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Clinical Research Article

Skeletal Muscle Myokine Expression in Critical Illness, Association With Outcome and Impact of Therapeutic Interventions

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Abstract

Context: Muscle expresses and secretes several myokines that bring about benefits in distant organs.

Objective: We investigated the impact of critical illness on muscular expression of irisin, kynurenine aminotransferases, and amylase; association with clinical outcome; and impact of interventions that attenuate muscle wasting/weakness.

Methods: We studied critically ill patients who participated in 2 randomized controlled trials (EPaNIC/NESCI) and documented time profiles in critically ill mice. Included in the study were 174 intensive care unit (ICU) patients (day 8 ± 1) vs 19 matched controls, and 60 mice subjected to surgery/sepsis vs 60 pair-fed healthy mice. Interventions studied included 7-day neuromuscular electrical stimulation (NMES), and withholding parenteral nutrition (PN) in the first ICU week (late PN) vs early PN. The main outcome measures were *FNDC5* (irisin- precursor), *KYAT1, KYAT3*, and amylase mRNA expression in skeletal muscle.

Results: Critically ill patients showed 34% to 80% lower mRNA expression of *FNDC5*, *KYAT1*, and amylases than controls ($P < .0001$). Critically ill mice showed time-dependent reductions in all mRNAs compared with healthy mice ($P \leq .04$). The lower *FNDC5* expression in patients was independently associated with a higher ICU mortality ($P = .015$) and ICU-acquired weakness ($P = .012$), whereas the lower amylase expression in ICU survivors was independently associated with a longer ICU stay ($P = .0060$). Lower amylase expression was independently associated with a lower risk of death ($P = .048$), and lower *KYAT1* expression with a lower risk of weakness ($P = .022$). NMES increased *FNDC5* expression compared with unstimulated muscle ($P = .016$), and late PN patients had a higher *KYAT1* expression than early PN patients ($P = .022$).

Conclusion: Expression of the studied myokines was affected by critical illness and associated with clinical outcomes, with limited effects of interventions that attenuate muscle wasting or weakness.

Key Words: critical illness, myokine, irisin, FNDC5, kynurenine aminotransferase, amylase

Abbreviations: BMI, body mass index; ICU, intensive care unit; EN, enteral nutrition; FNDC5, fibronectin type III domain-containing protein 5; KYAT, kynurenine aminotransferase; LPS, lipopolysaccharide; MRC, Medical Research Council; NMES, neuromuscular electrical stimulation; PN, parenteral nutrition.

Patients who are critically ill, requiring admission to an intensive care unit (ICU) for vital organ support, are bedridden [1]. The resulting immobilization contributes to mechanical unloading of the muscles [2]. Furthermore, characteristic hormonal changes, with low levels of anabolic peripheral effector hormones such as insulin-like growth factor I and high levels of the catabolic hormone cortisol, typically induce a catabolic state [3]. As a result, pronounced loss of muscle mass ensues, particularly when critical illness is prolonged [4, 5]. These are only a few of the pathophysiological factors linked with the high risk of developing clinically significant muscle weakness in the ICU, which in turn has been associated with increased risk of death and prolonged dependency on mechanical ventilation and other intensive care [6].

Disturbances in muscle mass and activity may not only be important for muscle function itself, but may also have much farther reaching consequences through endocrine and paracrine actions. Indeed, skeletal muscle produces and secretes a variety of proteins through which communication with other organs is mediated, such as the brain, liver, gut, adipose tissue, pancreas, bone, the cardiovascular system, and also the immune system [7–10]. As such, these so-called

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"myokines" may be important for the health of distant organs, and loss of skeletal muscle mass and reduced levels of physical activity thus may contribute to disease [7, 8].

Hundreds of proteins are secreted from muscle cells during proliferation and differentiation or in response to muscle contractions [8, 10]. Some of them have been identified as myokines [8]. The muscular expression of several of them has already been studied quite extensively in the context of critical illness, such as that of myostatin, myogenin, and certain interleukins, among others. This contrasts with some other proteins identified in the literature as (plausible) myokines, including irisin, kynurenine aminotransferases (KYATs), and amylases. Some information is available on serum/plasma concentrations of these proteins or affected metabolites and their association with outcome of critical illness. However, data on muscular expression of these myokines in this context are virtually lacking [11, 12].

Irisin has been extensively studied in the past decade in other contexts. This myokine is released from muscle after cleavage of the membrane-bound precursor protein fibronectin type III domain-containing protein 5 (FNDC5) [13]. Irisin is induced by exercise and has shown promise as a potential therapeutic agent in the prevention or treatment of various diseases. Exercise also induces KYATs, which shift peripheral metabolism of the tryptophan-derived kynurenine to kynurenic acid [14, 15]. This shift locally increases energy efficiency and fatigue resistance in muscle, but also mediates distant beneficial effects on energy utilization in adipose tissue and confers neuroprotection [8, 15–17]. Recently, a new myokine has been identified in *Drosophila*, the amylase "amyrel" [18]. Amyrel was found to be released from muscle in response to muscle proteasome stress, resulting in signaling to the brain which attenuated brain aging via preservation of brain proteostasis. Amyrel is not present in humans or mice. However, both species express several amylases that, although normally confined to the digestive system, can also be expressed and secreted by skeletal muscle and respond when proteostasis is challenged [18, 19].

In this study, we investigated whether human critical illness affects muscular expression of FNDC5/irisin, KYATs, and amylases, whether their expression associates with patient outcomes or histological features in muscle, and whether 2 interventions that have shown to attenuate muscle wasting or weakness affect them. In addition, we investigated the time course of myokine expression in muscle harvested from critically ill mice suffering from surgery and sepsis.

