

## Case Report

# Comprehensive GC-MS Measurement of Amino Acids, Metabolites, and Malondialdehyde in Metformin-Associated Lactic Acidosis at Admission and during Renal Replacement Treatment

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**Abstract:** Metformin is the most widely used drug in type 2 diabetes. Regular metformin use has been associated with changes in concentrations of amino acids. In the present study, we used valid stable-isotope labeled GC-MS methods to measure amino acids and metabolites, including creatinine as well as malondialdehyde (MDA), as an oxidative stress biomarker in plasma, urine, and dialysate samples in a patient at admission to the intensive care unit and during renal replacement treatment because of metformin-associated lactic acidosis (MALA, 21 mM lactate, 175  $\mu$ M metformin). GC-MS revealed lower concentrations of amino acids in plasma, normal concentrations of the nitric oxide (NO) metabolites nitrite and nitrate, and normal concentrations of MDA. Renal tubular reabsorption rates were altered on admission. The patient received renal replacement therapy over 50 to 70 h of normalized plasma amino acid concentrations and their tubular reabsorption, as well as the tubular reabsorption of nitrite and nitrate. This study indicates that GC-MS is a versatile analytical tool to measure different classes of physiological inorganic and organic substances in complex biological samples in clinical settings such as MALA.

**Keywords:** amino acids; GC-MS; intoxication; MALA; oxidative stress; renal replacement therapy



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## 1. Introduction

Metformin (*N,N*-dimethylbiguanide) is the most commonly prescribed oral antihyperglycemic drug to treat type 2 diabetes [1]. Metformin is eliminated in urine in its unchanged form via renal secretion [2]. Patients with metformin intoxication can develop metformin-associated lactic acidosis (MALA), a life-threatening condition. MALA is defined as a plasma pH < 7.35, a plasma lactate concentration >5 mM, and a metformin concentration >5 mg/L [3]. The pathophysiology of MALA is characterized by reduced lactate clearance due to impaired gluconeogenesis combined with inhibition of systemic mitochondrial respiration, causing an increase in anaerobic metabolism [4–7]. Patients with MALA are treated in the intensive care unit (ICU), requiring comprehensive supportive care in combination with renal replacement therapy (RRT), such as intermittent hemodialysis (HD) or continuous RRT [8].

Metformin use has been associated with changes in concentrations of amino acids and their metabolites, including homoarginine [9,10]. The effects of metformin-induced post-translational modifications (PTM) at toxic concentrations are scarce [11,12]. In urine of a patient with MALA, we have previously found an accumulation of hydroxy-proline

(OH-Pro) [13]. The aim of the present study was to determine the status of other amino acids, including arginine and some of its metabolites, and of oxidative stress in the MALA patient at ICU admission and during RRT. Free L-arginine (Arg) is the physiological substrate of nitric oxide synthase (NOS) isoforms [14]. Of particular interest were the major metabolites and measures of nitric oxide (NO), namely nitrate and nitrite. Post-translational dimethylation of Arg residues in proteins leads to symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA), which are endogenous inhibitors of NOS activity [14]. As dimethyl amine (DMA) is the major urinary metabolite of ADMA [15], it was of interest to investigate potential effects of MALA on this ADMA metabolite. Eventually, malondialdehyde (MDA) was measured as a biomarker of oxidative stress [16] in the MALA patient.

Mass spectrometry coupled to gas chromatography (GC-MS) is a widely used instrumental technique for the reliable quantitative determination of numerous physiological compounds, drugs, and their metabolites in biological samples such as plasma and urine. In the present work, we used previously described validated GC-MS methods for the measurement of the above-mentioned analytes [17–24]. These methods include use of suitable derivatization techniques for the analytes and their stable-isotope labeled analogs ( $^2\text{H}$ ,  $^{15}\text{N}$ ), which serve as internal standards. All GC-MS analyses in this study were performed in the negative-ion chemical ionization (NICI) mode and by using selected-ion monitoring (SIM) of analyte-specific ions.

## 2. Materials and Methods

### 2.1. Patient

The case report on metformin intoxication and RRT treatment has been described elsewhere in detail [13] and is briefly summarized below.

The 70-year old female patient (bodyweight, 84 kg) was oliguric, and the plasma creatinine concentration was 606  $\mu\text{M}$ , indicating acute renal failure. The patient was transferred to our hospital to start RRT. At ICU admission, the blood metformin concentration was 175  $\mu\text{M}$ . During hemodialysis, the metformin plasma concentration rapidly decreased, and the acidosis improved, with an increase in pH from 7.26 to 7.40 in approximately 2 h. Likewise, lactate concentration decreased from 21 mM to <2 mM within 12 h. After hemodialysis (HD) was stopped, continuous veno-venous hemodiafiltration (CVVHDF) and, subsequently, continuous veno-venous hemofiltration (CVVH) was applied. Heparin was used as an anticoagulant during CVVHDF, whereas citrate was used during CVVH. After 12 h of ICU admission, the metformin concentration decreased to 39  $\mu\text{M}$ , which is generally considered to be non-toxic [25]. Heparinized plasma, urine, and dialysate samples were centrifuged and stored at  $-80^\circ\text{C}$ . Written informed consent was obtained to collect residual material and for publication of this case report. Ethical approval was given by the institutional review board (METc 2014-552).

### 2.2. GC-MS Analyses in Plasma, Urine, and Dialysate Samples

Metformin, amino acids, nitrate, nitrite, and MDA were analyzed by GC-MS in plasma, urine, and dialysate (effluent) samples after suitable derivatization, as described previously [17–24]. All amino acids were measured simultaneously as methyl ester pentafluoropropionyl derivatives. Nitrate, nitrite, creatinine, and MDA were measured simultaneously as pentafluorobenzyl derivatives. DMA was analyzed after extractive derivatization as a pentafluorobenzamide derivative. In all analyses, commercially available and de novo synthesized stable-isotope labelled analogs were used as internal standards for each analyte.

