastrointestinal dysmotility and cachexia from childhood, double knockout mice treated with the nucleosides did not show loss of body mass until the latter half (age 12–17 months) of normal lifespans. We did not observe signs of vomiting and diarrhoea in the treated mutants, implying that the progressive gastrointestinal dysmotility, which is characteristic of MNGIE, is mild in the animal model. However, mortality of double mutant increased with nucleoside treatment up to 74% at 24 months in contrast to only 16% in untreated knockout mice indicating that the increased levels of thymidine and deoxyuridine shortens lifespans. The causes of death in the treated mutants could not be determined.

In patients with MNGIE, chronic intestinal dysmotility and pseudo-obstruction has been attributed to visceral myopathy, associated with mitochondrial DNA depletion, a mosaic pattern of COX-deficiency in smooth muscle cells, and interstitial fibrosis (Giordano et al., 2008). Analogous to intestinal pathology observed in patients with MNGIE, in nucleoside-treated double knockout mice, the small intestine showed depletion of mitochondrial DNA and histological alterations including COX-deficiency in the muscularis propria (the external muscle layer responsible for peristalsis) with reduced number of smooth muscle cells and increased fibrosis. The muscularis propria of the small intestine may be vulnerable, because of its exposure to high levels of supplemental nucleosides, as they are absorbed through nucleoside transporters in the intestinal tract (Young et al., 2013). The histological alterations associated with high levels of nucleosides strengthen the hypothesis that toxic accumulation of thymidine and deoxyuridine leads to the gradual accumulation of abnormal mitochondrial DNA over years, particularly in post-mitotic tissues such as muscle.

Although asymptomatic in ~80% of cases, leukoencephalopathy is a major criterion for diagnosis of MNGIE and typically manifests in brain MRIs as diffuse involvement of the cerebral hemispheres, with frequent involvement of basal ganglia, cerebellum and brainstem (Nishino et al., 2000; Garone et al., 2011). In brain of Tpp
−/−Upp
−/− mice, mitochondrial DNA depletion was mild (27%) at age 6-months and more severe (50–61%) at ages 14–18 months whereas leukoencephalopathy was detected by MRI in a 22-month-old mutant animal (Lopez et al., 2009). In contrast, after 1 year of thymidine and deoxyuridine administration to the Tpp
−/−Upp
−/− mice, MRI images of brain revealed slight but definite diffuse FLAIR signal hyperintensity, which increased with higher doses and longer durations of treatment. These observations support the hypothesis that the insidious onset of the disease is a result of the accumulation of somatic mitochondrial DNA alterations before clinical manifestations appear.

Thymidine and deoxyuridine treatment increased the levels of the mitochondrial dTTP in brain, most significantly in double knockout mice, but also mildly in wild-type animals. The excess of dTTP was associated with reduction of mitochondrial DNA only in brain, which harbours post-mitotic neurons that are dependent on pyrimidine and purine salvage pathways to generate dNTPs. The negative effect of excess dTTP on mitochondrial DNA replication has been well documented (Ferraro et al., 2005; Pontarini et al., 2006); however, more recent studies have noted that in association with excess dTTP, decreased dCTP reduces synthesis of mitochondrial DNA (Gonzalez-Vioque et al., 2011). We analysed both the absolute amounts of the four different mitochondrial dNTPs and their relative proportions. Although the absolute amount of dCTP was not decreased in analysed tissues (liver and brain) of the treated mutant, the relative proportion of dCTP was most severely reduced (<50% of normal) in brain where marked mitochondrial DNA depletion was observed. These findings support the hypothesis that the unbalanced mitochondrial dNTP ratios, rather than their absolute concentrations, alter the replication of the mitochondrial DNA, and that the combination of increased proportion of dTTP together with relatively less dCTP, decreases mitochondrial DNA synthesis and steady-state levels.