Materials and Methods

Studies Investigating Myokine Expression in Human Muscle Biopsies

In the first study, we performed a secondary analysis of participants of the Early Parenteral Nutrition Completing Enteral Nutrition in Adult Critically Ill Patients (EPaNIC) trial (ClinicalTrials.gov-NCT00512122) [20]. In this trial, 4640 adult ICU patients were randomly assigned to 1 of 2 nutritional strategies related to timing of initiating supplemental parenteral nutrition (PN) when enteral nutrition (EN) failed to deliver recommended macronutrient doses. In patients randomized to early PN, supplemental PN was initiated within 48 hours after ICU admission to complete insufficient EN early, allowing early full feeding. In patients randomized to late PN, supplemental PN was withheld in the first week in ICU, thus accepting any macronutrient deficit resulting from EN intolerance (late PN). Both groups received EN as soon as possible, insulin infusions to maintain normoglycemia, parenteral trace elements, minerals, and vitamins, and standard physiotherapy [20, 21]. Muscle strength was assessed with the Medical Research Council (MRC) sum score after ensuring that patients were awake and co-operative, with a score <48 diagnosing clinically relevant muscle weakness [21]. The protocol and primary outcomes have been published [20–22]. The Institutional Review Board approved the study (ML4190). Written informed consent was acquired from all patients or next of kin. Separate, additional written informed consent was obtained for harvesting of in vivo percutaneous needle biopsies from the vastus lateralis muscle of a subgroup of the patients (n = 188). Muscle biopsies were taken on ICU day 8 ± 1, at mid-thigh level (Bergström technique) after local anesthesia (lidocaine 2%). Patients with pre-existing neuromuscular diseases, patients with coagulation disorders, and patients receiving therapeutic anticoagulant drugs were a priori excluded. Using the same technique, muscle biopsies were collected from 20 controls in parallel with the patients, after written informed consent (ML4190). Controls had age, sex, and body mass index (BMI) distributions similar to the patients, but had never been admitted to an ICU prior to the biopsy. Controls were asked not to engage in rigorous physical activity before the biopsy collection, so that they had at least an overnight resting period, but no feeding restrictions were imposed. Sufficient, good quality RNA (median RNA integrity number 7.2, interquartile range 6.9-7.6) could be obtained for 162 patients and 19 controls (Fig. 1) [23]. The participants' characteristics are shown in Table 1.

In a second study, we performed a secondary analysis of patients who participated in the Neuromuscular Electrical Stimulation in Critically Ill Patients (NESCI) trial (ClinicalTrials.gov-NCT02133300). The detailed study design, procedures, and primary outcomes have been published [24]. The NESCI study included 50 adult patients between day 2 and 4 after ICU admission, who all received physiotherapy and early mobilization according to a standardized local "Start to move as soon as possible" protocol [21]. Randomization determined whether the quadriceps of the dominant or the nondominant leg was selected for stimulation. Neuromuscular electrical stimulation (NMES) consisted of a 1-hour daily NMES session (Chattanooga Physio NMES device, DJO Global, Herentals, Belgium), applied for 7 consecutive days, and continued on the general ward in case of earlier ICU discharge. During a 5-minute warming-up phase, the frequency was set at 4 Hz and the intensity was increased until a clear muscle response was visible. The muscle was then stimulated for the next 50 minutes at the highest tolerable intensity, obtaining the best possible muscle response without discomfort. Settings during the 8.5-second contraction phase were 45 Hz, pulse duration 350 μs, 1.5-second ramp up, and 1-second ramp down, whereas the 12-second rest phase consisted of 4 Hz contractions. In the 5-minute cooling down phase, settings were the same as those used in the warming-up phase. A good contraction upon stimulation was defined as a clearly visible and palpable contraction. The nonstimulated quadriceps muscle did not receive a sham treatment. During a period of administration of neuromuscular blocking agents, the NMES session did not take place. The Institutional Review Board approved the study (ML10058). Written informed consent was acquired from