GC-MS analyses were performed on a single quadrupole mass spectrometer model ISQ, equipped with a Trace 1210 series gas chromatograph, and an AS1310 autosampler (ThermoFisher, Dreieich, Germany). A fused-silica capillary column Optima 17 (15 m length, 0.25 mm I.D., 0.25  $\mu\text{m}$  film thickness) was used (Macherey-Nagel, Düren, Germany). Methane was used as the reagent gas (flow rate of 2.4 mL/min) for NICI. Quantitative analyses were performed in the SIM mode. The GC-MS method for amino acids measures the

sum of citrulline (Cit) and ornithine (Orn), asparagine (Asn) and aspartate (Asp), glutamine (Gln) and glutamate (Glu), leucine (Leu) and isoleucine (Ile). The concentrations of these amino acids are reported as Orn, Asp, Glu, and Leu, respectively. Analyte concentrations in urine were corrected for urinary creatinine excretion and are reported as  $\mu\text{mol}$  analyte per  $\text{mmol}$  creatinine. Creatinine in plasma and urine was also analyzed by GC-MS, as described elsewhere [24]. All analyses were performed at the same time in one laboratory by a single skillful person.

### 2.3. Calculations and Statistical Analyses

Fractional excretion (FE, %) values were calculated for all analytes by dividing the concentration ratio of creatinine (Crea) in plasma (P) and urine (U), i.e.,  $[\text{Crea}]_P/[\text{Crea}]_U$ , by the concentration ratio of an analyte (A) in plasma and urine, i.e.,  $[A]_P/[A]_U$ , measured at a certain time point, and by multiplying the result by 100 (see Formula (1)). Tubular reabsorption values (T, %) of analytes were calculated by subtracting the FE values from 100 (see Formula (2)).

$$\text{FE (\%)} = ([\text{Crea}]_P/[\text{Crea}]_U)/([A]_P/[A]_U) \times 100\% \quad (1)$$

$$\text{T (\%)} = 100 - \text{FE (\%)} \quad (2)$$

Renal clearance was calculated by dividing the elimination rate in urine from the previous time-point, at which urine was collected to the current time-point by the corresponding plasma concentration. The instantaneous plasma clearance by dialysis was calculated by dividing the product of dialysate flow and concentration in the dialysate (i.e., elimination rate) by the plasma concentration. Because the blood samples were not simultaneously obtained together with the effluent samples, the concentration in plasma was linearly interpolated using data from four time-points with the Trend function in Excel (Microsoft, Redmond, WA, USA). Calculating plasma clearance by dialysis using the AV method was not possible because no simultaneous inflow and outflow samples were obtained. To present the average clearance of each compound during CVVHDF and CVVH, respectively, the time-weighted mean clearance was calculated, which is more representative of the true clearance than the arithmetic mean, as it assumes a linear trend between each individual measurement [26].

Data analyses were performed with GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA) and R version 4.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

### 3. Results and Discussion

The concentrations of the analytes measured in the samples of the study are presented in Tables 1 and 2 and in Figures 1 and 2. For the sake of clarity and better readability, the results are presented separately for the amino acids and the other analytes. For comparison of analyte concentrations measured in the patient's samples, we used concentrations of the analytes as measured in studies on healthy subjects [17–24,27–37]. These data are summarized in Table 3. The reference interval for plasma creatinine was reported to be 53–115  $\mu\text{M}$  [36].

**Table 1.** (a) Concentrations of metformin, creatinine, and metabolites (all in  $\mu\text{M}$ ) in plasma samples of the patient during (time in h) RTR. (b) Concentrations of metformin (mM), creatinine (mM), and metabolites (all in  $\mu\text{M}$ ) in urine samples of the patient during (time in h) RTR. (c) Concentrations of metformin and metabolites (all in  $\mu\text{M}$ ) in effluent samples of the patient during (time in h) RTR.

| (a)  |           |            |         |         |      |      |      |      |
|------|-----------|------------|---------|---------|------|------|------|------|
| Time | Metformin | Creatinine | Nitrate | Nitrite | MDA  | GAA  | ADMA | SDMA |
| 5.5  | 75        | 388        | 57      | 2.64    | 1.16 | 1.42 | 0.22 | 1.48 |
| 7.2  | 73        | 385        | 59      | 3.09    | 0.90 | 3.46 | 0.53 | 1.76 |

Table 1. Cont.

