Methionine supplementation for multi-organ dysfunction in MetRS-related pulmonary alveolar proteinosis

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Shareable abstract (@ERSpublications)
Pulmonary alveolar proteinosis related to mutations in *MARS1* is a rare and severe lung disease with early onset and no curative treatment to date. In four affected children we showed that methionine supplementation is an effective treatment. https://bit.ly/2WlbcOz

Cite this article as: Hadchouel A, Drummond D, Pontoizeau C, *et al*. Methionine supplementation for multi-organ dysfunction in MetRS-related pulmonary alveolar proteinosis. *Eur Respir J* 2022; 59: 2101554 [DOI: 10.1183/13993003.01554-2021].

This article has an editorial commentary: https://doi.org/10.1183/13993003.02971-2021

Received: 2 June 2021
Accepted: 17 Aug 2021

Abstract

Introduction Pulmonary alveolar proteinosis related to mutations in the methionine tRNA synthetase (*MARS1*) gene is a severe, early-onset disease that results in death before the age of 2 years in one-third of patients. It is associated with a liver disease, growth failure and systemic inflammation. As methionine supplementation in yeast models restored normal enzymatic activity of the synthetase, we studied the tolerance, safety and efficacy of daily oral methionine supplementation in patients with severe and early disease.

Methods Four patients received methionine supplementation and were followed for respiratory, hepatic, growth and inflammation-related outcomes. Their course was compared to those of historical controls. Reactive oxygen species production by patient monocytes before and after methionine supplementation was also studied.

Results Methionine supplementation was associated with respiratory improvement, clearance of the extracellular lipoproteinaceous material and discontinuation of whole-lung lavage in all patients. The three patients who required oxygen or noninvasive ventilation could be weaned off within 60 days. In addition, liver dysfunction, inflammation and growth delay improved or resolved. At a cellular level, methionine supplementation normalised the production of reactive oxygen species by peripheral monocytes.

Conclusion Methionine supplementation was associated with important improvements in children with pulmonary alveolar proteinosis related to mutations in the *MARS1* gene. This study paves the way for similar strategies for other tRNA synthetase deficiencies.

Introduction

Pulmonary alveolar proteinosis (PAP) is characterised by alveolar accumulation of lipoproteinaceous material derived from surfactant [1]. It has multiple causes. We previously described a specific type of PAP prevalent on the island of La Réunion, characterised by early onset, associated liver involvement, systemic inflammation, frequent progression to lung fibrosis and poor prognosis [2]. Mortality reached 59%. Half of the deaths occurred before the age of 2 years, despite repetitive and frequent whole-lung lavage (WLL) and other treatments, such as high-dose intravenous steroids that were used in severely affected patients who displayed important inflammation. Mutations in *MARS1* were subsequently identified as disease-causing [3] and the phenotype was described as interstitial lung and liver disease in the Online Mendelian Inheritance in Man database (#615486). *MARS1* encodes the cytosolic methionine tRNA synthetase (MetRS), which plays a critical role in protein biosynthesis by charging tRNAs with methionine, leading to the formation of methionyl-tRNA. The double homozygous Ala393Thr/Ser567Leu mutations found in the "Réunion" patients are located in the catalytic domain of MetRS and severely impair growth and enzymatic activity in yeast, which is restored by methionine supplementation [3]. Enzymatic preparations purified from transfected *Escherichia coli* have confirmed the significant impact of the mutations on the rate of the aminoacylation reaction (reduction of the catalytic constant by five- to six-fold relative to wild-type), especially for methionine affinity, as shown by a significant increase in the Michaelis constant for methionine in mutants [4]. Patients of other ethnicities have been described, with other mutations, but a very similar phenotype [3, 5–10]. As enzymatic activity can be restored by methionine supplementation in yeast, we aimed to treat successive patients with standardised methionine supplementation to assess safety and tolerance of such supplementation, and to compare the evolution of the treated patients to that of our historical cohort.

Material and methods

Study design

This study was approved by the Comité de Protection des Personnes Est-II (CPP18/11/28/42028) and registered at clinicaltrials.gov (NCT03887169). It aimed to determine the efficacy, safety and tolerance of daily oral supplementation of methionine in patients presenting with PAP due to Ala393Thr/Ser567Leu mutations in *MARS1*. Patients were referred to the paediatric pulmonology unit at Necker Hospital (Paris, France) for their care. Written informed consent was obtained from the parents. Outcomes were assessed at 2 months of treatment. The supplementation was pursued if it was efficient and well tolerated. Data are presented at three time-point assessments: before starting the supplementation (D0), 2 months after the supplementation was started (M2) and at last follow-up (LFU). In France, methionine has the status of a nutritional supplement and is widely and freely available.

Administration scheme

Methionine was given as L-methionine powder diluted in water and was administrated orally or enterally, every 6 h, starting at 80 $mg \cdot kg^{-1}$ per day and progressively increased until plasma concentrations between 45 and 500 µM were obtained at residual and peak states (1 h after intake). Methods to determine the doses, targeted ranges and frequency of supplementation are detailed in the supplementary material.