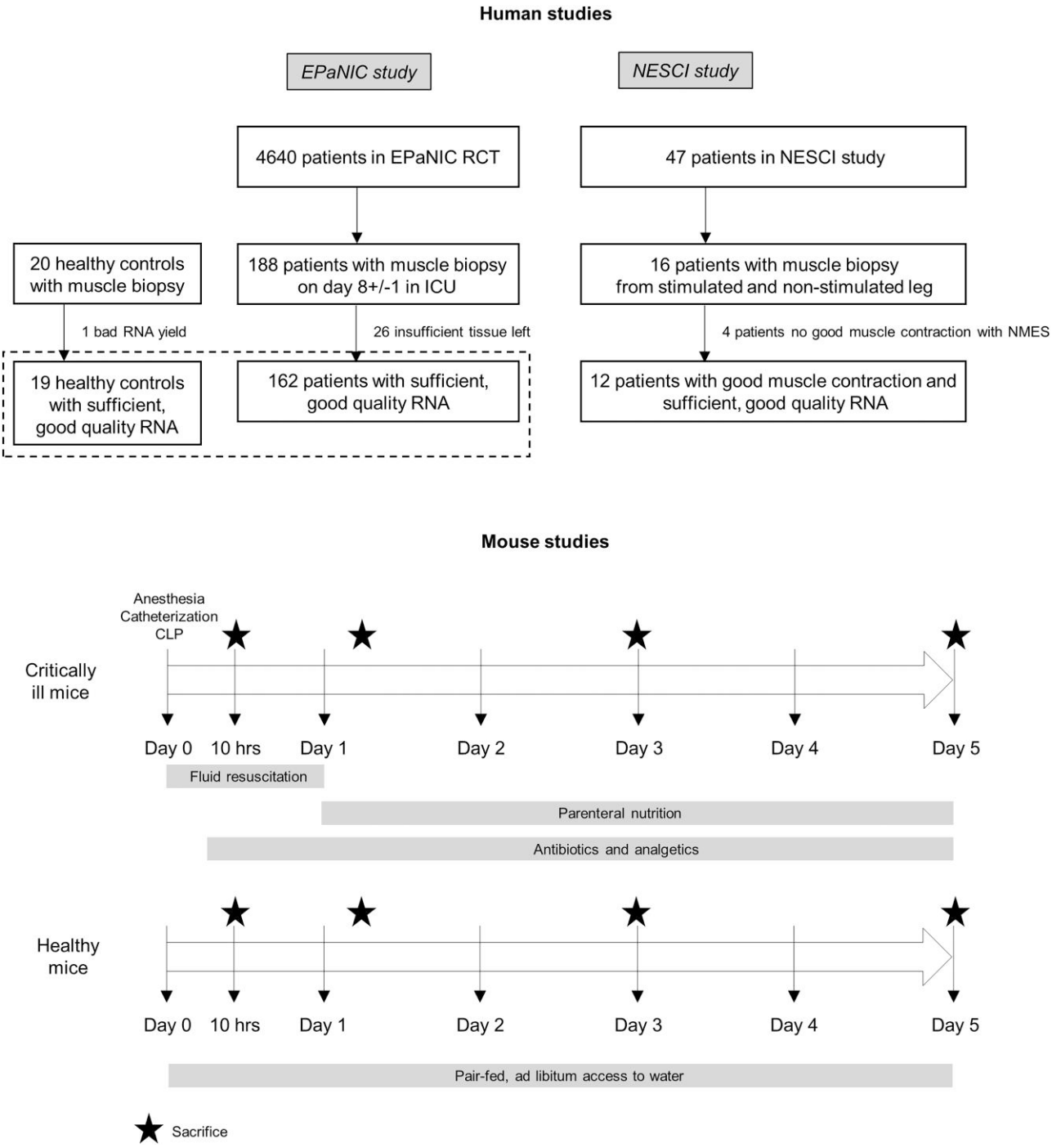

Figure 1. Flowchart and study design of human patient and mouse studies. CLP, cecal ligation and puncture; EPaNIC, Early Parenteral Nutrition Completing Enteral Nutrition in Adult Critically Ill Patients trial; NESCI, Neuromuscular Electrical Stimulation in Critically Ill Patients trial; NMES: neuromuscular electrical stimulation.

all patients or next of kin. Separate, additional, written informed consent was obtained for harvesting of in vivo bilateral percutaneous needle biopsies from the musculus vastus lateralis of a subgroup of the patients (n = 16). These were taken at the level of the mid-thigh after the intervention period, on the day of the last stimulation session or on the day after. Patients with pre-existing neuromuscular diseases, patients with coagulation disorders, and patients receiving therapeutic anticoagulant drugs were a priori excluded. For this study, we selected the 12 patients who showed a good muscle contraction upon neuromuscular electrical stimulation (Fig. 1). The patients' characteristics are shown in Table 1.

Both studies were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its amendments.

Study of the Myokine Expression Time Course in Mouse Muscle Biopsies

Twenty-four week old male C57BL/6J mice were obtained from Janvier (Le Genest-Saint-Isle, France). To induce critical

Table 1. Demographics, baseline characteristics, and muscle weakness of participants

	EPaNIC study						NESCI study
Characteristic	Controls (n = 19)	Patients (n = 162)	*P*	Early-PN (n = 66)	Late-PN (n = 66)	*P*	Patients (n = 12)
Male sex, n (%)	13 (68.4)	105 (64.8)	.75	46 (69.7)	45 (68.2)	.85	7 (58.3)
Age (year), mean ± SEM	59.3 ± 2.2	61.7 ± 1.8	.34	63.4 ± 1.6	61.7 ± 1.9	.50	63.0 ± 3.2
BMI (kg/m^2), mean ± SEM[a]	25.8 ± 0.8	26.9 ± 0.4	.24	26.8 ± 0.7	26.4 ± 0.6	.59	25.9 ± 1.6
Diabetes mellitus, n. (%)		27 (16.7)		9 (13.6)	10 (15.2)	.80	0 (0.0)
History of malignancy, n. (%)		48 (29.6)		24 (36.4)	19 (28.8)	.35	1 (8.3)
NRS score ≥5, n (%)		54 (33.3)		24 (36.4)	23 (34.9)	.85	NA
APACHE-II score upon admission, mean ± SEM		34.0 ± 0.7		34.7 ± 1.0	33.5 ± 1.1	.52	25.7 ± 2.5
Emergency admission, n. (%)		145 (89.5)		60 (90.9)	58 (87.9)	.57	11 (91.7)
Diagnostic category at admission, n. (%)						.86	
Cardiac surgery		14 (8.6)		5 (7.6)	5 (7.6)		2 (16.7)
Elective other surgery		7 (4.3)		2 (3.0)	4 (6.1)		0 (0.0)
Emergency other surgery		91 (56.2)		38 (57.6)	37 (56.1)		3 (25.0)
Medical disease		50 (30.9)		21 (31.8)	20 (30.3)		7 (58.3)
No enteral feeding instructed by surgeon, n (%)		32 (19.8)		17 (25.8)	13 (19.7)	.40	2 (16.7)
Sepsis upon admission, n (%)		94 (58.0)		39 (59.1)	38 (57.6)	.85	6 (50.0)
Randomization to late PN, n (%)		77 (47.5)					NA
Muscle weakness, n (%)				22 (64.7)	16 (43.2)	.068	NA[b]
Weak (MRC sum score <48)		44 (27.16)					
Not weak (MRC sum score ≥48)		38 (23.46)					
Not assessable		80 (49.38)					