| (a)  |           |            |         |         |         |            |       |      |      |
|------|-----------|------------|---------|---------|---------|------------|-------|------|------|
| Time | Metformin | Creatinine | Nitrate | Nitrite | MDA     | GAA        | ADMA  | SDMA |      |
| 9.0  | 63        | 350        | 56      | 3.07    | 1.25    | 3.63       | 0.48  | 1.98 |      |
| 10.7 | 55        | 297        | 55      | 2.83    | 0.93    | 5.30       | 0.71  | 2.38 |      |
| 11.3 | 50        | 307        | 58      | 3.24    | 0.89    | 5.31       | 0.50  | 1.88 |      |
| 12.6 | 44        | 267        | 51      | 3.34    | 0.83    | 4.09       | 0.53  | 1.30 |      |
| 15.9 | 35        | 202        | 53      | 3.25    | 0.07    | 2.83       | 0.32  | 0.98 |      |
| 30.8 | 19        | 183        | 57      | 4.38    | 1.18    | 5.85       | 0.48  | 1.42 |      |
| 35.0 | 16        | 167        | 53      | 3.66    | 0.96    | 4.72       | 0.44  | 1.73 |      |
| 40.3 | 13        | 145        | 51      | 2.96    | 1.14    | 1.93       | 0.30  | 1.03 |      |
| 48.8 | 10        | 135        | 58      | 3.12    | 0.81    | 4.74       | 0.48  | 1.03 |      |
| 53.3 | 10        | 190        | 53      | 2.92    | 1.03    | 2.82       | 0.35  | 2.25 |      |
| 57.0 | 9         | 219        | 57      | 3.08    | 1.23    | 2.62       | 0.46  | 1.34 |      |
| 62.0 | 7         | 250        | 53      | 3.29    | 0.94    | 1.96       | 0.50  | 1.18 |      |
| 63.0 | 7         | 226        | 56      | 3.02    | 0.90    | 1.91       | 0.51  | 1.34 |      |
| 64.7 | 6         | 234        | 56      | 3.29    | 0.92    | 3.35       | 0.33  | 2.15 |      |
| 66.6 | 4         | 230        | 60      | 3.08    | 0.82    | 2.28       | 0.42  | 1.52 |      |
| (b)  |           |            |         |         |         |            |       |      |      |
| Time | Metformin | Creatinine | DMA     | Nitrate | Nitrite | MDA        | GAA   | ADMA | SDMA |
| 5.5  | 1.46      | 2.5        | 126     | 239     | 10.5    | 1.08       | 13.2  | 1.32 | 12.9 |
| 7.2  | 1.17      | 1.88       | 84      | 137     | 10.3    | 0.98       | 12.2  | 1.8  | 4.3  |
| 9.0  | 1.44      | 2.23       | 124     | 107     | 9.3     | 1.79       | 13.6  | 2.14 | 7.2  |
| 10.7 | 1.23      | 2.11       | 102     | 119     | 7.4     | 1.09       | 11.0  | 1.07 | 5.0  |
| 11.3 | 1.25      | 2.19       | 105     | 91      | 5.1     | 1.08       | 11.8  | 1.23 | 4.8  |
| 12.6 | 2.58      | 4.84       | 213     | 112     | 9.8     | 15.07      | 39.1  | 2.68 | 11.8 |
| 15.9 | 3.37      | 6.14       | 279     | 81      | 5.1     | 19.52      | 59.6  | 4.39 | 17.8 |
| 30.8 | 3.5       | 4.88       | 321     | 216     | 24.1    | 7.31       | 146.1 | 7.65 | 33.7 |
| 35.0 | 2.87      | 4.49       | 231     | 84      | 10.6    | 9.66       | 147.8 | 4.93 | 14.8 |
| 40.3 | 4.02      | 6.29       | 367     | 78      | 7.5     | 6.64       | 196.4 | 6.71 | 15.1 |
| 46.5 | 5.11      | 6.39       | 423     | 83      | 5.4     | 6.88       | 212.8 | 8.45 | 15.6 |
| 48.8 | 3.28      | 5.43       | 360     | 84      | 1.9     | 5.28       | 248.2 | 5.78 | 13.3 |
| 50.8 | 2.04      | 3.33       | 230     | 86      | 1.8     | 3.53       | 198.4 | 4.15 | 6.1  |
| 53.3 | 1.17      | 2.56       | 191     | 93      | 2.0     | 10.81      | 179.7 | 3.42 | 5.4  |
| 57.0 | 0.95      | 2.61       | 148     | 108     | 2.2     | 4.22       | 97.5  | 3.17 | 5.7  |
| 62.0 | 0.68      | 3.17       | 153     | 127     | 2.1     | 1.81       | 79.7  | 3    | 5.1  |
| 63.0 | 0.64      | 3.6        | 157     | 137     | 3.3     | 1.85       | 81.1  | 2.79 | 5.7  |
| 64.7 | 0.6       | 3.85       | 162     | 141     | 3.1     | 1.7        | 59.8  | 3.15 | 6.0  |
| 66.6 | 0.5       | 4.04       | 233     | 150     | 2.3     | 2.07       | 59.4  | 3.85 | 7.8  |
| (c)  |           |            |         |         |         |            |       |      |      |
| Time | Metformin | DMA        | Nitrate | Nitrite | MDA     | Creatinine | GAA   | ADMA | SDMA |
| 5.0  | 28.6      | 3.41       | 31.1    | 9.49    | 0.19    | 155        | 1.67  | 0.16 | 0.54 |
| 8.8  | 80.9      | 10.5       | 32.2    | 2.70    | 0.27    | 344        | 2.00  | 0.40 | 1.52 |
| 11.0 | 69.6      | 9.09       | 25.9    | 4.48    | 0.25    | 427        | 2.49  | 0.32 | 1.22 |
| 12.0 | 60.7      | 7.73       | 29.0    | 4.13    | 0.29    | 384        | 2.94  | 0.31 | 1.39 |
| 13.0 | 54.9      | 7.80       | 24.3    | 4.12    | 0.27    | 423        | 3.97  | 0.32 | 1.31 |
| 15.3 | 45.2      | 7.92       | 27.5    | 3.89    | 0.23    | 415        | 3.04  | 0.31 | 1.36 |
| 17.6 | 37.1      | 6.86       | 25.3    | 3.92    | 0.26    | 344        | 2.38  | 0.28 | 0.93 |
| 19.5 | 32.5      | 6.96       | 21.4    | 4.10    | 0.22    | 256        | 3.85  | 0.31 | 1.09 |
| 20.8 | 29.7      | 6.14       | 24.7    | 4.08    | 0.20    | 256        | 2.36  | 0.32 | 1.01 |
| 24.3 | 23.3      | 4.50       | 21.7    | 3.62    | 0.23    | 188        | 4.32  | 0.36 | 0.76 |
| 29.1 | 20.6      | 7.16       | 29.6    | 3.29    | 0.21    | 190        | 6.12  | 0.33 | 1.30 |
| 30.5 | 18.2      | 5.68       | 28.3    | 2.97    | 0.23    | 286        | 5.90  | 0.30 | 1.16 |
| 33.4 | 16.2      | 7.81       | 24.4    | 2.93    | 0.20    | 245        | 8.83  | 0.33 | 1.27 |
| 35.0 | 15.6      | 6.11       | 32.1    | 5.25    | 0.22    | 267        | 7.48  | 0.25 | 1.43 |
| 40.3 | 12.1      | 6.93       | 24.3    | 4.17    | 0.48    | 259        | 3.44  | 0.28 | 1.07 |
| 42.6 | 11.9      | 5.15       | 24.5    | 3.82    | 0.23    | 252        | 6.38  | 0.35 | 0.94 |
| 46.5 | 10.0      | 5.54       | 23.5    | 3.94    | 0.19    | 224        | 10.73 | 0.25 | 1.37 |
| 48.9 | 9.6       | 5.83       | 23.8    | 4.75    | 0.24    | 220        | 5.25  | 0.30 | 1.84 |
| 50.0 | 18.0      | 5.95       | 25.4    | 2.67    | 0.24    | 277        | 5.73  | 0.39 | 1.58 |

**Table 2.** (a) Concentrations of amino acids (all in  $\mu\text{M}$ ) in plasma samples of the patient during (time in h) the RTR. (b) Concentrations of amino acids (all in  $\mu\text{M}$ ) in urine samples of the patient during (time in h) RTR. (c) Concentrations of amino acids (all in  $\mu\text{M}$ ) in effluent samples of the patient during (time in h) RTR.