Measured outcomes

Efficacy of the treatment was evaluated based on the respiratory, hepatic, inflammatory and growth status. Respiratory assessment included regular clinical evaluation, chest computed tomography (CT) at D0, M2 and at LFU, pathological aspects of bronchoalveolar lavage (BAL) fluid and the possibility to discontinue the WLL. Liver status was assessed by clinical examination, liver ultrasound and liver function tests. Growth and nutritional status were assessed by monitoring growth curves and albuminemia. Systemic inflammation was assessed by measuring C-reactive protein, the erythrocyte sedimentation rate, blood neutrophil count and IgG levels.

Patients were monitored for potential adverse effects that included liver dysfunction and central nervous system abnormalities, especially a risk of cerebral oedema as described in congenital hypermethioninaemia at methionine plasma levels >800 µM [11]. Other possible adverse events were variations in arterial blood pressure, nausea, vomiting, dizziness and polyuria, as described in subjects receiving a loading dose of methionine to study the relationship between homocysteine levels and cardiovascular disease. Those effects were mild and transient [12]. Plasma concentrations of homocysteine, which derived from methionine, were monitored. Supplementation with vitamins B6, B9 and B12 was initiated when homocysteine exceeded 30 µM to favour the remethylation of homocysteine back to methionine [13].

Comparison to the historical controls

We compared the course of treated patients to that of patients reported by Enaud *et al.* [2] as well as seven patients who were diagnosed since that publication. All those patients harboured the Ala393Thr/Ser567Leu genotype.

Western blot

MetRS protein expression in peripheral blood mononuclear cells was assessed by Western blot. Methods are detailed in the supplementary material.

Priming of reactive oxygen species production by peripheral monocytes

Function of peripheral monocytes was assessed by quantifying reactive oxygen species (ROS) production, as detailed in the supplementary material.

Statistical analyses

For historical controls, data were expressed as median and interquartile range. We computed the difference between the values for each continuous variable at D0 and at LFU for each treated patient, as well as between diagnosis and 6 months to 1 year from diagnosis for the historical controls. Differences were compared between groups using Mann–Whitney tests. For each categorical variable (*i.e.* weaning from oxygen and enteral nutrition), we compared the proportion of patients who were weaned from such support at the second assessment between groups using Fisher's exact test. A p-value <0.05 was considered statistically significant.

Results

Disease course and efficacy of methionine supplementation

The patients' characteristics before treatment are presented in table 1 and disease course for each patient is summarised in table 2.

Patient 1

Patient 1 (P1) was referred to her local hospital at 4 months of age for vomiting, failure to thrive, enlarged liver and tachypnoea. At that time, chest CT showed only discrete lesions (figure 1). However, BAL fluid was macroscopically opalescent and its pathological examination confirmed the diagnosis of PAP (figure 1). Molecular diagnosis of *MARS1* mutations was made subsequently. Before starting methionine supplementation, P1 had severe growth failure and hypotonia, required continuous supplemental oxygen, enteral nutrition and experienced chronic vomiting. Laboratory parameters showed anaemia, cholestasis, mild elevated aspartate transaminase (AST), hypoalbuminemia, inflammation and high IgG level (table 2). Ultrasound showed hepatomegaly with hyperechoic parenchyma. Brain magnetic resonance imaging (MRI) was normal. Supplementation with methionine was started at 6 months of age. She underwent seven therapeutic WLLs from D7 to D61 of treatment. She was weaned from oxygen on D42 and enteral nutrition on D54, with resolution of vomiting. On M2, all clinical and biological features were improved (table 2). Chest CT showed improvement. Echogenicity of the liver normalised. We decided to pursue the treatment. She was discharged home on D71. She was admitted for a new assessment at 9 months of age; 1 month after the last WLL. The BAL showed improvement, with total clearance of the extracellular lipoproteinaceous material and a marked decrease in the proportion of vacuolised Oil Red O (ORO)-positive macrophages (figure 1). At the last follow-up 10 months later, she was asymptomatic. Her weight had reached the mean on the growth curve (table 2). There was no neurological impairment nor developmental delay. She was not taking any other treatment apart from methionine (30 $mg \cdot kg^{-1}$ per 6 h) and no therapeutic WLL had been performed since D61. Treatment every 6 h led to reproducible residual and peak values of methionine plasma concentrations (supplementary figure S1). Apart from a moderately persistent elevated sedimentation rate, all her laboratory parameters had returned to normal (table 2). Size and echogenicity of the liver normalised. Her chest CT showed very discrete postero-basal ground-glass opacities, with no signs of fibrosis (figure 1).

