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Combined treatment with oral metronidazole and *N*-acetylcysteine is effective in ethylmalonic encephalopathy

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Ethylmalonic encephalopathy is caused by mutations in ETHE1, a mitochondrial matrix sulfur dioxygenase, leading to failure to detoxify sulfide, a product of intestinal anaerobes and, in trace amounts, tissues. Metronidazole, a bactericide, or *N*-acetylcysteine, a precursor of sulfide-buffering glutathione, substantially prolonged the lifespan of *Ethe1*-deficient mice, with the combined treatment being additive. The same dual treatment caused marked clinical improvement in five affected children, with hardly any adverse or side effects.

Accumulated sulfide (H_2S) due to ETHE1 impairment (**Supplementary Fig. 1**) inhibits cytochrome *c* oxidase, short-chain acyl CoA dehydrogenase (COX, also known as SCAD)[1] and possibly other enzymatic activities as well, damages intestinal mucosa and endothelia and alters the vessel tone[2], resulting in hemorrhagic diarrhea, episodes of petechial purpura with edematous acrocyanosis and progressive neurological failure, which are the hallmarks of ethylmalonic encephalopathy. H_2S is mainly released by bacterial anaerobes of the large intestine, but endogenous production in trace amounts occurs in virtually all organs[2], making H_2S a physiological 'gasotransmitter'[3].

We used an *Ethe1*$^{-/-}$ mouse model[1] to test a therapeutic strategy aiming at decreasing bacterial production of H_2S and neutralizing toxic H_2S. Metronidazole[4] is a bactericidal nitroimidazole effective against anaerobic bacteria. We administered the recommended daily dosage for children, 30 mg per kg body weight, by two intraperitoneal injections in six *Ethe1*$^{-/-}$ mice from post-partum day 18 (P18). The median survival shifted from 27 to 54.5 d (**Fig. 1a**).

Mitochondrial glutathione (GSH)[5] can accept the sulfur atom of H_2S through the action of sulfide-CoQ reductase[6] (**Supplementary Fig. 1**), forming nontoxic GSSH persulfide (which can, in turn, become a substrate of ETHE1 (refs. 1,6)). We added *N*-acetylcysteine[7–9], a cell-permeable precursor of GSH[10], to drinking water (1%, pH 7.0) in a second group of six *Ethe1*$^{-/-}$ mice starting on P18, resulting in a daily intake of 5 g per kg body weight. The median survival shifted to 49 d (**Fig. 1a**). Combined treatment led the median survival of a third group of six *Ethe1*$^{-/-}$ mice to shift to 71.5 d, indicating an additive effect (**Fig. 1a**). Although the serum concentration of thiosulfate, a stable and readily measurable index of H_2S, was still much higher than normal, it significantly ($P < 0.0015$, two-tailed, unpaired Student's *t* test) decreased in treated *Ethe1*$^{-/-}$ mice at P30 compared to untreated *Ethe1*$^{-/-}$ mice (**Supplementary Fig. 2**), suggesting that the combined therapy acted specifically against accumulation of H_2S and H_2S-related compounds. Quantitative motor tests performed at P30 showed significantly ($P < 0.016$, two-tailed, unpaired Student's *t* test) lower activity of untreated *Ethe1*$^{-/-}$ mice as compared to control littermates, whereas values in treated *Ethe1*$^{-/-}$ mice overlapped those of the controls (**Supplementary Fig. 3**). At P45, treated *Ethe1*$^{-/-}$ mice actually showed significantly higher motor activity than control littermates ($P < 0.016$, two-tailed, unpaired Student's *t* test) indicating a possible hyperactive state (**Supplementary Fig. 3**). In sharp contrast with untreated *Ethe1*$^{-/-}$ mice, treated *Ethe1*$^{-/-}$ mice had an increase in body weight similar to, albeit less than, control littermates (**Supplementary Fig. 4**). Although the histochemical reaction to COX was equally low in muscle and brain of treated and untreated *Ethe1*$^{-/-}$ mice at P30 (**Supplementary Fig. 5**) and remained low in a treated *Ethe1*$^{-/-}$ mouse at P45 (data not shown), the severe COX depletion in colonocytes of untreated *Ethe1*$^{-/-}$ mice at P30 disappeared completely and persistently in the treated *Ethe1*$^{-/-}$ mice (**Fig. 1b**). This result suggests that recovery of COX proficiency varies widely between rapidly proliferating cells such as colonocytes and postmitotic cells such as muscle fibers and neurons, possibly because of the combined effect of H_2S-mediated inhibition and structural degradation of COX[1] and slow turnover of mitochondrial respiratory chain complexes in mouse muscle[11] and neurons[12]. COX deficiency in essential tissues may ultimately contribute to early death of treated mice, in spite of the marked delay associated with therapy.