| (a)  |     |     |     |     |      |      |     |      |      |      |      |       |      |     |      |      |      |
|------|-----|-----|-----|-----|------|------|-----|------|------|------|------|-------|------|-----|------|------|------|
| Time | Ala | Thr | Gly | Val | Ser  | Sar  | Leu | Asp  | Gln  | Met  | Orn  | Phe   | Tyr  | Lys | Arg  | hArg | Trp  |
| 5.5  | 149 | 53  | 82  | 141 | 52   | 1.12 | 143 | 32   | 247  | 35   | 18   | 36    | 31   | 96  | 17   | 1.12 | 5.7  |
| 7.2  | 281 | 106 | 168 | 296 | 90   | 1.98 | 322 | 70   | 662  | 67   | 38   | 82    | 66   | 203 | 37   | 2.1  | 9.9  |
| 9.0  | 217 | 91  | 163 | 231 | 80   | 1.45 | 276 | 63   | 560  | 57   | 39   | 75    | 55   | 163 | 25   | 1.52 | 13.5 |
| 10.7 | 197 | 88  | 173 | 191 | 85   | 1.41 | 249 | 69   | 533  | 57   | 30   | 72    | 50   | 162 | 29   | 1.48 | 16.9 |
| 11.3 | 190 | 87  | 167 | 178 | 75   | 1.32 | 243 | 63   | 512  | 55   | 29   | 72    | 49   | 160 | 30   | 1.35 | 16.5 |
| 12.6 | 194 | 93  | 183 | 185 | 87   | 1.36 | 243 | 69   | 534  | 56   | 32   | 77    | 50   | 180 | 34   | 1.28 | 13.2 |
| 15.9 | 190 | 107 | 197 | 192 | 117  | 1.1  | 259 | 66   | 478  | 54   | 36   | 88    | 57   | 205 | 36   | 1.12 | 16.3 |
| 30.8 | 280 | 152 | 277 | 303 | 116  | 1.72 | 359 | 92   | 610  | 62   | 77   | 113   | 92   | 220 | 30   | 0.65 | 28.0 |
| 35.0 | 268 | 116 | 222 | 240 | 128  | 1.3  | 263 | 78   | 496  | 53   | 56   | 98    | 68   | 173 | 29   | 0.48 | 27.4 |
| 40.3 | 223 | 102 | 184 | 224 | 98   | 1.28 | 254 | 57   | 419  | 47   | 62   | 96    | 62   | 169 | 25   | 0.40 | 31.9 |
| 48.8 | 240 | 121 | 190 | 298 | 92   | 1.23 | 330 | 72   | 420  | 50   | 56   | 95    | 64   | 174 | 31   | 0.36 | 24.3 |
| 53.3 | 301 | 102 | 228 | 271 | 110  | 1.34 | 246 | 80   | 483  | 54   | 54   | 99    | 55   | 174 | 28   | 0.38 | 29.0 |
| 57.0 | 263 | 84  | 202 | 234 | 97   | 1.23 | 199 | 70   | 433  | 49   | 50   | 88    | 46   | 143 | 18   | 0.36 | 27.5 |
| 62.0 | 211 | 73  | 189 | 206 | 86   | 1.09 | 170 | 67   | 399  | 47   | 42   | 92    | 42   | 137 | 22   | 0.36 | 25.8 |
| 63.0 | 178 | 67  | 177 | 190 | 94   | 1.01 | 176 | 52   | 373  | 44   | 43   | 87    | 41   | 130 | 22   | 0.32 | 19.3 |
| 64.7 | 202 | 87  | 187 | 280 | 92   | 1.09 | 269 | 69   | 423  | 50   | 52   | 97    | 49   | 160 | 31   | 0.35 | 14.3 |
| 66.6 | 217 | 101 | 273 | 296 | 175  | 1.25 | 301 | 81   | 496  | 55   | 96   | 105   | 58   | 169 | 34   | 0.42 | 34.3 |
| (b)  |     |     |     |     |      |      |     |      |      |      |      |       |      |     |      |      |      |
| Time | Ala | Thr | Gly | Val | Ser  | Sar  | Leu | Asp  | Gln  | Met  | Orn  | Phe   | Tyr  | Lys | Arg  | hArg | Trp  |
| 5.5  | 175 | 116 | 206 | 95  | 105  | 1.7  | 122 | 81   | 652  | 54.5 | 16.1 | 46.4  | 81   | 224 | 14.9 | 0.27 | 12.9 |
| 7.2  | 135 | 155 | 246 | 91  | 112  | 1.8  | 107 | 101  | 823  | 63.8 | 21.0 | 55.7  | 82   | 226 | 13.9 | 0.24 | 20.4 |
| 9.0  | 126 | 200 | 309 | 80  | 141  | 1.5  | 136 | 134  | 850  | 66.6 | 20.7 | 61.7  | 90   | 252 | 15.2 | 0.22 | 23.8 |
| 10.7 | 284 | 139 | 199 | 227 | 101  | 2.0  | 174 | 81   | 762  | 61.6 | 24.3 | 54.2  | 99   | 365 | 14.0 | 0.52 | 14.1 |
| 11.3 | 290 | 150 | 217 | 219 | 108  | 2.4  | 182 | 88   | 847  | 66.4 | 25.5 | 58.0  | 108  | 380 | 15.9 | 0.53 | 15.5 |
| 12.6 | 147 | 281 | 506 | 74  | 206  | 2.0  | 234 | 250  | 953  | 69.2 | 23.4 | 88.9  | 102  | 282 | 30.5 | 0.23 | 25.1 |
| 15.9 | 179 | 397 | 745 | 94  | 302  | 2.5  | 332 | 553  | 1202 | 83.1 | 37.6 | 125.9 | 135  | 421 | 46.8 | 0.27 | 35.2 |
| 30.8 | 320 | 524 | 985 | 132 | 420  | 4.2  | 530 | 946  | 947  | 77.5 | 64.0 | 198.9 | 208  | 480 | 85.6 | 0.22 | 78.6 |
| 35.0 | 258 | 283 | 659 | 129 | 279  | 3.3  | 376 | 631  | 745  | 62.6 | 38.0 | 136.5 | 150  | 293 | 57.2 | 0.18 | 43.9 |