Abbreviations: APACHE, Acute Physiology And Chronic Health Evaluation; BMI, body mass index; ICU, intensive care unit; MRC, Medical Research Council; NA, not applicable or not available; NRS, nutritional risk screening; PN, parenteral nutrition; SEM, standard error of the mean.
[a]Height and weight of the patients were documented upon hospital admission, as per standard practice, from which BMI was calculated.
[b]MRC score was only determined for the quadriceps muscle and was below 4 for 1 patient out of 8 patients who were assessable.

illness, mice were subjected to a double insult comprising the combination of surgery and induction of polymicrobial sepsis, considering that surgery and sepsis often co-occur in critically ill patients. Under ketamine–xylazine anesthesia (intraperitoneally injected, 100 mg/kg–13 mg/kg), a catheter was placed in the central jugular vein, followed by cecal ligation and puncture to induce polymicrobial sepsis (Fig. 1) [25, 26]. The latter procedure comprised ligation of the cecum at 50% of its length and a single through-and-through puncture with an 18G needle. After surgery, mice were fasted and received intravenous fluid resuscitation with Plasmalyte A Viaflo (Baxter) and 6% hydroxyethyl starch (Fresenius Kabi, Schelle, Belgium) (5:1 ratio; 0.3 mL/hour), to mimic the clinical setting in which PN is initially withheld. Mice exposed to critical illness for longer than 1 day received standard mixed PN (0.2 mL/hour, 5.8 kcal/day; Olimel N7E, Baxter, Lessines, Belgium) starting 24 hours after surgery. Throughout the study, mice received antibiotics (0.5 mg imipenem/cilastatine; Merck Sharp & Dohme BV, Haarlem, The Netherlands) and analgesics (4.5-9 µg buprenorphine; Vetergesic, Patheon UK Ltd, Covingham, UK) injected subcutaneously twice daily, starting 6 hours after the surgery. Mice were sacrificed 10 hours, 30 hours ("1 day"), 3 days, or 5 days after surgery, covering the acute and prolonged phase of critical illness [27, 28]. At the point of sacrifice, blood was withdrawn by cardiac puncture, followed by decapitation and removal of organs, including the gastrocnemius and soleus muscles, which were snap frozen in liquid nitrogen and stored at −80 °C. As controls, individually caged healthy mice receiving standard chow at the same daily caloric intake as critically ill mice (pair-fed) were included for each time point. The study was continued until at least 15 surviving animals had been included at each time point. Survival of septic mice was 100% at 10 hours and 30 hours, and 88% at 3 and 5 days. Study approval was obtained from the Institutional Ethical Committee for Animal Experimentation (P134/2013, P093/2014). Animals were treated according to the Principals of Laboratory Animal Care formulated by the U.S. National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health.

RNA Extraction and Real-Time Polymerase Chain Reaction (for Both Human and Mice Samples)

Extraction of RNA from human vastus lateralis muscle samples was performed with use of an in-house protocol consisting of tissue lysis with QIAzol Lysis Reagent (Qiagen, Venlo, The Netherlands) and isopropanol precipitation. Extraction of RNA from mouse gastrocnemius and soleus muscles was performed with Qiazol and the RNeasy mini RNA isolation kit (QIAGEN, Venlo, The Netherlands). DNAse treatment removed genomic DNA. RNA was reverse transcribed to cDNA using random hexamers. Real-time polymerase chain reaction

Table 2. Gene expression assays

Human gene expression		Mouse gene expression	
Gene	Assay ID	Gene	Assay ID
FNDC5	Hs00401006_m1	*Fndc5*	Mm01181543_m1
KYAT1	Hs00187858_m1	*Kyat1*	Mm00549584_m1
KYAT3	Hs00219725_m1	*Kyat3*	Mm00620553_m1
Amylase[a]	Hs00420710_g1	*Amy1*	Mm00651524_m1
CASC3	Hs00201226_m1	*Rn18s*	Mm03928990_g1

[a]Quantifies expression of *AMY1A*, *AMY1B*, *AMY1C*, *AMY2A* and *AMY2B*. Total amylase expression was quantified in human muscle in view of unavailability of assays for the different individual genes.

(QuantStudio3, Applied Biosystems, Carlsbad, CA) for gene expression analysis was performed with use of TaqMan chemistry. Gene expression assays (Applied Biosystems, Foster City, CA) are listed in Table 2. We quantified gene expression of *FNDC5/Fndc5*, of the KYATs *KYAT1/Kyat1* and *KYAT3/Kyat3*, and of total amylase or *Amy1* in human or mouse muscle, respectively. Relative gene expression was determined with the $2^{-\Delta\Delta Ct}$ method using Cancer Susceptibility Candidate Gene 3 (CASC3, for human muscle) or 18S ribosomal RNA (*Rn18s*, for mouse muscle) as the housekeeping gene. Housekeeping genes were selected based on comparable expression for the critically ill vs control condition. RNA extraction, reverse transcription, and real-time polymerase chain reactions were done in batches for all samples per study.