Given the results in the mouse model, we treated an Italian patient (subject 1), carrying a homozygous mutation in *ETHE1* (ref. 13) encoding an L55P substitution. He had manifested the clinical and biochemical signs typical of ethylmalonic encephalopathy since 6 months of age (**Fig. 1c** and **Supplementary Fig. 6**). Despite dietary manipulations consisting of reducing intake of monosaccharides, which have a stronger osmotic effect, in favor of polysaccharides and consuming only hydrolyzed proteins, as well as coenzyme Q10 supplementation (50 mg per day), progression of his main clinical features occurred in the following months. A magnetic resonance imaging (MRI) scan at 28 months showed brain atrophy, leukodystrophy and patchy lesions of the basal nuclei (**Fig. 1c**). Diffuse petechiae of the trunk and limbs and acrocyanosis were worsened, as observed by the patient's physician.

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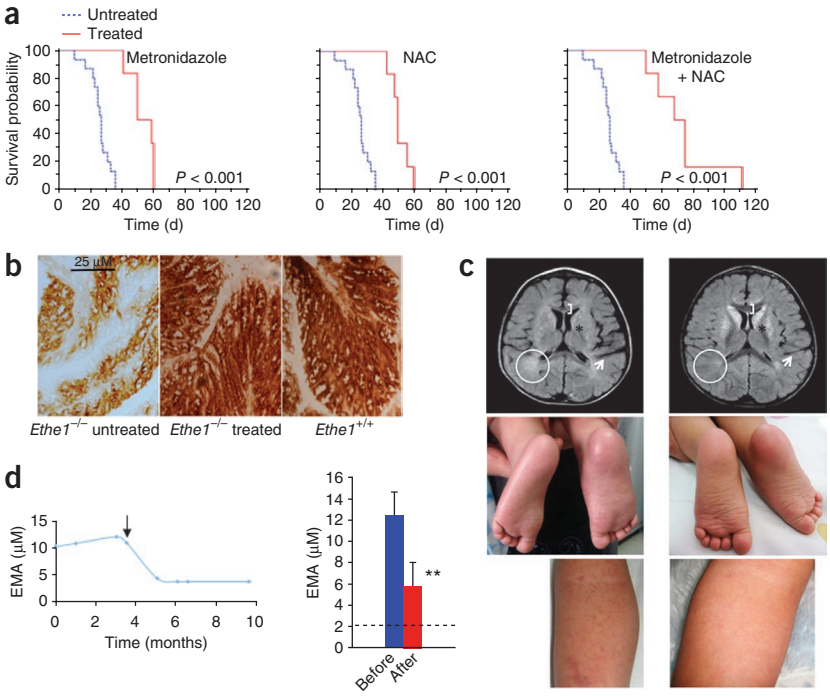

Figure 1 Clinical, morphological and biochemical features of *Ethe1*$^{-/-}$ mice and human subjects. (**a**) Kaplan-Meyer survival probability curves of untreated versus treated *Ethe1*$^{-/-}$ mice. The *P* values refer to the results of the log rank test. NAC, *N*-acetylcysteine. (**b**) COX histochemical reaction in the large intestine of P30 mice. In the untreated *Ethe1*$^{-/-}$ specimen (left) COX is absent in mucosal cells facing the lumen and reduced in cryptal cells, whereas a comparable reaction is present in treated *Ethe1*$^{-/-}$ (middle) and control (right) *Ethe1*$^{+/+}$ specimens. (**c**) Neuroradiological and cutaneous features in patients before and during metronidazole and *N*-acetylcysteine combined treatment. Top left, fluid attenuated inversion recovery MRI of a transverse section of subject 1's brain at 28 months of age, one month before starting the treatment; top right, the same sequence as at left performed at 36 months of age, 7 months after starting the treatment. Compared to the image at left, the right one shows reduction of brain atrophy (see the difference in size of the sylvian fissure, indicated by an arrow, and lateral ventricles), regression of white matter changes (see the encircled area of the centrum semiovale) and increased thickness of the genu of the corpus callosum (bracket), but increased abnormalities in the neostriatum (asterisk). Middle left, severe plantar acrocyanosis and swelling in subject 2 before treatment (age 30 months); middle right, the same frame shot as at left of subject 2 after treatment (age 36 months) shows the resolution of both acrocyanosis and edema. Bottom left, petechial rash in subject 3 (age 17 months); bottom right, the rash resolved after treatment (age 23 months). (**d**) Plasma EMA before and after treatment. Left, serial measurements of plasma EMA in subject 1 before (mean value = 10.5 ± 1.5 μM) and after (mean value = 4.2 ± 0.3 μM) the start (arrow) of combined metronidazole and *N*-acetylcysteine treatment (unpaired Student's *t* test $P = 3.65 \times 10^{-6}$). Right, plasma EMA in subjects 1–5 before and after the start of metronidazole and *N*-acetylcysteine treatment. $^{**}P = 0.003$ (paired, two-tailed Student's *t* test). Informed consent was obtained from the human subjects' parents for the use of biological material from all human subjects. Mouse studies were approved by the Ethics Committee of the Foundation 'Carlo Besta' Neurological Institute, in accordance with guidelines of the Italian Ministry of Health. The use and care of mice followed the Italian Law D.L. 116/1992 and the EU directive 86/609/CEE. Standard food and water were given *ad libitum*.