| 40.3 | 361 | 233 | 740 | 172 | 315  | 5.0  | 507 | 761  | 928  | 65.7 | 39.4 | 185.0 | 179  | 248 | 87.5 | 0.19 | 57.8 |
| 46.5 | 515 | 194 | 854 | 137 | 293  | 8.0  | 539 | 939  | 932  | 63.1 | 65.2 | 176.8 | 169  | 235 | 66.2 | 0.2  | 71.4 |
| 48.8 | 432 | 165 | 724 | 113 | 248  | 5.6  | 338 | 820  | 784  | 51.3 | 46.7 | 135.1 | 135  | 167 | 41.8 | 0.13 | 66.6 |
| 50.8 | 277 | 152 | 500 | 84  | 206  | 3.1  | 204 | 579  | 572  | 43.4 | 31.3 | 77.5  | 91   | 143 | 38.8 | 0.1  | 44.0 |
| 53.3 | 223 | 67  | 405 | 26  | 134  | 2.5  | 63  | 421  | 374  | 34.8 | 20.1 | 32.0  | 50   | 35  | 9.3  | 0.08 | 24.7 |
| 57.0 | 237 | 96  | 469 | 43  | 165  | 2.4  | 81  | 412  | 444  | 39.9 | 15.5 | 35.6  | 53   | 56  | 11.4 | 0.08 | 17.9 |
| 62.0 | 148 | 77  | 429 | 35  | 142  | 2.9  | 84  | 312  | 425  | 37.3 | 14.7 | 37.0  | 48   | 70  | 12.2 | 0.07 | 22.5 |
| 63.0 | 137 | 82  | 437 | 36  | 149  | 2.8  | 91  | 264  | 421  | 39.6 | 13.3 | 39.2  | 48   | 75  | 14.6 | 0.07 | 15.8 |
| 64.7 | 118 | 69  | 407 | 31  | 127  | 3.0  | 78  | 164  | 427  | 40.1 | 14.0 | 39.0  | 46   | 81  | 12.4 | 0.07 | 23.3 |
| 66.6 | 107 | 93  | 439 | 34  | 158  | 3.1  | 102 | 199  | 479  | 44.5 | 20.0 | 40.6  | 55   | 118 | 13.6 | 0.09 | 27.7 |
| (c)  |     |     |     |     |      |      |     |      |      |      |      |       |      |     |      |      |      |
| Time | Ala | Thr | Gly | Val | Ser  | Sar  | Leu | Asp  | Gln  | Met  | Orn  | Phe   | Tyr  | Lys | Arg  | hArg | Trp  |
| 5.0  | 71  | 17  | 28  | 66  | 11.8 | 0.58 | 55  | 9.2  | 104  | 15.3 | 4.7  | 14.1  | 13.3 | 31  | 7.4  | 0.59 | 2.1  |
| 8.8  | 203 | 77  | 115 | 238 | 43.7 | 2.68 | 242 | 40.2 | 541  | 38.9 | 21.3 | 67.0  | 58.4 | 138 | 38.5 | 1.71 | 10.4 |
| 11.0 | 140 | 64  | 111 | 166 | 35.5 | 1.37 | 161 | 35.1 | 432  | 35.2 | 16.2 | 56.6  | 40.8 | 103 | 28.5 | 1.36 | 6.5  |
| 12.0 | 125 | 60  | 109 | 137 | 34.5 | 1.45 | 139 | 33.2 | 421  | 33.7 | 15.0 | 52.7  | 38.3 | 108 | 27.0 | 1.20 | 6.8  |
| 13.0 | 135 | 72  | 135 | 153 | 40.4 | 1.45 | 166 | 41.9 | 482  | 36.8 | 18.1 | 63.5  | 42.8 | 133 | 33.0 | 1.21 | 7.7  |
| 15.3 | 156 | 88  | 157 | 160 | 51.5 | 2.02 | 196 | 49.2 | 486  | 39.0 | 22.1 | 71.4  | 50.5 | 148 | 43.0 | 1.22 | 10.0 |
| 17.6 | 145 | 90  | 152 | 154 | 53.0 | 1.35 | 192 | 47.8 | 426  | 35.8 | 21.0 | 72.0  | 50.4 | 148 | 43.9 | 0.98 | 9.7  |
| 19.5 | 181 | 106 | 158 | 179 | 60.6 | 1.19 | 223 | 52.4 | 395  | 34.3 | 25.4 | 79.8  | 60.5 | 163 | 50.7 | 0.84 | 11.4 |
| 20.8 | 158 | 125 | 143 | 191 | 65.9 | 1.58 | 240 | 57.0 | 418  | 35.1 | 26.9 | 83.8  | 70.8 | 181 | 54.3 | 0.80 | 12.3 |
| 24.3 | 207 | 129 | 170 | 204 | 68.3 | 1.25 | 249 | 58.2 | 375  | 34.4 | 28.5 | 84.4  | 76.7 | 175 | 60.3 | 0.70 | 13.2 |
| 29.1 | 205 | 121 | 165 | 210 | 61.9 | 1.75 | 240 | 52.9 | 371  | 34.6 | 33.2 | 83.5  | 77.4 | 141 | 46.8 | 0.64 | 14.9 |
| 30.5 | 182 | 123 | 168 | 220 | 62.4 | 1.88 | 259 | 52.4 | 369  | 35.6 | 35.7 | 85.6  | 73.0 | 140 | 48.9 | 0.58 | 15.1 |
| 33.4 | 208 | 112 | 156 | 221 | 58.4 | 1.91 | 227 | 52.2 | 358  | 34.9 | 34.1 | 84.1  | 65.7 | 121 | 41.3 | 0.48 | 13.6 |
| 35.0 | 187 | 96  | 140 | 181 | 51.1 | 1.69 | 180 | 45.6 | 317  | 31.6 | 28.3 | 77.8  | 54.4 | 107 | 34.6 | 0.46 | 11.8 |
| 40.3 | 153 | 83  | 124 | 177 | 46.1 | 1.28 | 173 | 36.3 | 251  | 28.0 | 27.8 | 74.6  | 46.6 | 105 | 32.6 | 0.33 | 11.2 |
| 42.6 | 195 | 107 | 139 | 250 | 51.1 | 1.64 | 246 | 47.3 | 318  | 32.8 | 47.4 | 91.6  | 65.0 | 130 | 35.3 | 0.40 | 14.3 |
| 46.5 | 180 | 95  | 128 | 215 | 45.1 | 1.44 | 204 | 46.1 | 291  | 29.9 | 41.8 | 85.1  | 56.9 | 109 | 28.7 | 0.33 | 14.5 |
| 48.9 | 157 | 101 | 128 | 223 | 52.1 | 1.13 | 257 | 48.8 | 295  | 33.7 | 37.6 | 81.5  | 51.4 | 116 | 40.1 | 0.36 | 17.3 |
| 50.0 | 198 | 122 | 162 | 234 | 63.9 | 1.66 | 267 | 52.9 | 367  | 37.9 | 36.3 | 89.5  | 71.5 | 136 | 49.0 | 0.56 | 14.2 |