Histological Analyses

Morphological features in human muscle were evaluated on 5-µm paraffin sections stained with hematoxylin–eosin. These included the presence of adipocytes among the myofibers, pronounced endomysial connective tissue/fibrosis, and signs of inflammation and necrosis [4]. Myofiber type and cross-sectional area distribution were analyzed after immunohistochemical staining for type I and type II myofibers, as previously described and reported in detail [21]. As previously, the proportion of myofibers with a size smaller than 2500 µm^2 (corresponding to the peak in the myofiber size distribution) were used as a marker of myofiber size distribution to study the association between myokine expression and myofiber size [21].

Statistical Analyses

Data are presented as mean (SEM) or number (percentage) as indicated. Proportions were compared with chi-square tests and continuous data with t tests, if necessary, after transformation to obtain a near normal distribution. Multivariable logistic regression analyses adjusting for age, BMI, type and severity of illness, nutritional risk screening (NRS) score, and randomization to late PN vs early PN were performed to assess independent associations of myokine expression with clinical outcomes of critically ill patients. Investigated clinical outcomes comprised ICU mortality, prolonged need (>7 days) of intensive care after collection of the muscle biopsy in ICU survivors, and development of ICU-acquired weakness (MRC sum score <48). Pearson correlation coefficients were calculated to study correlations between myokine expression and myofiber type and size distribution.

For studying the impact of late PN vs early PN on expression of the studied genes, a propensity score–matched subgroup of patients was selected with the "MatchIt" package in R4.0.3 with age, sex, BMI, nutritional risk, type and severity of illness, emergency vs elective admission, history of diabetes or malignancy, preadmission dialysis, and sepsis upon ICU admission as covariates, and based on 1 to 1 nearest neighbor matching without replacement, with use of caliper 0.4. This propensity score matching was performed to avoid potential selection bias, since the intervention had an impact on the length of ICU stay. The characteristics of the retained matched patients (66 late PN, 66 early PN) are shown in Table 1.

Analyses were performed with JMPpro16.1.0 (SAS Institute Inc., Cary, NC). Two-sided *P* values lower than .05 were considered statistically significant. No corrections were performed for multiple comparisons.

Results

Impact of Critical Illness on Myokine Expression in Human Vastus Lateralis and Mouse Gastrocnemius and Soleus Muscle

In critically ill patients, mRNA expression of *FNDC5*, *KYAT1*, and total amylases (*AMY1A*, *AMY1B*, *AMY1C*, *AMY2A*, and *AMY2B*) in muscle was 34% to 80% lower than expression in controls, whereas no significant effect was observed for *KYAT3* (Fig. 2).

In muscle of critically ill mice, significant reductions were observed for all myokines compared with healthy mice, in myokine- and time-specific patterns, in both gastrocnemius and soleus muscle (Fig. 2). *Fndc5* expression was reduced on day 3 (54-63%) and day 5 (44-69%), and *Kyat1* expression was reduced from day 1 onwards (18–25%) up to day 3 (51-52%) and day 5 (21-44%) in both muscles, the decrease being somewhat larger in soleus than in gastrocnemius muscle. Whereas the patterns for *Fndc5* and *Kyat1* expression were comparable for both muscles, time-dependent changes were more muscle dependent for *Kyat3* and *Amy1* expression, with stronger reductions observed in soleus than in gastrocnemius muscle. *Kyat3* expression dropped 20% at 10 hours of critical illness, was normal at day 1 and day 3, and increased at day 5 in gastrocnemius muscle. In soleus muscle, the expression was normal at 10 hours and at day 1, and 28% to 29% reduced at day 3 and day 5. *Amy1* expression had decreased 30% after 1 day of critical illness but was restored thereafter in gastrocnemius muscle. In soleus muscle, expression progressively reduced from day 1 onwards (26%) up to day 3 (55%) and day 5 (71%).

Association of Vastus Lateralis Myokine Expression With Outcome of Critically Ill Patients

ICU nonsurvivors showed lower *FNDC5* expression than ICU survivors in a univariable analysis, whereas the other myokines did not significantly differ according to survival status (Fig. 3). Lower *FNDC5* expression remained independently associated with a higher risk of death in the ICU in multivariable analysis, whereas the inverse association was observed for amylase expression (Table 3).

ICU survivors who needed more than 7 days of intensive care after collection of the muscle biopsy had a lower expression of *FNDC5* and of amylases than ICU survivors who needed a shorter ICU stay after the biopsy, with similar trends observed for *KYAT1* and *KYAT3* (Fig. 3). In multivariable analysis, only

