Muscular myostatin gene expression and plasma concentrations are decreased in critically ill patients

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Methods
MSTN expression levels frozen tissue was homogenized and total RNA was isolated according to the manufacturer's instructions of Trizol Reagent (Invitrogen, CA, USA). RNA samples were stored at −80 °C until assayed. Complementary DNA (cDNA) synthesis was done according to manufacture manual (High Capacity RNA-to-cDNA Kit; Applied Biosystems, Foster City, CA). Samples were analyzed in triplicate with Power SYBR Green PCR Master Mix (Applied Biosystems). Real-time quantitative polymerase chain reaction was performed using an ABI PRISM 7300 System (using SDS 1.4 system software, Applied Biosystems). The expression level of cyclophyllin A was used as an internal control. The used primer sequences were: cyclophyllin A (forward: TGTGAAGTCACCACCCTGACACAT; reverse: AGACAAGGTCCCAAAGACAGCAGA) and myostatin (forward GGCTCAAACAACCTGAATCC; reverse: TCCCTTCTGGATCTTTTTGG; Life technologies). Cycle threshold values were used to calculate the amount of amplified polymerase chain reaction product in comparison to the housekeeping gene cyclophyllin A. The relative amounts of each transcript were analyzed using the 2−ΔC(t) method [1].

References
1. Mai K, Klug L, Rakova N, Piper SK, Mahler A, Bobbert T, Schulz-Menger J, Spranger J, Boschmann M, Luft FC: Hypoxia and exercise interactions on skeletal muscle insulin sensitivity in obese subjects with metabolic syndrome: results of a randomized controlled trial. Int J Obes (Lond) 2020, 44(5):1119-1128.
Inclusion flow chart depicting included and excluded respectively enrolled and analyzed patients. IDDM = insulin dependent diabetes mellitus; ICU = Intensive Care Unit; SOFA-Score = Sepsis-related Organ Failure Assessment score; sPT = standard physiotherapy; pPT = protocol-based physiotherapy; pPT+adMeas = protocol-based physiotherapy + additional muscle activating measures.
Figure A2 Correlation between myostatin plasma levels on day 8 and muscle strength measured via Medical Research Council score at first awakening

Myostatin plasma levels on day 8 show a significant positive correlation to muscle strength measured via MRC score at first adequate awakening.

MRC = Medical Research Council Score

See Table A3 for correlation coefficient and significance value.
Figure A3 Differences in MSTN gene expression and myostatin plasma trajectory in patients according to electrophysiological diagnosis

a) MSTN gene expression was significantly lower in all critically ill patients except those diagnosed with CIP.

b) Myostatin plasma concentration was significantly lower in all critically ill patients irrespective of the electrophysiological diagnosis but the recovery over time did not reach statistical significance [GLM: \( p = 0.106; n = 15 \) patients classified as noCIPCIM, \( n = 8 \) patients classified as CIM, \( n = 2 \) patients classified as CIP and \( n = 2 \) patients classified as CIPCIM with values from all three timepoints were analyzed]

c) Patients with myostatin plasma levels on day 14 at or above healthy controls did not show any signs of CIP, CIM or CIPCIM in their electrophysiological evaluation.

GLM = general linear model for the factor “time” in critically ill; mRNA = messenger ribonucleic acid; CIM = Critical Illness Myopathy; CIP = Critical Illness Polyneuropathy; CIPCIM = Critical Illness Polyneuropathy and Myopathy

\( **: p < 0.010 \) for Kruskal-Wallis test between healthy controls and critically ill

\( *: p < 0.05 \) and \( ***: p < 0.001 \) for post-hoc test comparison to healthy controls
a) All critically ill patients show a typical pattern of molecular parameters with upregulation of muscle protein degradation and inflammation. No difference between the two clusters of Myostatin plasma levels were present.

b) Myocyte cross sectional area also showed no difference between the two Myostatin groups.

