Methionine supplementation for multiorgan dysfunction in MetRS-related pulmonary alveolar proteinosis

Online Supplement

METHODS

ADMINISTRATION SCHEME AND TARGETED RANGES

The frequency of medication was based on the known half-life of the molecule [1] and the peak was determined by performing kinetic measurements on the patients during the first day of supplementation. The initial dose was determined based on the usual mean methionine intake in alimentation for infants and children in France (available at https://www.anses.fr/fr/system/files/NUT-Ra-Proteines.pdf), with the initial aim to double methionine intake. The targeted plasma concentrations were defined according to available published data on normal methionine concentrations in children and on congenital disorders leading to hypermethioninemia and its potential toxicity. The normal fasting concentration should not exceed 45 µM [2]. Congenital hypermethioninemia is described notably in patients with methionine adenosyltransferase I/III (MAT I/III) or cystathionine beta-synthase deficiency. The consequences of high blood levels of methionine are liver dysfunction and central nervous system (CNS) abnormalities, especially with a risk of cerebral oedema. In a large series of patients with MAT I/III deficiency, CNS abnormalities were observed for those with mean plasma methionine values > 800 µM, whereas those with mean plasma methionine values < 800 µM generally did not have such abnormalities [3]. No instances of hepatic malfunction were detected in the aforementioned series. The authors recommended considering a dietary methionine restriction when plasma levels exceeded 500 µM and to only clinically monitor the patients with levels below 500 µM [3]. We thus decided to target methionine plasma

levels between 45 and 500 µM to obtain levels above the normal range but below the toxic range.

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Neutrophils were isolated from the blood of Patient 1 and a control as described previously [4] After hypotonic lysis of erythrocytes, the neutrophil pellets were collected and washed in PBS. Neutrophils (10^7 cells in 500 µl HBSS) were then incubated with the proteinase inhibitor DFP (2.5 mM), followed by lysis with 125 µl concentrated modified Laemmli sample buffer (5X) containing 50 µg/mL pepstatin, 50 µg/mL leupeptin, 25 mM NaF, 12.5 mM Na3VO4, 12.5 mM EDTA, 12.5 mM EGTA, 6.25 mM p-NPP, and 50 µg/mL aprotinin.[5] Samples were denatured in boiling water (100°C) for 3 min and stored at −80°C until use. Samples were thawed and sonicated for 10 s before use and then subjected to classical 10% SDS–PAGE[5]. The separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in a mixture of tris-buffered saline and Tween-20. The membranes were then incubated overnight at 4°C in a solution containing a specific anti-MARS antibody (Abnova H00004141-B01P), followed by incubation in secondary antibodies (Santa Cruz, Heidelberg, Germany). Blots were visualized using ECL Western blotting reagents (Amersham Pharmacia).

PRIMING OF ROS PRODUCTION BY PERIPHEAL PHAGOCYTES/MONOCYTES

Whole blood from Patients 1 and 3 and a control was collected from lithium heparinized tubes (500 µl) and incubated for 15 min at 37°C with dihydrorhodamine 123(DHE) (Sigma-Aldrich). Samples were then treated for 1 h at 37°C with GM-CSF (10 ng/ml; R&D Systems), followed by stimulation for 5 min with fMLF (10-5M; Sigma-Aldrich). The reaction was stopped by adding 1 ml ice-cold lysis solution (BD Biosciences) and incubating for 5 min on ice. Samples were then washed with PBS (Sigma-Aldrich) and the pellets resuspended in 300 µl FacsFlow

solution (BD Biosciences). Samples were analysed by flow cytometry on a FACSCanto II (BD Biosciences). Phagocytes (neutrophils and monocytes) were selected on an FSC-SSC dot plot. Events (50,000) were recorded at a constant PMT voltage. Results are expressed as the index of stimulation (MIF DHE GM-CSF + fMLP/DHE GM-CSF alone).