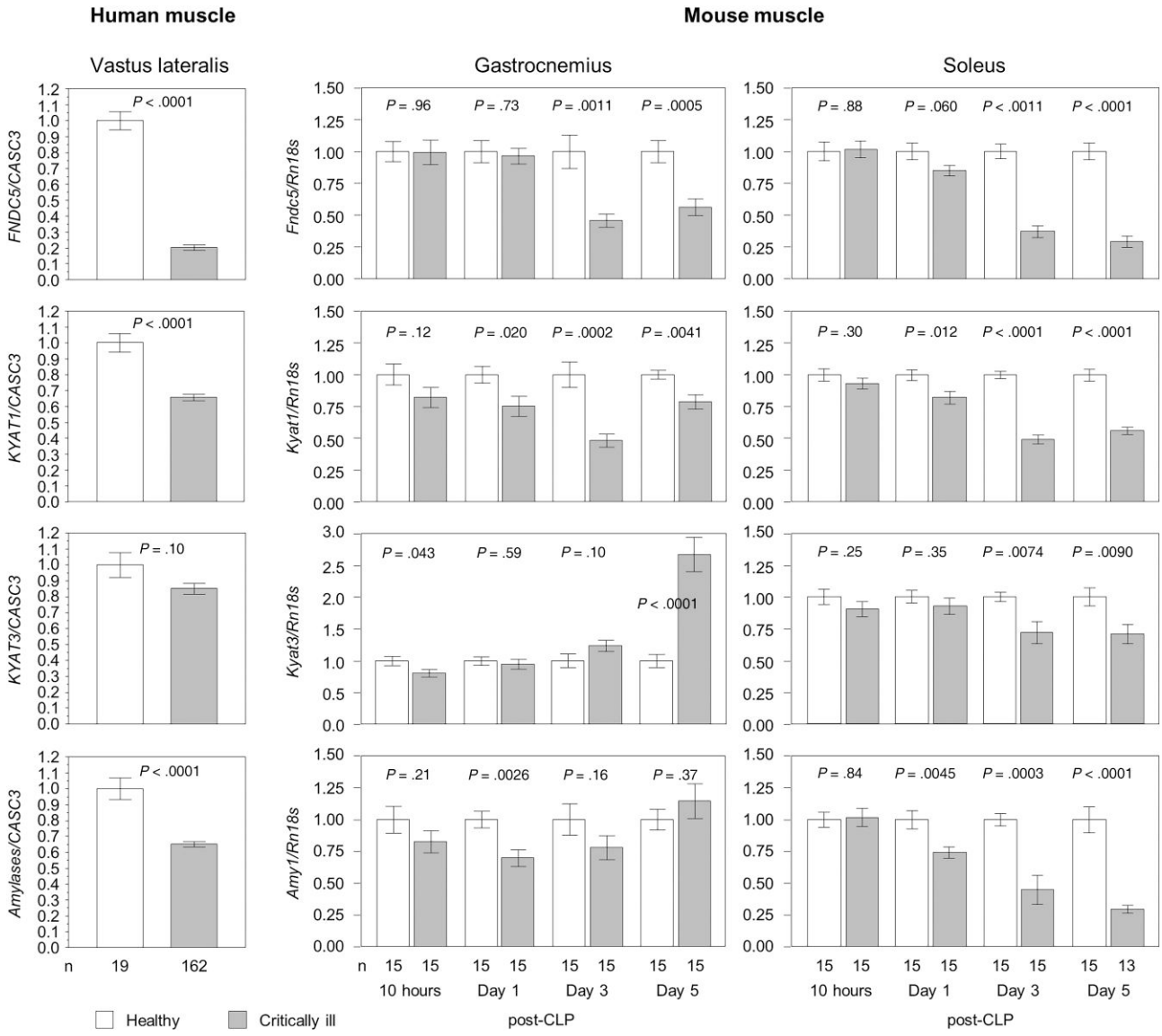

Figure 2. Impact of critical illness on human and mouse muscle myokine expression. Myokine expression is compared for critically ill patients on day 8 ± 1 vs matched controls and for critically ill mice vs pair-fed healthy mice sacrificed after 10 hours, 1 day, 3 days, or 5 days. *FNDC5* expression in human muscle was square root transformed for statistical testing, raw data are shown in the graphs. Data are presented as bar graphs, with bars representing the mean and whiskers representing SEM. SEM, standard error of the mean.

lower amylase expression remained independently associated with a longer subsequent need for intensive care (Table 3).

Patients who acquired clinically relevant muscle weakness in the ICU had a lower expression of *FNDC5* than patients who did not develop such weakness (Fig. 3). In multivariable analysis, lower *FNDC5* expression remained independently associated with a higher risk of weakness, whereas the inverse association was observed for *KYAT1* expression (Table 3).

Association of Vastus Lateralis Myokine Expression With Histological Features in Patient Biopsies

Histological features were investigated in muscle biopsies of 18 controls and 101 patients. Compared with controls, patient muscles showed a remarkable presence of adipocytes (26.7% vs 0.0%, $P = .0014$) and connective endomysial tissue/fibrosis (63.4% vs 5.6%, $P < .0001$), as well as signs of inflammation (72.3% vs 0.0%, $P < .0001$), whereas signs of necrosis were rarely observed (4.0% vs 0.0%, $P = .24$). Patients also showed a lower percentage of type I myofibers ($47 \pm 2\%$ vs $57 \pm 4\%$, $P = .019$) and a higher percentage of myofibers smaller than 2500 µm^2 ($30 \pm 2\%$ vs $21 \pm 4\%$, $P = .044$) than controls.

In muscle biopsies of patients, *FNDC5* expression was lower in muscles that showed signs of inflammation than in muscles not showing these signs (Fig. 4). *FNDC5* expression in patients' muscle correlated inversely with the proportion of myofibers smaller than 2500 µm^2 (Table 4). No associations were found between myokine expression and the presence of adipocytes, connective endomysial tissue/fibrosis, signs of necrosis, or the proportion of type I myofibers.