The depletion of mitochondrial DNA in nucleoside-treated Tpp
−/−Upp
−/− mice observed in brain and small intestine (two organs prominently affected in MNGIE) caused histochemically detectable COX deficiency in cells. Although the mitochondrial DNA depletion and COX deficiency were mild overall in the organs, these tissues harbour a variety of cell types that appear to be differentially affected by elevated levels of pyrimidine nucleosides. Post-mitotic cells in the brain and small intestine are more dependent on the mitochondrial pyrimidine salvage pathway than mitotically active cells, like microglia or epithelial cells, which express enzymes of the cytosolic salvage and de novo deoxynucleotide synthetic pathways during the S-phase (Ferraro et al., 2005). Alternatively, susceptibility to thymidine and deoxyuridine accumulation may be related to cell-specific expression of membrane nucleoside transporters (Minuesa et al., 2009; Parkinson et al., 2011; Koczor et al., 2012). In either scenario, selective vulnerability of subpopulations of cells in the brain and intestines likely accounts for the observed leukoencephalopathy and gastrointestinal dysmotility. In patients with MNGIE, mitochondrial DNA depletion was reported in the small intestine, more marked in the external layer of muscularis propria (93%) than in other areas of the small intestine [i.e. internal layer (43%) and ganglion cells (79%)] (Giordano et al., 2008), supporting the notion that severe mitochondrial DNA depletion arises in a specific cell type of the tissues with thymidine phosphorylase deficiency. Likely
consequences of the mitochondrial DNA depletion in the muscularis propria are COX-deficient smooth cells and fibrosis, which are observed in both patients with MNGIE and in thymidine and deoxyuridine treated $\text{Tpp}^{-/-} \text{Upp}^{-/-}$ mice.

In contrast to patients with MNGIE, who manifest abnormal multiple deletions in brain, muscle, kidney and liver (Nishigaki et al. 2003), brain and muscle of 24-month-old treated and untreated wild-type and $\text{Tpp}^{-/-} \text{Upp}^{-/-}$ mice, showed similar levels of deleted mitochondrial DNAs that are likely due to ageing rather than thymidine phosphorylase deficiency (Pikó et al., 1988; Tanhauser and Laipis, 1995; Eimon et al., 1996).

With treatment, mutant mice showed phenotypic changes including decreased latency time in Rotarod tests, indicating loss of coordination, balance, strength or a combination of these factors. Motor coordination is a complex behavioural domain and can reflect balance, muscle strength, and patterned gait, as well as sensory competence, often associated with cerebellar dysfunction; ataxia can also be caused by impairment of motor cortex, striatum, or the spinal cord (Massaquoi and Hallett, 1998). Treated $\text{Tpp}^{-/-} \text{Upp}^{-/-}$ mice also showed decreased muscle strength by the grip test. The absence of morphologically recognizable myopathic, and neurogenic abnormalities in the muscle of treated or untreated $\text{Tpp}^{-/-} \text{Upp}^{-/-}$ mice suggests that the weakness might be due to CNS dysfunction.

It is noteworthy that in contrast to the double knockouts, normal mice treated with thymidine and deoxyuridine did not show overt side-effects or reduced lifespan. These observations may serve as preliminary data for long-term oral administration of nucleosides as therapies for other mitochondrial diseases in which the nucleotide levels are decreased, such as TK2 or DGUOK deficiencies.

In conclusion, the stress of exogenous pyrimidine nucleosides in our $\text{Tpp}^{-/-} \text{Upp}^{-/-}$ mice augments their clinical manifestations including: weight loss, muscle weakness, small intestine muscularis propria pathology, leukoencephalopathy, decreased survival, mitochondrial DNA depletion, and mitochondrial respiratory chain dysfunction, mimicking MNGIE disease. These finding support the pathogenic roles of thymidine and deoxyuridine in MNGIE and represent an important model to study pathogenic mechanisms and new therapeutic approaches.

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**Supplementary material**

Supplementary material is available at Brain online.

**References**


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