Patient 2

In patient 2 (P2), *MARS1*-related PAP was diagnosed at 5 months of age. She had already undergone 25 WLLs. She received monthly *i.v.* steroid pulses (300 $mg^{-1} \cdot m^{-2}$ per day for 3 days each) and daily oral steroids from the age of 11 months in order to control her respiratory and inflammatory status. She became steroid-dependent with respiratory relapses when decreasing steroids, and developed several complications with systemic arterial hypertension and osteoporotic fractures. She was started on mycophenolate mofetil (MMF) at the age of 21 months, which allowed tapering then stopping of the steroids at the age of 25 months, and spacing the WLL every 6 months. She was the first patient treated with MMF. She still showed feeding difficulties, refusing oral feeding, presenting regular vomiting and requiring total enteral

TABLE 1 Patient characteristics at baseline

	Patient 1	Patient 2	Patient 3	Patient 4
Ethnic origin	Réunion	Mayotte	Réunion	Comoros
Gender	Girl	Girl	Girl	Boy
Age at diagnosis (months)	4	5	3	3
Age at treatment initiation (months)	6	35	6	21
Respiratory status				
Oxygen dependency	Yes	No	Yes	Yes
Ventilatory support	No	No	No	Yes
Growth and nutrition				
Weight (kg) (z-score)	4.2 (−3.4)	14.9 (+1)	4.1 (−3.7)	8.4 (−2.7)
Regimen	Enteral (65%) + oral (35%)	Complete enteral nutrition	Enteral (70%) + oral (30%)	Complete parenteral nutrition
Vomiting	Yes	Yes	Yes	Yes
Neurodevelopmental assessment				
Clinical impairment	Yes, hypotonia	No	Yes, hypotonia	Yes, sat with support, vocalises syllables, no words
Brain MRI[#]	Normal	Periventricular cysts	Periventricular cysts	Normal
Liver status				
Enlarged liver	Yes	Yes	Yes	Yes
Elevated AST/ALT	Yes	No	Yes	No
Elevated GGT	Yes	No	Yes	Yes
Haematological status				
Anaemia	Yes	No	Yes	Yes
High neutrophil count	Yes	No	Yes	Yes
Thrombocytosis	Yes	No	No	No
Inflammation[¶]	Yes	Yes	Yes	Yes
Daily methionine intake at baseline (mg)	231	650	256	297
Fasting methionine plasma concentration (μM)[+]	18	33	13	23

MRI: magnetic resonance imaging; AST: aspartate transaminase; ALT: alanine transaminase; GGT: γ-glutamyltransferase. [#]: performed at diagnosis for patients 1, 2 and 3, and at 6 and 22 months for patient 4; [¶]: diagnosed by the presence of a high level of C-reactive protein, a high sedimentation rate or both; [+]: normal laboratory values: 17–45 μM.

nutrition using a gastrostomy. At the time methionine supplementation was started, she had WLL every 6 months, and had been taking MMF for 15 months. She displayed discrete persistent inflammation (slightly elevated values of the sedimentation rate and IgG) that resolved at the last follow-up (table 2). She underwent one WLL which showed only mild lipoproteinaceous material deposition. After 2 months of supplementation with methionine, she was starting to eat by herself and the nausea and vomiting had disappeared. We decided to pursue the supplementation. After 1 year of treatment (last follow-up), she showed a significant decrease in her feeding difficulties, along with satisfactory growth (table 2). Her chest CT, which was already greatly improved after MMF initiation, showed no further changes after methionine supplementation. There were no signs of fibrosis. WLL and MMF were discontinued. She was taking methionine at a dosage of 27 $mg^{-1} \cdot kg^{-1}$ per 6 h, with reproducible residual and peak values of methionine plasma concentrations (supplementary figure S1).

Patient 3

At diagnosis at 4 months of age, and before starting methionine supplementation, patient 3 (P3) displayed a similar clinical, biological and pathological presentation as P2 (table 2 and figure 1). Her chest CT showed a more pronounced crazy-paving aspect (figure 1). Ultrasound showed an enlarged liver. Brain MRI showed periventricular cysts. Supplementation with methionine was started at 6 months of age. She underwent two therapeutic WLLs on D16 and D45. Vomiting decreased from D10 and finally ceased on D31. She was weaned from oxygen on D47 and enteral nutrition on D71. On M2, all clinical and biological features were improved (table 2). Control chest CT showed a clear improvement, with regression of posterior consolidations and only persistent scattered subpleural pseudonodular consolidative lesions. The size of the liver decreased on ultrasound. Because of these results, we decided to pursue the treatment. She was discharged home at M2. She was admitted for a new assessment at 11 months old. Clinically, her respiratory and growth status continued to improve. Her chest CT showed new improvement, with almost complete regression of the subpleural pseudonodular consolidations. A subtle very low-density