Impact on Vastus Lateralis Myokine Expression of 2 Interventions that Attenuate Muscle Wasting or Weakness

In the NESCI study, NMES attenuated loss of muscle mass [24]. NMES that resulted in a good muscle contraction

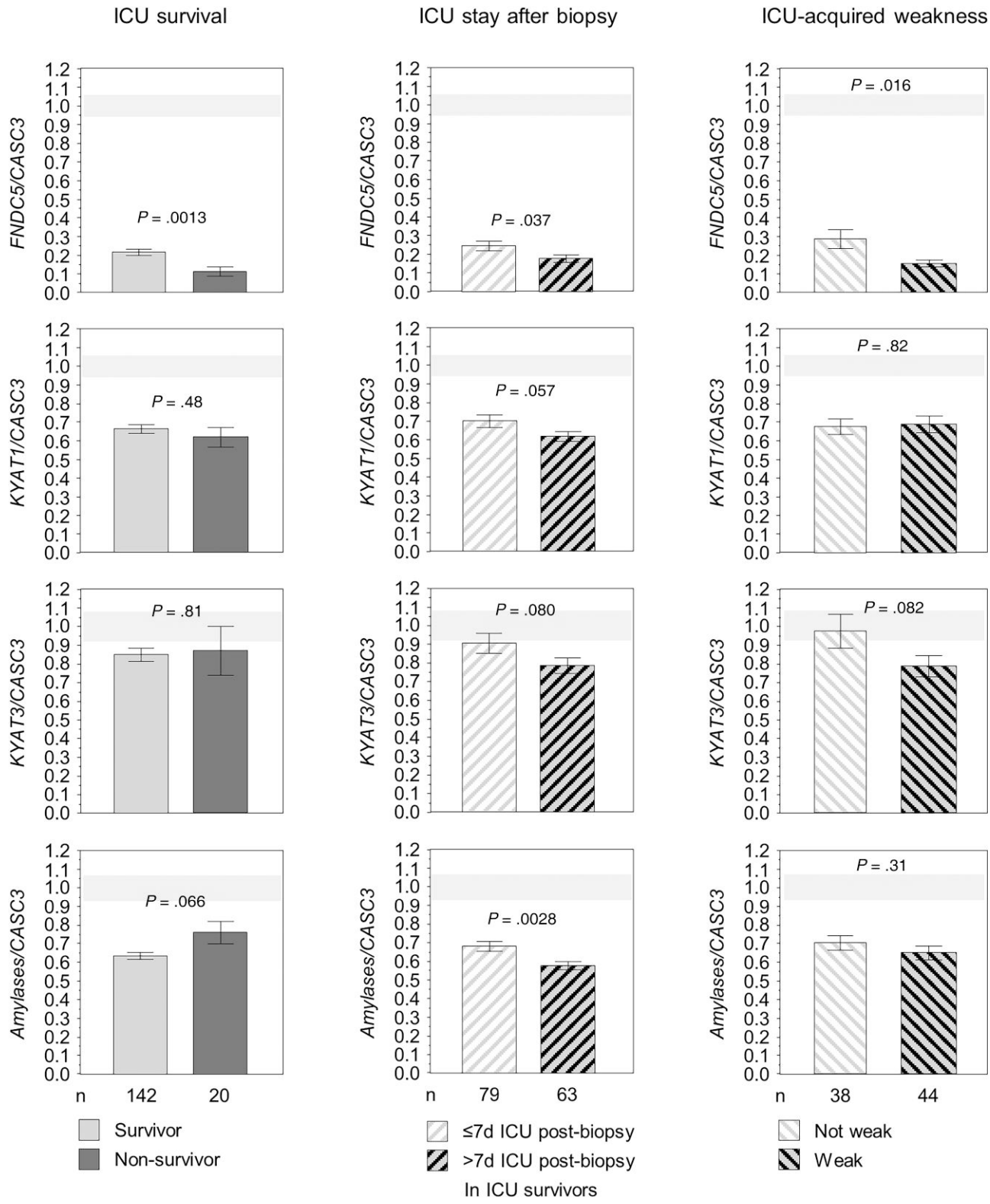

Figure 3. Association of muscle myokine expression with outcome of critically ill patients. Myokine expression is compared for ICU nonsurvivors vs survivors, for patients who needed more than 7 vs maximally 7 days of intensive care after the biopsy, and for patients who developed muscle weakness in the ICU vs those who did not. *FNDC5* expression was square root transformed for statistical testing, raw data are shown in the graphs. Data are presented as bar graphs, with bars representing the mean and whiskers representing SEM. SEM, standard error of the mean.

increased *FNDC5* expression compared with the unstimulated muscle, but did not affect the other myokines (Fig. 5).

The EPaNIC study has shown that late PN attenuated the risk of developing muscle weakness in the ICU compared with early PN [21]. Patients who had been assigned to the late PN group had a higher expression of *KYAT1* in muscle than patients who had been assigned to the early PN group (Fig. 5). The other myokines did

Table 3. Multivariable analyses associating myokine expression with clinical outcome

Outcome and gene	Odds ratio (95% CI)	*P*
Death in ICU vs ICU survival		
FNDC5/CASC3, per 0.1 square root unit added	0.551 (0.310-0.900)	**.015**
KYAT1/CASC3, per 0.1 unit added	0.976 (0.735-1.268)	.85
KYAT3/CASC3, per 0.1 unit added	1.013 (0.850-1.192)	.88
AMY/CASC3, per 0.1 unit added	1.336 (1.002-1.841)	**.048**
Long (7 days) vs short (≤7 days) need of intensive care after biopsy in ICU survivors		
FNDC5/CASC3, per 0.1 square root unit added	0.918 (0.695-1.199)	.53
KYAT1/CASC3, per 0.1 unit added	0.920 (0.755-1.115)	.39
KYAT3/CASC3, per 0.1 unit added	1.023 (0.903-1.159)	.71
AMY/CASC3, per 0.1 unit added	0.714 (0.540-0.912)	**.0060**
Muscle weakness vs no weakness		
FNDC5/CASC3, per 0.1 square root unit added	0.556 (0.312-0.889)	**.012**
KYAT1/CASC3, per 0.1 unit added	1.437 (1.050-2.093)	**.022**
KYAT3/CASC3, per 0.1 unit added	0.992 (0.821-1.190)	.93
AMY/CASC3, per 0.1 unit added	0.685 (0.427-1.035)	.073

P-values indicated in bold represent statistically significant associations.
Abbreviation: ICU, intensive care unit.

not show a differential expression according to timing of PN initiation.