RESULTS

METHIONINE ADMINISTRATION

Methionine plasma concentrations were measured to adapt the doses of the supplementation. Twenty-four-hour kinetic studies were performed on days (D) 1 and 3 of treatment and at least one time later before M2. After that, methionine plasma concentrations were regularly monitored at residual and peak states for one or two intakes. Within each patient, treatment every 6h led to reproducible residual and peak values for 24-h periods throughout days and months of treatment (Supplemental Figure S1).

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MetRS protein levels in PBMCs of P1 before starting methionine were normal relative to those of a control individual (Supplemental Figure S3).

PRIMING OF ROS PRODUCTION BY PERIPHEAL PHAGOCYTES/MONOCYTES

We assessed ROS production by peripheral monocytes of P1 and P3 before and after three months of treatment. Before treatment, GM-CSF priming of ROS production by peripheral monocytes (Supplemental Figure S3) stimulated by GM-CSF and fMLP was low (stimulation index relative to control of 46% for P1 and 58% for P3). After three months of treatment, the stimulation index relative to control normalized for P1 (109%) and improved for P3 (73%) (Supplemental Figure S3).

References:

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FIGURE LEGENDS:

Supplemental Figure S1. Kinetic data of residual (solid lines) and peak (dotted lines) plasma methionine values.

The peak was determined during a kinetic study to be 1 h after taking the medication. A and B: Complete kinetic study measuring the residual and peak concentrations at each intake over 24 h. Data are shown for Patient 1 after one year of treatment (A) with a methionine dose of 110 mg/kg/day and for Patient 3 on day 3 of the treatment (B) with a methionine dose of 80 mg/kg/day. C and D: Residual and peak plasma values on three different days under the same dosage. Data are shown for Patient 2 after 10 (M10), 11 (M11), and 13 (M13) months of treatment with a methionine dose of 80 mg/kg/day and for Patient 4 on three different days (D5, D10, and D11) during the same month (M2) of treatment with a methionine dose of 90 mg/kg/day. Within each patient, giving treatment every 6 hours allowed getting reproducible residual and peak value during 24-hour periods and also across days and months of treatment.

Supplemental Figure S2. Comparison of clinical and biological parameters with historical controls.

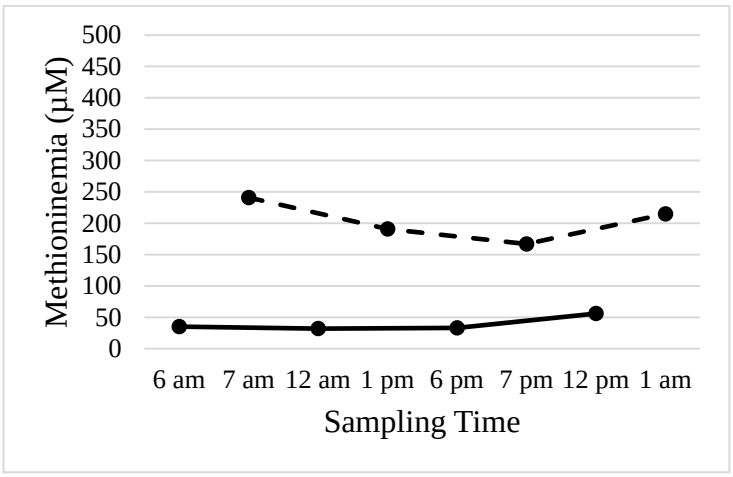
Comparison of the data between Patients 1, 3, and 4 (solid lines) and historical controls (median value and IQR, dotted line) are shown for SD of weight, respiratory rate (RR), haemoglobin (Hb), white blood cell (WBC), neutrophils, and platelets counts, CRP, albumin, prothrombin rate (PT), AST, GGT and total bilirubin (T Bilirubin). M0: D0 or diagnosis; M6-M12: last follow-up for treated patients and six months to one year of the progression of the disease for historical controls. The lower limit of the normal is designated by a horizontal dotted line for haemoglobin, albumin, and the prothrombin rate; the upper limit of normal is designated by a horizontal dotted line for WBC, neutrophils, and platelets counts, CRP, GGT, and total and conjugated bilirubin. 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 GGT (p=0.038).