https://doi.org/10.1183/13993003.01554-2021

TABLE 2 Disease course under methionine supplementation

	Patient 1			Patient 2			Patient 3			Patient 4		
	D0 6 months	M2	LFU 19 months	D0 3 years	M2	LFU 4.5 years	D0 6 months	M2	LFU 16 months	D0 21 months	M2	LFU 33 months
Clinical features												
Weight (kg) (z-score)	4.2 (−3.4)	4.9 (−3.3)	10.2 (0)	14.9 (+1)	15 (+1)	17.3 (+1)	4 (−3.7)	5.3 (−3)	8.4 (−1.6)	8.4 (−2.7)	10 (−1.5)	10.6 (−2)
Respiratory rate (cycles per min)	70	32	32	28	24	18	60	55	27	65	65	42
Oxygen dependency	1 L·min^{-1}	No	No	No	No	No	0.5 L·min^{-1}	No	No	2–4 L·min^{-1} +c-NIV	D: 0.5 L·min^{-1} N: 0.8 L·min^{-1}	No
Nutritional regimen	65% E 35% O	100% O	100% O	100% E	80% E 20% O	35% E 65% O	70% E 30% O	35% E 65% O	100% O	100% P	80% P 20% E	70% E 30% O
Nausea/vomiting	Yes	No	No	Yes	No	No	Yes	No	No	Yes	No	No
Psychomotor impairment	Hypotonia	None	None	None	None	None	Hypotonia	None	None	Important delay	Sat alone, said a few words	Walks
Biological features												
Haemoglobin (g·dL^{-1})	9.1	10.4	11.7	11	11.3	11	8.7	10.1	10.8	9.2	9.3	11.5
Leukocytes (cells·mm^{-3})	31 700	5100	6900	6400	9200	8300	20 900	13 500	15 700	18 820	8200	7700
Neutrophils (cells·mm^{-3})	18 100	1900	1400	1700	4500	2400	11 200	3100	3300	14 490	3100	2200
Platelets (cells·mm^{-3})	668 000	218 000	339 000	157 000	235 000	252 000	441 000	565 000	406 000	189 000	183 000	165 000
Sedimentation rate (mm)	56	46	25	11	7	7	87	43	58	34	27	44
CRP (mg·L^{-1})	53.8	8.3	<5	<5	<5	<5	15.8	<5	<5	71.4	<5	<5
IgG (g·L^{-1})	21.52	10.94	8.03	9.23	10.89	8.68	18.03	11.3	13.93	15.07	11.32	13.34
Albumin (g·L^{-1})	23.2	38.4	42.3	38	44.2	37.1	28.7	43	45.2	6	26.5	30
Prothrombin rate (%)	54	71	86	91	85	92	72	85	82	81	71	80
AST (IU·L^{-1})	81	44	52	45	28	31	101	51	48	50	108	57
ALT (IU·L^{-1})	23	16	25	28	22	23	65	29	22	7	44	24
GGT (IU·L^{-1})	64	44	13	16	17	12	406	18	16	198	214	71
T bilirubin (µM)	47	4	4	4	4	4	4	3	2	30	13	4
C bilirubin (µM)	36	2	0	0	0	0	4	0	0	13	0	0
Other treatments												
WLL	+	–	–	+	–	–	+	–	–	+	–	–
Steroids	–	–	–	–	–	–	–	–	–	+	–	–
MMF	–	–	–	+	+	–	–	–	–	–	–	–

D0: before starting the supplementation; M2: month 2; LFU: last follow-up; CRP: C-reactive protein; AST: aspartate transaminase; ALT: alanine transaminase; GGT: γ-glutamyltransferase WLL: whole-lung lavage; MMF: mycophenolate mofetil; c-NIV: continuous noninvasive ventilation; D: day; N: night; E: enteral; O: oral; P: parenteral.