*: p < 0.050, **: p < 0.010 and ***: p < 0.001 for Kruskal-Wallis test between healthy controls and critically ill

*: p < 0.05, **: p < 0.01 and ***: p < 0.001 for post-hoc test comparison to healthy controls
Figure A5 Insulin Sensitivity Index according to myostatin cluster and between myostatin plasma level on day 14 and Insulin Sensitivity Index

a) Critically ill patients with plasma Myostatin levels at or above healthy controls show a significantly higher Insulin Sensitivity Index.

b) Myostatin plasma levels on day 14 show a significant positive correlation to the Insulin Sensitivity Index.

See Table A5 for correlation coefficient and significance value.
| Myostatin plasma values (pg/ml) | Median   | Interquartile Range |
|--------------------------------|----------|---------------------|
| Healthy Controls               | 2990.3   | 2417.0 – 3609.9     |
| Day 4                          | 406.4    | 233.1 - 632.7       |
| Day 8                          | 707.3    | 314.8 - 1312.8      |
| Day 14                         | 1213.9   | 790.3 – 1842.5      |

**Table A1 Myostatin plasma values**

*Values for metric variables are presented as median and interquartile range.*
Table A2 Baseline characteristics for myostatin groups

Values for metric variables are presented as median and interquartile range and for categorical variables as count and percentages. Mann-Whitney U or Chi-Square Test were used to calculate statistical significance. BMI = Body Mass Index; ICU = Intensive Care Unit; ARDS = Acute Respiratory Distress Syndrome; CNS = Central Nervous System SOFA = Sepsis-related Organ Failure Assessment score; SAPS2 = Simplified Acute Physiology Score; RASS = Richmond Agitation Sedation Scale; +time shown is the time the patient received the actual physiotherapeutic intervention during which the muscle was stimulated not including preparation or documentation.
Correlations were assessed with Spearman’s rank correlation coefficient. 

*mRNA = messenger ribonucleic acid.*

### Table A3 Correlation MSTN, myostatin and muscle strength

|                          | MRC score first awakening | MRC score ICU discharge |
|--------------------------|---------------------------|-------------------------|
|                          | Correlation coefficient  | R²          | p     | Correlation coefficient | R²          | p     |
| **MSTN – relative mRNA expression (fold change)** | -0.089 | 0.001 | 0.586 | 0.212 | 0.014 | 0.189 |
| Myostatin day 4 – relative serum concentration (fold change) | 0.025 | 0.010 | 0.897 | -0.063 | 0.002 | 0.747 |
| Myostatin day 8 – relative serum concentration (fold change) | 0.339 | 0.109 | 0.020 | 0.169 | 0.058 | 0.256 |
| Myostatin day 14 – relative serum concentration (fold change) | 0.161 | 0.088 | 0.298 | 0.044 | 0.042 | 0.776 |
Table A4 Correlation MSTN, myostatin and direct muscle stimulation compound muscle action potential

Correlations were assessed with Spearman’s rank correlation coefficient. dmCMAP = direct muscle stimulation compound muscle action potential; mRNA = messenger ribonucleic acid.

|                          | dmCMAP     | R²          | p      |
|--------------------------|------------|-------------|--------|
| MSTN – relative mRNA expression (fold change) | 0.142      | <0.0001     | 0.358  |
| Myostatin day 4 – relative serum concentration (fold change) | 0.156      | 0.049       | 0.411  |
| Myostatin day 8 – relative serum concentration (fold change) | -0.043     | <0.0001     | 0.764  |
| Myostatin day 14 – relative serum concentration (fold change) | 0.135      | 0.002       | 0.366  |
|                                | Insulin Sensitivity Index (mg/kg/min)/(mU/l) | Correlation coefficient | R²  | p      |
|--------------------------------|---------------------------------------------|-------------------------|-----|--------|
| MSTN – relative mRNA expression (fold change) | -0.030                                      |                         | 0.071 | 0.847 |
| Myostatin day 4 – relative serum concentration (fold change) | 0.199                                       |                         | 0.077 | 0.341 |
| Myostatin day 8 – relative serum concentration (fold change) | 0.112                                       |                         | 0.021 | 0.463 |
| Myostatin day 14 – relative serum concentration (fold change) | 0.357                                       |                         | 0.228 | 0.015 |

**Table A5 Correlation MSTN, myostatin and Insulin Sensitivity Index**

Correlations were assessed with Spearman's rank correlation coefficient.

*mRNA = messenger ribonucleic acid.*