At 29 months of age, we added a daily therapy consisting of oral metronidazole (30 mg per kg body weight) and *N*-acetylcysteine (105 mg per kg body weight); the latter was administered orally but with the intravenous formulation to avoid collateral osmotic effects due to the sugar additive of the granular preparation. During the next 8 months, we observed an increase in body weight (+1.5 kg), a marked reduction and then virtual disappearance of diarrhea, petechial showers and acrocyanosis (**Table 1** and **Supplementary Fig. 6**) and marked neurological improvement, with a lower frequency of seizure attacks from several per day to less than one a week. The EEG showed the disappearance of slow-voltage waves. Truncal hypotonia became less severe, and spontaneous motor activity ensued at the lower limbs. A brain MRI at 36 months of age showed a reversion of brain atrophy and a reduction in leukodystrophy, although the lesions in the neostriatum had become more evident (**Fig. 1c**). Ethylmalonic acid (EMA) in urine and plasma dropped significantly and persistently (**Fig. 1d** and **Table 1**), and so did the concentrations of C4 acylcarnitines (**Table 1**). Occasional gastroesophageal reflux after ingestion of *N*-acetylcysteine responded well to ranitidine.

Table 1 Clinical and laboratory features of individuals with ethylmalonic encephalopathy

	Age (months) at treatment start	Treatment duration (months)	Body weight (kg)		Diarrhea[a]		Petechiae		Acrocyanosis		Tone		Seizures		Urinary EMA (mmol per mol Cr)[b]		Plasma EMA (μM)[c]		Plasma thiosulfate (μM)[d]		Plasma C4 carnitines (μM)	
			Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Subject 1	29	8	9	10.5	+++	–/+	Diffuse, spontaneous	None	Severe	None	Hypo	Less severe	Several per day	<1 per week	1066 ± 466	112 ± 15.2	10.5 ± 1.5	4.2 ± 0.3	137.1	13	8.4	1.95
Subject 2	30	6	8.5	10	+++	–/+	Diffuse, spontaneous	Occasional[e]	Severe	Occasional[e]	Hypo	Normal	None	None	ND	ND	11.4	4.3	30.7	14.3	ND	ND
Subject 3	17	6	8	9	++++	–/+	Diffuse, spontaneous	Rare[e]	Severe	Occasional[e]	Rigid	Marked improvement	None	None	ND	ND	11.9	6.3	51.6	29.5	ND	ND
Subject 4	10	3	7	7.3	+++	–/+	Diffuse, spontaneous	Rare[e]	Severe	None	Hypo	Marked improvement	None	None	ND	ND	16.1	5.8	17.6	9.1	ND	ND
Subject 5	10	3	7.8	8	++++	++	Diffuse, spontaneous	Rare[e]	Severe	Severe	Hypo	Marked improvement	None	None	ND	ND	12.9	8.9	172.4	67.2	ND	ND

Hypo, hypotonic; ND, not determined. Cr, creatinine.
[a]–/+, one or less than one bowel movement per day; ++, up to five; +++, 5–10; ++++, >10. [b]Two-tailed unpaired Student's *t* test, $P < 0.0007$. [c]For statistical analysis, see legend of **Figure 1**. [d]Two-tailed Wilcoxon test for paired samples, $P = 0.043$. [e]Upon excessive crying or by applying pressure.

We were encouraged enough by the long-lasting improvement of subject 1 to extend the same therapy to four Palestinian patients, a 32-month-old boy (subject 2), a 19-month-old boy (subject 3), a 12-month-old girl (subject 4) and a 10-month-old boy (subject 5), all formally unrelated but sharing the same homozygous 505+1G>T splice-site mutation in *ETHE1* (ref. 14). After >3 months of therapy, these patients showed clinical, biochemical and MRI improvements similar to those of subject 1 (**Table 1**, **Fig. 1c** and **Supplementary Fig. 7**). In all five subjects, plasma EMA and serum thiosulfate (**Fig. 1d** and **Table 1**) concentrations consistently decreased during treatment, indicating a specific and considerable effect of the combined therapy on the metabolic abnormality associated with ethylmalonic encephalopathy.

The noteworthy prolongation of lifespan in *Ethe1*$^{-/-}$ mice and the evidence-based clinical improvement in patients make the combined treatment with metronidazole and *N*-acetylcysteine the most effective therapy available for ethylmalonic encephalopathy. Prenatal screening of the *ETHE1* gene in at-risk fetuses, and of urinary EMA in neonates, could prompt pediatricians to apply this treatment in a timely manner to prevent or reduce irreversible brain damage.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

Study design: V.T. and M.Z.; clinical evaluation and sample collection: A.B.B. and I.D.; neuroradiological evaluation: M.S.; histochemical evaluation: C.L.; biochemical assays: A.B.B. and T.H.; mouse treatment and evaluation: C.V.; statistical analysis: C.V.; manuscript writing: M.Z.; critical revision of the manuscript: C.V., V.T. and M.Z.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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