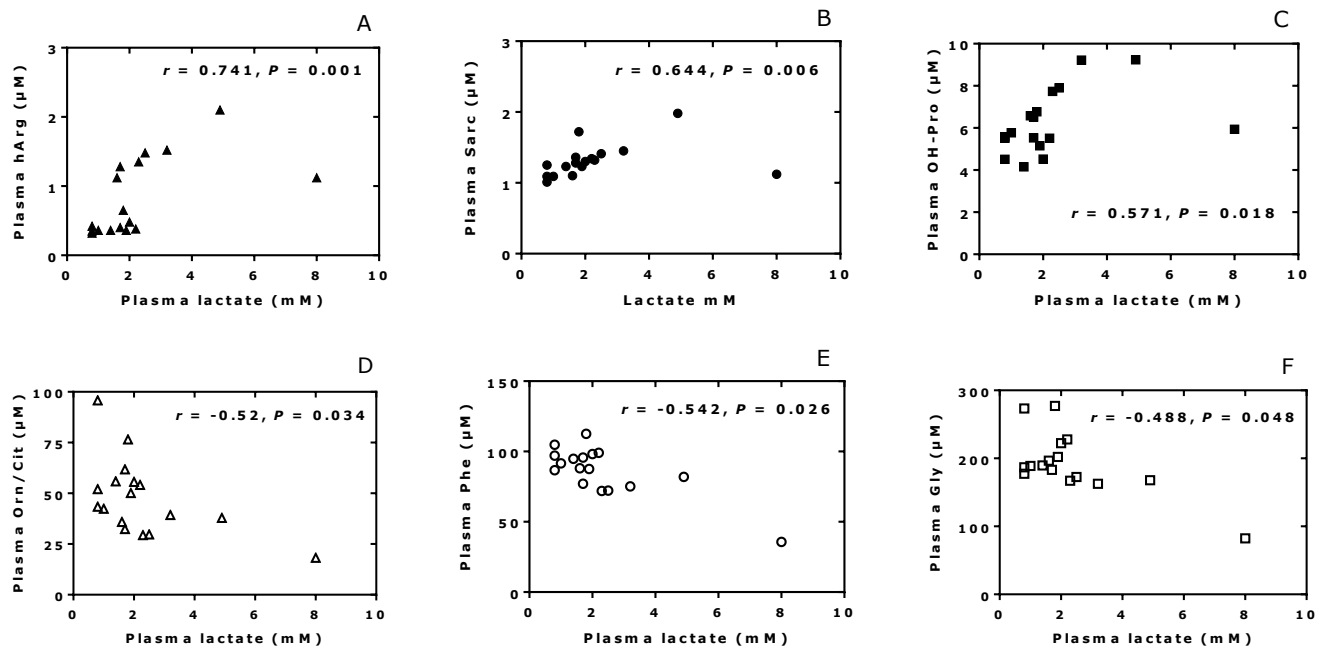


**Figure 1.** Correlations (after Spearman) between the plasma concentrations of the indicated amino acids and lactate measured in the MALA patient.

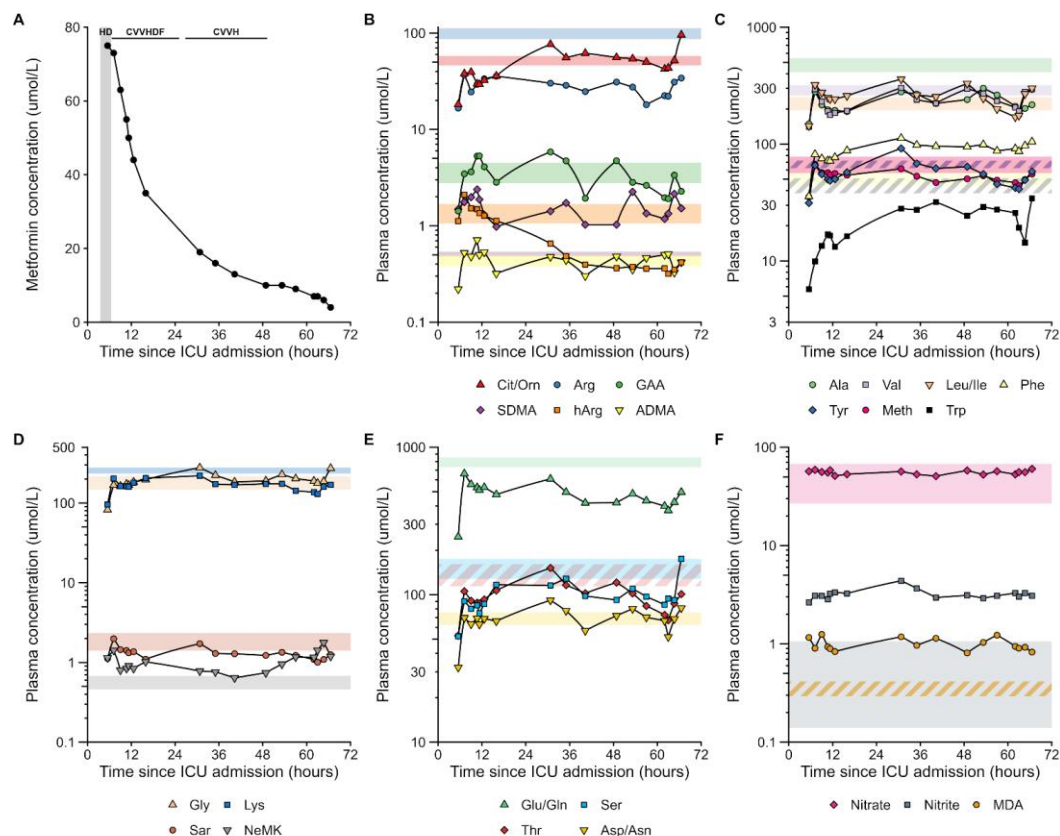


**Figure 2.** Time course of the plasma concentrations of metformin (A), the indicated amino acids (B–E), and nitrate, nitrite, and malondialdehyde (MDA) (F) during the renal replacement treatment (RRT) of the patient in the ICU since admission. HD, hemodialysis; CVVHDF, continuous veno-venous hemodiafiltration; CVVH, continuous veno-venous hemofiltration (CVVH). Shaded areas are reference values (interquartile range) obtained from healthy volunteers.