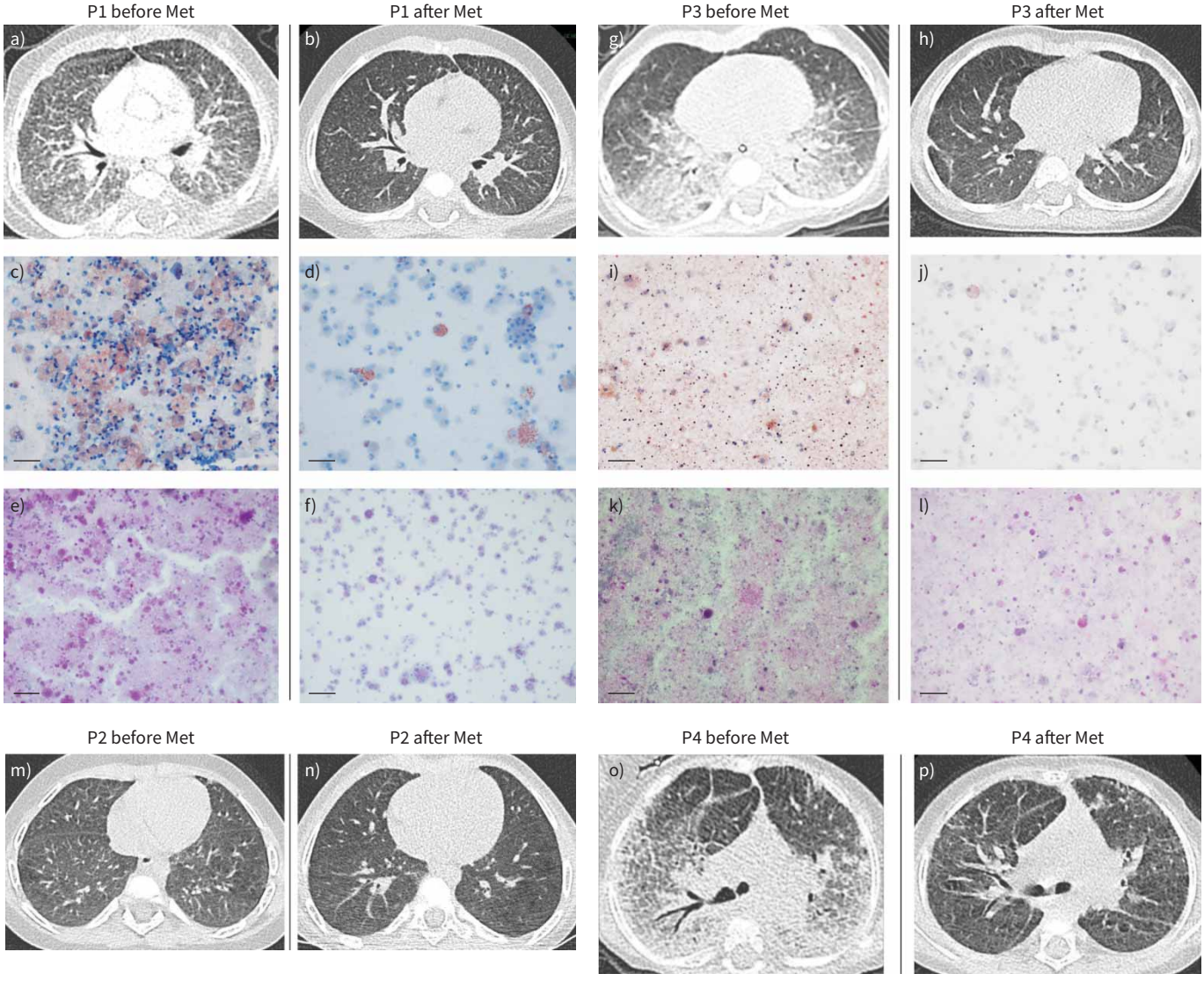

FIGURE 1 Comparison of imaging and pathological data before and after supplementation with methionine (Met). a–f) and g–l) computed tomography (CT) and bronchoalveolar lavage (BAL) fluid images for patients 1 (P1) and 3 (P3), respectively. a, g) CT at diagnosis; b, h) CT at the last follow-up; c–f, i–l) pathological aspects of BAL fluid; c, e, i, k) first whole-lung lavage (WLL); d, f, j, l) last BAL. c, d, i, j) Oil Red O (ORO) staining, ×200, scale bars=50 μm; e, f, k, l) Periodic Acid Schiff staining (PAS), ×100, scale bars=100 μm. a) The CT, which was performed very early in the course of the disease, showed discrete lesions with anterior hyperinflation, low-density ground-glass opacities, intralobular lines and thickened interlobular septa with an anteroposterior density gradient; b) after 13 months of treatment, the CT showed a disappearance of the crazy paving pattern; c, d) this was associated with a decrease in total cellularity of the fluid and in the number of ORO-positive macrophages, ranging from 100% before to 13% under treatment at day 90; e, f) PAS staining showed the complete disappearance of the abnormal extracellular lipoproteinaceous material, highly present before treatment, after 3 months of supplementation. g) The CT showed diffuse ground-glass opacities, thickened interlobular septa and intralobular lines (crazy-paving pattern), with an increasing gradient of density in posterior and inferior areas; h) after 11 months of treatment, the CT showed a clear improvement, with complete regression of the subpleural pseudonodular consolidations and of the crazy-paving pattern. A subtle crazy-paving pattern of very low density remained; i, j) this was associated with a decrease in cellularity of the fluid and in the number of ORO-positive macrophages ranging from 90% before treatment to 7% after 5 months of treatment; k, l) PAS staining showed partial regression of the abnormal extracellular lipoproteinaceous material, highly present before treatment, after 5 months of supplementation. m, n) Patient 2 (P2): m) chest CT before starting methionine; after 25 WLLs, several series of methylprednisolone pulses and 15 months of treatment with mycophenolate mofetil (MMF), showed no specific lesion apart from discrete ground-glass opacities that could be related to breathing movements; n) chest CT performed after 18 months of supplementation with methionine and after 6 months of discontinuation of MMF showed no specific lesion and especially no sign of fibrosis. o, p) Patient 4 (P4): o) chest CT before starting methionine; after 19 WLLs and four series of methylprednisolone pulses, CT showed a crazy-paving appearance with an increasing gradient of density in the posterior and inferior areas, along with subpleural and intraparenchymal microcystic lesions; p) chest CT performed after 12 months of supplementation with methionine showed an important regression of consolidations. Microcystic lesions remained stable.

crazy-paving pattern remained, with no signs of fibrosis. She underwent a BAL, which showed partial regression of the extracellular abnormal lipoproteinaceous material and a marked decrease in the number of vacuolised ORO-positive macrophages (figure 1). Size of the liver normalised on ultrasound. At the last follow-up 5 months later, she had been taking methionine for 10 months with no other treatment. Her current dosage was 28 $mg^{-1}\cdot kg^{-1}$ per 6 h with stable methionine plasma concentrations (supplementary figure S1). She had no respiratory symptoms and growth status continued to improve (table 2). She had no neurological impairment nor developmental delay and brain MRI was not controlled. Her chest CT showed new improvement with only discrete lesions (figure 1).