Supplemental Figure S3. Cellular analyses for Patients 1 and 3.

A: Analysis of MetRS protein expression for Patient 1. The MetRS protein was normally expressed in PBMCs relative to those of a control individual. B: GM-CSF priming of ROS production by peripheral monocytes before (M0) and after three months of treatment (M3) for Patient 1 (P1) and Patient 3 (P3). Priming of ROS production by peripheral monocytes stimulated by GM-CSF and fMLP was measured in patients and controls and the stimulation index expressed as the percentage of the control values. The control value was thus considered to be 100%. The stimulation index relative to control for both patients before treatment was low: 46% for P1 and 58% for P3. After three months of treatment, the stimulation index relative to control normalized for P1 (109%) and improved for P3 (73%).

A



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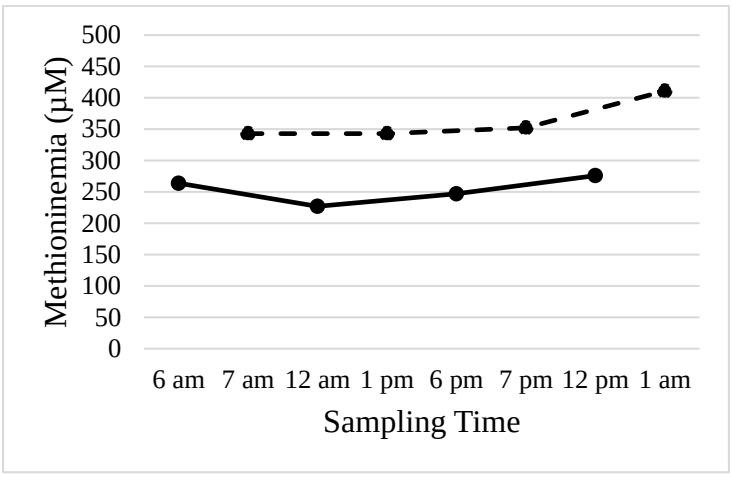

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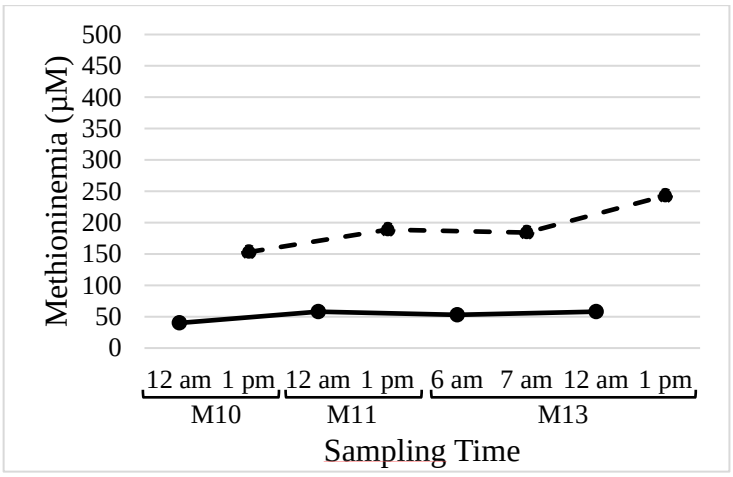