**Table 3.** Reported plasma/serum concentrations (range,  $\mu\text{M}$ ) in healthy humans for the analytes measured in the present study at admission to the ICU (5.5 h) until the end (66 h).

| Analyte            | Men [34]    | Women [33]       | MALA Patient<br>5.5 h–66 h | Fold Change | Refs. |
|--------------------|-------------|------------------|----------------------------|-------------|-------|
| Creatinine         | 82–101      | n.m.             | –230                       | –0.6        | [36]  |
| Malondialdehyde    | 0.31–0.46   | n.m.             | 1.16–0.82                  | –0.7        | [16]  |
| Metformin          | n.a.        | n.a.             | –4                         | –19.0       | n.a.  |
| Nitrate            | 38–55       | n.m.             | 57–60                      | +1.0        | [14]  |
| Nitrite            | 1.9–2.6     | n.m.             | 2.64–3.08                  | +1.2        | [14]  |
| <b>Amino acids</b> |             |                  |                            |             |       |
| ADMA               | 0.187–0.534 | n.m.             | 0.220–0.420                | +1.9        | [30]  |
| Alanine            | 254–392     | 203–443          | 149–217                    | +1.5        | [33]  |
| Arginine           | 50–90       | 50–119           | 17–34                      | +2.0        | [33]  |
| Asparagine         | 48–94       | 33–59            | 32–81                      | +2.5        | [33]  |
| Citrulline         | 34–48       | 18–42            |                            | +2.3        | [33]  |
| Glutamine          | 449–654     | 414–669<br>12–57 | 247–496                    | +2.0        | [33]  |
| Glycine            | 147–248     | 149–397          | 82.0–273                   | +3.3        | [33]  |
| Guanidinoacetate   | 1.77–4.57   | n.m.             | 1.42–2.28                  | +0.9        | none  |
| Homoarginine       | 0.66–1.74   | n.m.             | 1.12–0.42                  | –2.7        | [32]  |
| Hydroxyproline     | 5.8–10.9    | n.m.             | 5.94–5.58                  | –0.9        | none  |
| Leucine            | 155–254     | 74–125<br>35–66  | 143–301                    | +2.1        | [33]  |
| Lysine             | 110–171     | 115–223          | 96–169                     | +1.8        | [33]  |
| Methionine         | 41–59       | 17–30            | 35–55                      | +1.6        | [33]  |
| Ornithine          | see Cit     | 25–68            | 18–96                      | +5.3        | [33]  |
| Phenylalanine      | 39–69       | 42–66            | 36–105                     | +2.9        | [33]  |
| Proline            | 118–230     | 70–188           | 68–86                      | +1.3        | [33]  |
| Sarcosine          | 1.3–2.6     | n.m.             | 1.12–1.25                  | +1.1        | [33]  |
| SDMA               | n.m.        | n.m.             | 1.48–1.52                  | +1.4        | [30]  |
| Serine             | 106–210     | 80–157           | 52–175                     | +3.4        | [33]  |
| Threonine          | 105–162     | 68–169           | 53–101                     | +1.9        | [33]  |
| Tryptophan         | 6.3–20.4    | 41–68            | 5.7–34.3                   | +6.0        | [33]  |
| Tyrosine           | 36–64       | 40–74            | 31–58                      | +1.9        | [33]  |
| Valine             | 218–286     | 138–243          | 141–296                    | +2.1        | [33]  |
| N <sup>ε</sup> MK  | n.m.        | n.m.             | 1.14–1.19                  | +1.04       | [27]  |

n.a., not applicable; n.m., not measured.

In general, the plasma concentrations of the analyzed amino acids were in the lower range of reference intervals. The concentrations of nitrate, nitrite, and MDA measured in plasma and urine were within normal ranges. These observations suggest rather unaltered amino acid, NO homeostasis, and oxidative stress. The results of the study are discussed in detail as follows below for plasma, urine, and effluent (dialysate).

At admission, the plasma Arg concentration was lower compared to healthy humans (Tables 2 and 3). The plasma concentration of the Arg metabolite ADMA was also lower than in healthy adults. The SDMA plasma concentration was higher, probably due to de-

creased urinary excretion of this Arg metabolite, which is eliminated primarily unchanged by glomerular filtration. The plasma concentration of guanidinoacetate (GAA), an Arg metabolite from the arginine:glycine amidinotransferase (AGAT) pathway and the precursor of creatine, remained within normal ranges. The plasma concentration of hArg, another Arg metabolite from the AGAT pathway, was normal at ICU admission but decreased over time. The plasma concentrations of N<sup>ε</sup>-methyl lysine (N<sup>ε</sup>MK), a PTM metabolite of lysine (Lys) residues in proteins, were considerably higher than in healthy subjects, indicating a higher monomethylation extent of Lys in the MALA patient. At admission, the plasma concentrations of nitrate, nitrite, and MDA were within normal ranges (Tables 1 and 3). Thus, NO synthesis and oxidative stress were not elevated in the MALA patient and did not change with renal replacement treatment.

Except for hydroxy-proline (−0.9-fold) and homoarginine (−2.7-fold), at the end of the renal replacement treatment, the plasma amino acid concentrations were up to 6-fold higher compared to those at admission to the ICU (Table 3). The highest increase was observed for tryptophan (6-fold).

We tested for potential correlations between the plasma concentrations of lactate and other analytes. The plasma lactate concentration values correlated positively with the plasma concentrations of homoarginine (hArg;  $r = 0.741$ ,  $p = 0.0007$ ), sarcosine (Sar;  $r = 0.644$ ,  $p = 0.005$ ), and hydroxy-proline (OH-Pro;  $r = 0.5707$ ,  $p = 0.0184$ ), yet not of proline [13] (Figure 1). Lactate has been shown to inhibit proline oxidase activity [35]. The correlation was negative for phenylalanine (Phe;  $r = -0.521$ ,  $p = 0.025$ ), ornithine (Orn;  $r = -0.520$ ,  $p = 0.032$ ), and glycine (Gly;  $r = -0.488$ ,  $p = 0.047$ ) (Figure 1). The plasma lactate concentrations did not correlate with the plasma concentrations of nitrite, nitrate, or MDA. These associations could result from effects of lactate of enzyme activities involved in the metabolism of amino acids [35], but this remains to be investigated in other cases of MALA. Lack of correlation between plasma concentrations of nitrite, nitrate, or MDA and lactate was also observed in healthy young men [34].

The urinary excretion rate of ADMA was lower than in healthy adults. The excretion rate of the ADMA metabolite DMA was higher than in healthy adults. This observation suggests increased metabolism of ADMA to DMA, presumably due to elevated activity of dimethylarginine dimethylaminohydrolase (DDAH) that hydrolyses ADMA to DMA and citrulline.