Patient 4

In patient 4 (P4), *MARS1*-related PAP was diagnosed at 3 months of age. Before starting methionine, despite repetitive WLL (n=19) and monthly *i.v.* steroid pulses, P4 was severely affected by chronic respiratory insufficiency, requiring continuous noninvasive ventilation (NIV) with oxygen, and growth failure and recurrent vomiting necessitating exclusive parenteral nutrition (table 2). He had a severe psychomotor delay, was not able to sit without support and vocalised a few syllables. Laboratory parameters showed anaemia, cholestasis, hypoalbuminemia, inflammation and high IgG level. He was dependent on blood transfusions and albumin perfusions. Liver was enlarged and hyperechoic on ultrasound. Chest CT showed a crazy-paving appearance, with an increasing posterior and inferior gradient of density, along with microcystic lesions suggestive of early-stage fibrosis (figure 1). Brain MRI was normal on two occasions. The last WLL and the last *i.v.* steroid pulses had been performed 1 month before the beginning of methionine supplementation. After starting methionine at 21 months of age, he was weaned from NIV on D38, with a progressive decrease in oxygen supply. He was weaned from parenteral nutrition on D87. The last blood transfusion and albumin perfusion were performed on D79 and D56, respectively. A chest CT performed after 2 months of treatment showed a marked decrease in the density and extension of consolidations; microcystic lesions remained stable. On ultrasound, size of the liver was stable, but echogenicity returned to normal. P4 has not undergone therapeutic WLL nor received steroids or other treatment since the beginning of methionine supplementation. At the last follow-up at 33 months of age, he was taking methionine at a dosage of 20 $mg\cdot kg^{-1}$ per 6 h with stable plasma concentrations (supplementary figure S1). There was a marked catch-up in growth, his anaemia and cholestasis had resolved and the albumin plasma levels had improved. He was completely weaned off oxygen. He has started to eat by himself. Furthermore, there was a catch-up in psychomotor milestones, as he had a vocabulary of more than 10 words, understood and followed simple directions, and walked. Chest CT showed further improvement with an important regression of consolidations; microcystic lesions remained stable.

Comparison to historical controls

The cohort of historical controls included 41 patients. 25 (61%) died from terminal respiratory failure at a median age of 3.5 (1.1–16.5) years. We compared the course of treated patients from D0 (“M0”) to LFU (“M6–M12”) to that of the historical controls from diagnosis (“M0”) to 6 months to 1 year of progression of the disease or at the last evaluation if they died within 6 months of diagnosis (“M6–M12”). The first time point considered for the historical controls was diagnosis, because it corresponds to the beginning of WLL, which used to be the standard of care for those patients. Thus, for patients treated with methionine and for the historical controls, initial data correspond to status before starting treatment. Among the historical controls, data were available for comparison at the two assessment points for 20 (11 of whom are dead). Their characteristics at diagnosis were similar to those of the patients treated with methionine. The median age at diagnosis was 4 (3.3–6.8) months. During the course of their disease, they were treated with repetitive WLL alone (nine out of 20) or WLL and systemic steroids (11 out of 20). The median number of therapeutic WLL from diagnosis to the second assessment was 13 (10–19). No patient received MMF. At diagnosis, 15 patients required supplemental oxygen and 18 required enteral nutrition. Before starting methionine, three patients required oxygen and four an enteral or parenteral nutrition. At the second assessment, one out of 15 had been weaned off oxygen and none off enteral nutrition, *versus* three out of three patients treated with methionine for oxygen and two out of four for enteral nutrition (p=0.005 for oxygen weaning, p=0.026 for enteral nutrition weaning). Among the five historical controls who did not initially require oxygen, two worsened and required oxygen at the second assessment. Among the two historical controls who did not initially require enteral nutrition, one required nutritional support at the second assessment. In historical controls, repetitive WLL and steroids did not lead to significant improvement in chest CT images and BAL fluid composition, as illustrated in figure 2 for two patients. The patient presented in figure 2a and b was diagnosed at 5 months of age. Chest CT and BAL are presented at diagnosis and 5 months later after nine WLLs. By that time, he was dependent on oxygen and NIV. He is currently 8.5 years old and still requires oxygen and nocturnal NIV. The patient presented in figure 2c and d was diagnosed at 3 months of age. Chest CT and BAL are presented 12 months later after