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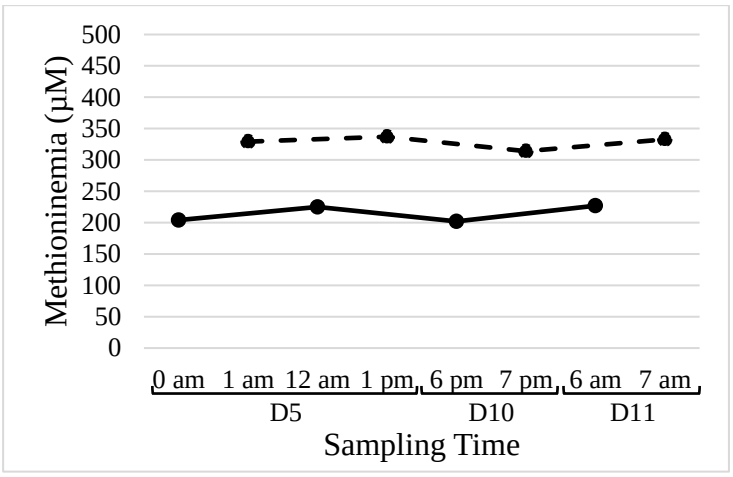
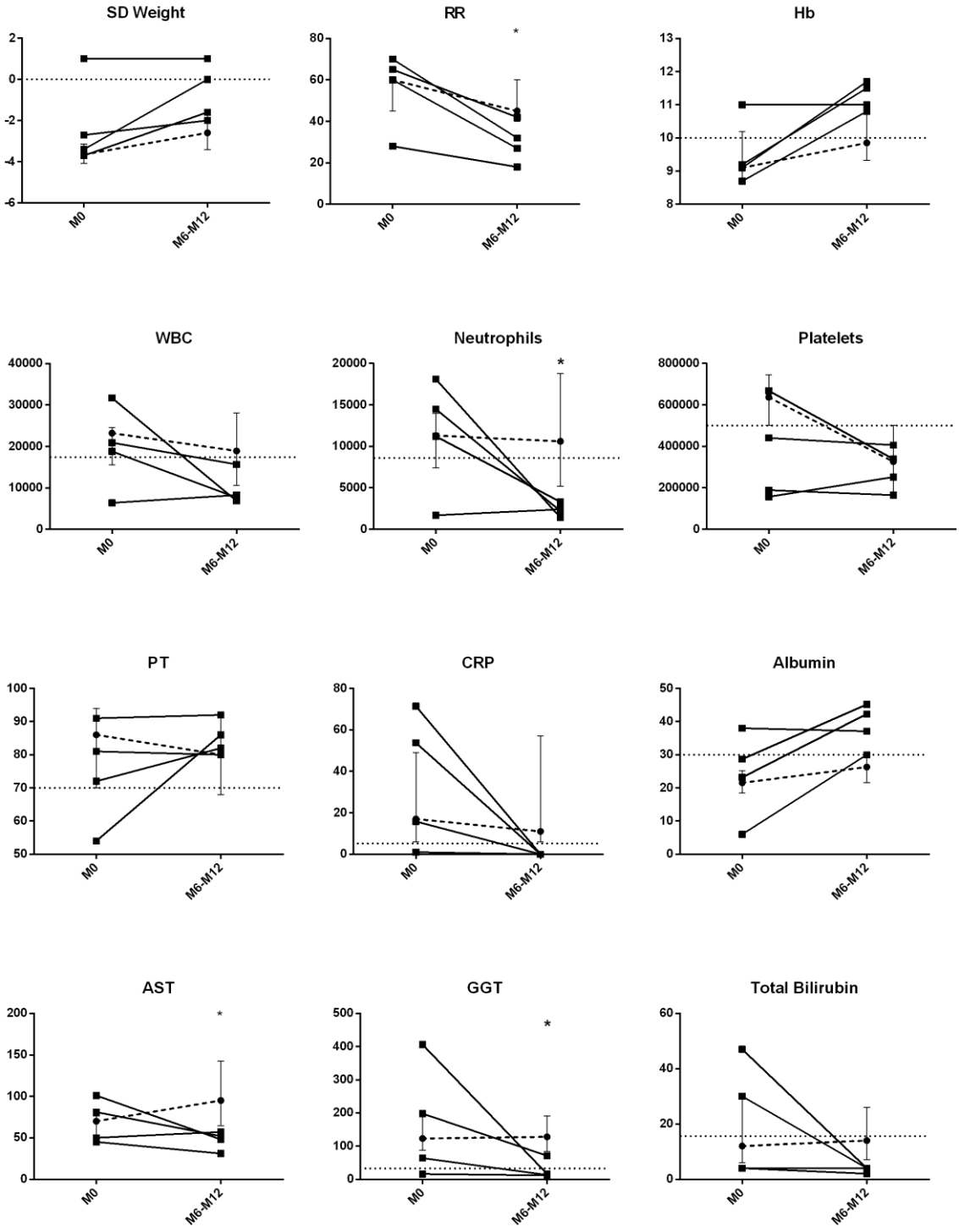