The tubular reabsorption values of most amino acids (Figure 3A–E) and of nitrite and nitrate (Figure 3F) were low and increased within the first hours of extracorporeal treatment to reach normal values. The tubular reabsorption values of GAA and Asn/Asp differed from those of the other amino acids. The negative tubular reabsorption values seen for GAA (Figure 3A) and Asn/Asp (Figure 3D) are indicative of renal secretion of these amino acids. During ICU admission, urinary Lys and N<sup>ε</sup>MK excretion decreased temporarily (Figure 3E), suggesting improved tubular reabsorption over time. The low tubular reabsorption values of nitrate and nitrite within the first hours of treatment indicate altered renal management of these NO metabolites in the MALA patient.

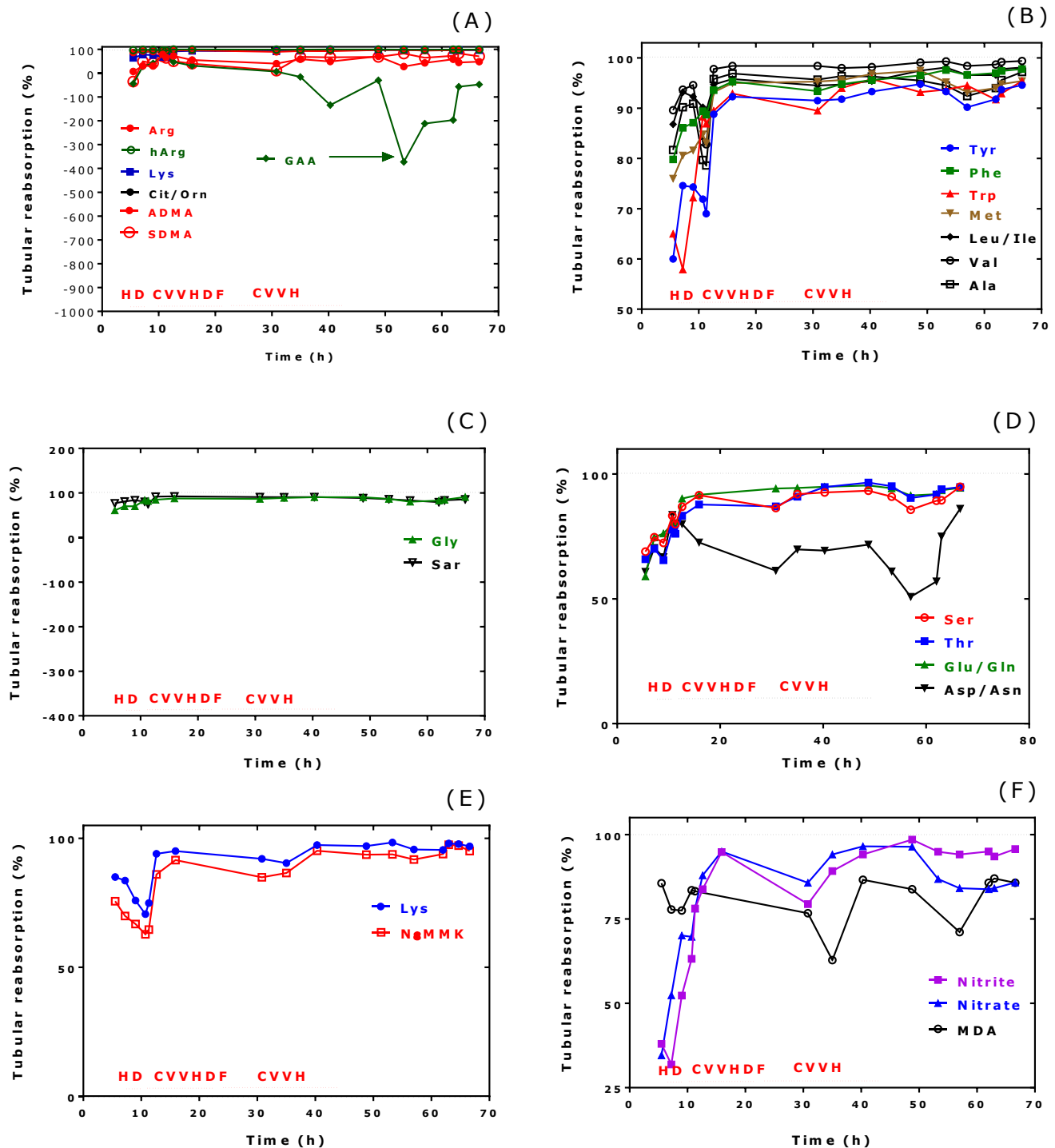


**Figure 3.** Time course of the calculated tubular reabsorption rates of the indicated amino acids, nitrate, nitrite, and malondialdehyde (MDA) during the renal replacement treatment (RRT) of the patient in the ICU since admission. HD, hemodialysis; CVVHDF, continuous veno-venous hemodiafiltration; CVVH, continuous veno-venous hemofiltration (CVVH).

#### 4. Conclusions

Stable-isotope dilution GC-MS allows quantitative analysis of structurally different physiological inorganic (nitrite, nitrate), organic hydrophilic (amino acids and metabolites), and lipophilic (MDA) substances, as well as drugs (metformin) in complex biological samples such as plasma and urine. Our present and previous [13] study suggest that metformin-intoxication is not associated with greatly altered amino acid homeostasis,

except for an elevated PTM of proline by prolyl-hydroxylation. This PTM seems to be specific for the MALA patient since other PTM metabolites, including those of Arg (i.e., ADMA, SDMA) and Lys (i.e., N<sup>ε</sup>MK), were not elevated at admission and did not change remarkably during the renal replacement treatment. That the plasma MDA concentration was not elevated in the MALA patient at admission and did not change during the renal replacement therapy suggests that oxidative stress is not associated with MALA. Extracorporeal renal replacement therapy is efficient in MALA, both with respect to metformin elimination and normalization of the renal management of solutes. In this case report, we have analyzed a single MALA patient. This is a study limitation. Confirmation of the observations in this patient is warranted by analyzing plasma, urine, and dialysate samples from additional metformin-intoxicated patients.

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**Institutional Review Board Statement:** Ethical approval was given by the institutional review board Medisch Ethische Toetsingscommissie Universitair Medisch Centrum Groningen (METc UMCG) (code 2014-552, approved on 13 January 2015).

**Informed Consent Statement:** Written informed consent was obtained from the patient admitted with MALA to the ICU to collect residual material and for the publication of this case report according to the CARE guidelines.

**Data Availability Statement:** Data are available on request.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

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