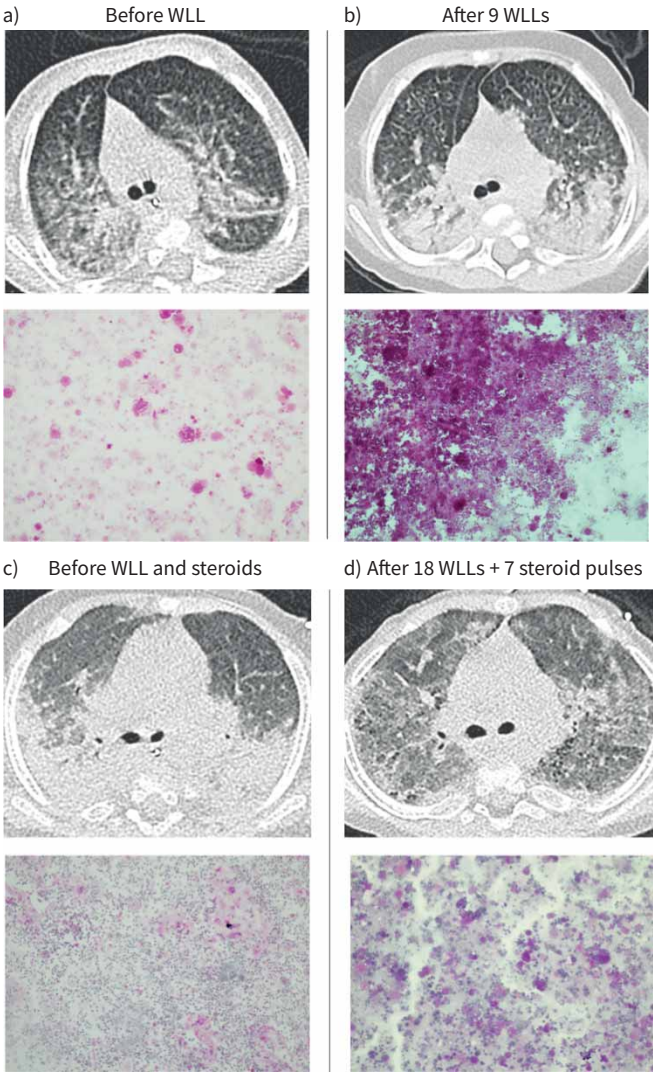

FIGURE 2 Chest computed tomography (CT) and pathological images for historical controls. a, b) Chest CT images and Periodic Acid Schiff staining (PAS) staining of bronchoalveolar lavage (BAL) fluid from a 5-month-old boy a) at diagnosis and b) 6 months later after nine whole-lung lavages (WLLs). Chest CT and BAL images show worsening of consolidations and an increasing amount of PAS-positive material over time, respectively. c, d) Chest CT images and PAS staining of BAL fluid for a 3-month-old boy c) at diagnosis and d) 12 months later after 18 WLLs and seven series of intravenous steroid pulses. Chest CT images show the persistence of the crazy-paving pattern, with partial regression of consolidations and the appearance of signs of fibrosis with microcystic lesions. BAL cytological analyses show the persistence of abundant extracellular and intramacrophage PAS-positive material.

18 WLLs and seven series of *i.v.* steroid pulses. By that time, he was dependent on oxygen. He died 1 month later. Regarding other clinical and biological features, differences between values at M0 and M6–M12 were statistically significant between historical controls and treated patients for respiratory rate (p=0.025), blood neutrophils (p=0.034), AST (p=0.004) and γ-glutamyltransferase (p=0.038) (supplementary figure S2), showing a greater improvement of these parameters for patients treated with methionine than for the historical controls. These results suggest efficacy of methionine not only in improving the respiratory status, but also inflammation, nutrition and liver status.

Safety of the treatment

Methionine supplementation was well tolerated during the protocol and afterwards. Nausea and vomiting occurred in the four patients, but pre-existed the treatment and stopped after its initiation. P3 presented

initially mild elevated transaminases (tables 1 and 2), which normalised on D5 of treatment. On D21 she presented a new episode of elevated transaminases (greater than three-fold relative to the upper limit of normal), and a reappearance of vomiting, feeding difficulties and weight loss. Infectious work-up was negative. These alterations were associated with a parallel rapid decrease in methionine plasma concentration, probably explained by a rapid weight gain (+500 g between D1 and D21 of treatment, *i.e.* a 12.5% increase in the patient's weight). As liver failure with elevated transaminases is itself one of the features of *MARS1*-related PAP, the observed decrease in methionine plasma level was hypothesised to be the cause of these alterations. A complementary analysis of the literature found data supporting this hypothesis: in animal models, methionine restriction induces steatohepatitis [14–16]. Methionine doses were increased and resulted in an increase in methionine plasma levels, along with a rapid improvement in AST and ALT values, resolution of vomiting, resumption of oral feeding, and weight gain. No recurrence of elevated transaminases has occurred since. Plasma homocysteine never reached the threshold of 30 µM for any of the patients.

Cellular assays

MetRS protein levels in PBMCs of P1 before starting methionine were normal relative to those of a control individual (supplementary figure S2). GM-CSF priming of ROS production by peripheral monocytes was measured in P1 and P3. It was low at baseline and improved after 3 months of supplementation with methionine (supplementary figure S2).

Discussion

We report the results of supplementation with methionine for PAP and multi-organ dysfunction caused by hereditary MetRS deficiency. The treatment led to an important improvement in clinical, laboratory, imaging and pathological parameters and was well tolerated. Peripheral monocytes showed an initially altered function which improved under treatment.

Drawing on our preliminary results [3], two other groups reported methionine use in patients harbouring other *MARS1* mutations. In the case reported by Rips *et al.* [7], methionine supplementation was briefly mentioned as leading to a clinical improvement, but no details on the administration modality, doses, plasma concentrations or potential cellular assays were provided. Lenz *et al.* [10] published a report on two brothers. Although they provide some data on the doses and plasma concentrations of methionine, they are insufficient to assume that methionine plasma levels were within the target range during 24-h periods or for days or months of treatment. The index case simultaneously received methionine and other treatments (*i.v.* immunoglobulins, hydroxychloroquine, steroids and antibiotics). Neither chest CT nor BAL examination comparing before and after treatment is given. His older brother was diagnosed during familial screening, but was paucisymptomatic. Thus, the efficacy of methionine supplementation is difficult to determine from these articles. The publication of isolated case reports reflects the difficulty of drawing powerful conclusions for therapeutic innovations in this orphan disease.