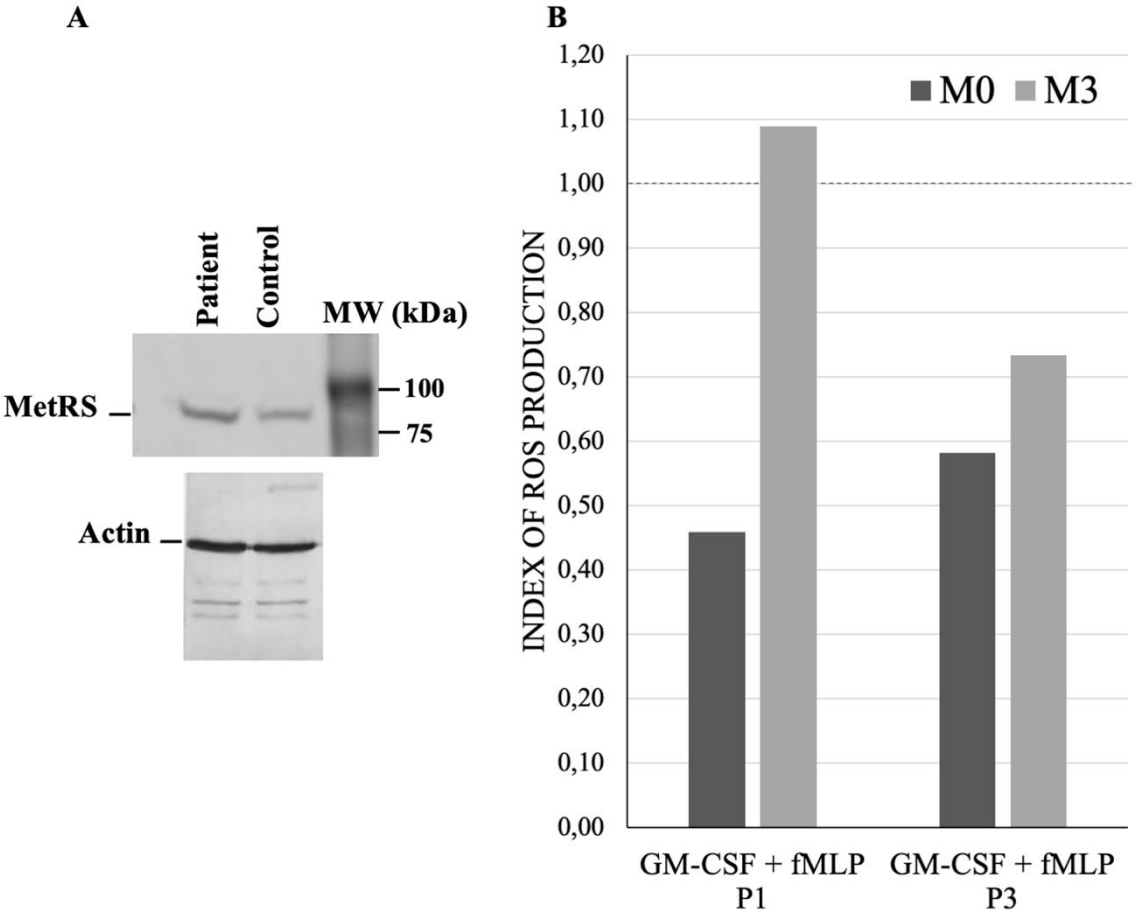


Supplemental Figure S1



Supplemental Figure S2



Supplemental Figure S3