Almost all patients harbouring the "Réunion" mutations have been followed in our reference centre, allowing precise comparison of the treated patients to historical controls. Collected data were compared to patients that had had the standard of care for this disease, including repetitive WLL. Such a comparison showed significant differences in the evolution of respiratory, nutritional, liver and inflammatory status. P1 and P3 are the first to attain complete respiratory remission at their age. The relapse of hepatic and digestive signs in P3 when methionine plasma concentration decreased and the disappearance of those symptoms once plasma concentrations reached the target range again also argue for the accountability of methionine supplementation on the favourable outcome in the treated patients.

The small sample size and the heterogeneity of the four patients are limitations of this study. *MARS1*-related PAP is a rare disease with an incidence of 1 in 10 000 newborns in Réunion and nearby, and only a few case reports have described this disease in other ethnic groups [3, 5–10], explaining the small sample size. P2 and P4 did not achieve complete remission of their liver involvement at the last follow-up, with a persistent enlarged liver. Treatment was started later than for P1 and P3. This could explain, in part, the discrepancy in their responses to treatment. A prospective follow-up of these patients and the treatment of other children will make it possible to determine the best scheme for treatment and predict its efficacy at various stages of the disease. The results obtained for P1 and P3 argue for starting methionine supplementation as soon as possible.

One strength of this study is the correction of the phenotype at the cellular level by the methionine supplementation. As for other forms of PAP, *MARS1* mutations are hypothesised to induce alveolar macrophage dysfunction, leading to altered pulmonary surfactant metabolism. However, this has not yet

been proven. We thus quantified the oxidative burst by the peripheral blood monocytes from two patients, as this would provide indirect information on alveolar macrophage function. Priming of ROS production by monocytes was very low before treatment and improved after 3 months of treatment. These results are the first to suggest macrophage dysfunction subsequent to *MARS1* mutations and to show such an improvement after treatment.

Tolerance of the treatment was good, consistent with studies on congenital hypermethioninaemia, which did not reveal toxicity at the plasma methionine concentrations observed in our study [11, 12]. Oral intake at fixed intervals resulted in stable blood concentrations within each patient. In the era of personalised medicine, which promotes the development of costly and highly specific targeted drugs, L-methionine is highly affordable. WLL, which remains currently the standard of care for these patients, is a heavy and risky procedure performed under general anaesthesia and requires repetitive hospitalisations. In addition, this treatment has never enabled complete remission in the past and appears to have no influence on the long-term prognosis [2]. Thus, methionine appears to be a highly promising and cost-effective treatment.

This study paves the way for similar strategies for other aminoacyl-tRNA synthetase (ARS) deficiencies. Fuchs *et al*. [17] reviewed 112 patients with diverse ARS deficiencies that share common features, including lung and liver disease and failure to thrive. The authors proposed a pathophysiological model in which disorders result from insufficient aminoacylation activity to meet translational demand in specific organs. They have suggested supplying the corresponding amino acid for each specific ARS deficiency as a therapeutic approach. Our results provide a first proof of concept for this strategy.

In conclusion, oral methionine supplementation in four children with PAP and multisystemic dysfunction related to *MARS1* mutations led to full remission of the disease in two patients and a clear ongoing improvement in the two others. These promising results will fundamentally change the prognosis of this severe and often fatal disease. In addition, they offer promising therapeutic perspectives for similar strategies in other ARS deficiencies.

Acknowledgements: We thank Christophe Merlette, the technical director of the mass spectrometry platform at Necker Hospital (Paris, France), for his invaluable contribution to the efficient implementation of the study. We thank Laurent Enaud (CHU Réunion site Sud, Service de Pédiatrie, Saint Pierre, France), who has been long involved in the care of numerous families with this disease on the island of Réunion. We thank Joel Schlatter, Sylvain Auvity and the pharmaceutical assistants from the Pharmacie Hospitalière at Necker Hospital for preparation of the methionine capsules. Finally, we thank the patients and their families for their participation.

Author contributions: A. Hadchouel, D. Drummond, C. Pontoizeau, M-M. Hurtado-Nedelec, J. El Benna, M. Schiff, T.J. Molina, L. Berteloot, C. Ottolenghi, J-M. Tréluyer, J. de Blic and C. Delacourt contributed to the conception and design of the study, the acquisition, analysis and interpretation of data for the study. L. Aoust, E. Gachelin, C. Perisson, C. Vigier, F. Lacaille and S. Renolleau contributed to the acquisition of the data for the study and revised the draft of the manuscript. A. Hadchouel, D. Drummond and C. Delacourt drafted the work and revised it after revision by the other authors. All authors approved the final version to be published.

Conflict of interest: A. Hadchouel reports grants from APHP and Fonds de Recherche en Santé Respiratoire–Fondation du Souffle, during the conduct of the study; and has a patent EP 21 305 689.8 pending. The remaining authors have nothing to disclose.

Support statement: This work was funded by La Fondation du Souffle et le Fonds de Recherche en Santé Respiratoire (SRC2017) and La Cellule Recherche et Innovation de l'APHP, Hôpital Necker. Funding information for this article has been deposited with the Crossref Funder Registry.

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