Discussion

Critically ill patients show remarkably low vastus lateralis expression of *FNDC5*, KYAT, and amylase mRNA after 1 week in the ICU compared with healthy subjects. Largely similar time-dependent reductions were observed in gastrocnemius and soleus muscle of critically ill mice compared with healthy pair-fed mice, somewhat more pronounced in soleus than in gastrocnemius. In patients, lower vastus lateralis *FNDC5* expression was associated with higher risk of death and weakness, and lower amylase expression with a longer dependency on intensive care. Lower amylase or *KYAT1* expression was associated with a lower risk of death or weakness, respectively. NMES was able to increase *FNDC5* expression, whereas withholding early PN increased *KYAT1* expression.

Muscular expression of *FNDC5* was lowered after prolonged critical illness, both in patients with heterogeneous diagnoses and in mice subjected to surgery and cecal ligation and puncture–induced sepsis. This is in line with reduced muscular *Fndc5* expression that has been reported in mice given lipopolysaccharide (LPS) [11] or after ischemic stroke [12]. The observed low *FNDC5/Fndc5* expression may be in agreement with the more abundant data on its secreted cleavage product irisin. Indeed, serum or plasma irisin concentrations have been shown to be lower in patients and mice with sepsis [11], in patients after ischemic stroke [29] or with acute pancreatitis [30], and in elderly surgical patients [31]. Low serum irisin concentrations have been shown to correlate with higher disease severity in sepsis and with adverse outcomes of patients with varying underlying medical problems [11, 29, 31, 32] but associations for muscular expression had not been studied. We observed associations of low muscular *FNDC5*/irisin expression with a higher risk of dying in the ICU and of developing ICU-acquired muscle weakness. Studies on irisin treatment may support causality of this association. In a mouse model of acute pancreatitis, irisin treatment improved survival and mitigated pancreatic, intestinal, liver, and lung injury [30, 33]. Vital organ–protective effects of irisin treatment have in fact been shown in many animal studies of sepsis [11, 34–36], ischemia-reperfusion–induced organ injury [37–41], and critical brain insults [42, 43], among others. Regarding the association of low *FNDC5*/irisin expression with risk of ICU-acquired weakness, it is interesting to note that lower preoperative plasma irisin levels in elderly patients correlated with preoperative bedrest time as a surrogate marker of muscle wasting, and were associated with lower independence for performing activities of daily living [31], whereas irisin treatment of mice exerted muscle-protective effects. Indeed, this treatment induced significant hypertrophy and enhanced grip strength of uninjured muscle, improved regeneration and rescued loss of muscle mass of denervation-induced injured muscle, and showed beneficial effects in hind limb ischemia–reperfusion injury [44, 45]. We also observed an association of lower *FNDC5* expression in muscle with signs of inflammation in muscle and with a higher proportion of smaller myofibers. The inverse association with inflammation may be in line with an anti-inflammatory effect of irisin. In that regard, the exercise-induced increase in muscle irisin expression was shown to exert anti-inflammatory effects in mice on a high-fat diet [46], and irisin reduced inflammation in hind limb ischemia–reperfusion injury in mice [45]. However, inflammatory cytokines may also reduce *Fndc5* expression in muscle, as shown in rodent models of chronic heart failure [47]. The association with myofiber size may reflect its promyogenic role [44].

KYATs shift peripheral kynurenine metabolism away from the neurotoxic kynurenine toward kynurenic acid, which cannot cross the blood–brain barrier [14]. Expression of *KYAT1* is highest in muscle and brain, whereas *KYAT3* shows a wide tissue distribution and is expressed at a medium level in muscle [48]. In muscle of prolonged critically patients, we observed a decreased expression of *KYAT1* mRNA, which was also seen from 1 day of critical illness onwards in mice. *KYAT3* mRNA showed no significant difference in patients, only a very transient decrease in gastrocnemius muscle of mice 10 hours after the insult which gradually switched to increased expression after 5 days, and a later decrease in soleus muscle of mice 3 and 5 days after the insult. *KYAT1* and *KYAT3* expressions had not previously been compared directly between critically ill patients and healthy subjects. One study did perform an in silico gene expression analysis, though for whole blood, that pointed to a lower *KYAT1* but higher *KYAT3* expression in critically ill trauma patients who developed venous thromboembolism vs those who did not [49]. We found here that muscular KYAT expression in critically ill patients did not independently associate with clinical outcome, apart from an association of lower *KYAT1* expression with a lower risk of weakness. However, this association seems counterintuitive in view of the beneficial effects on muscle of rerouting kynurenine to kynurenic acid under healthy conditions [16] and the reported association of higher serum kynurenine levels with frailty and worse physical function [50]. Whereas the literature lacked data on muscular KYAT

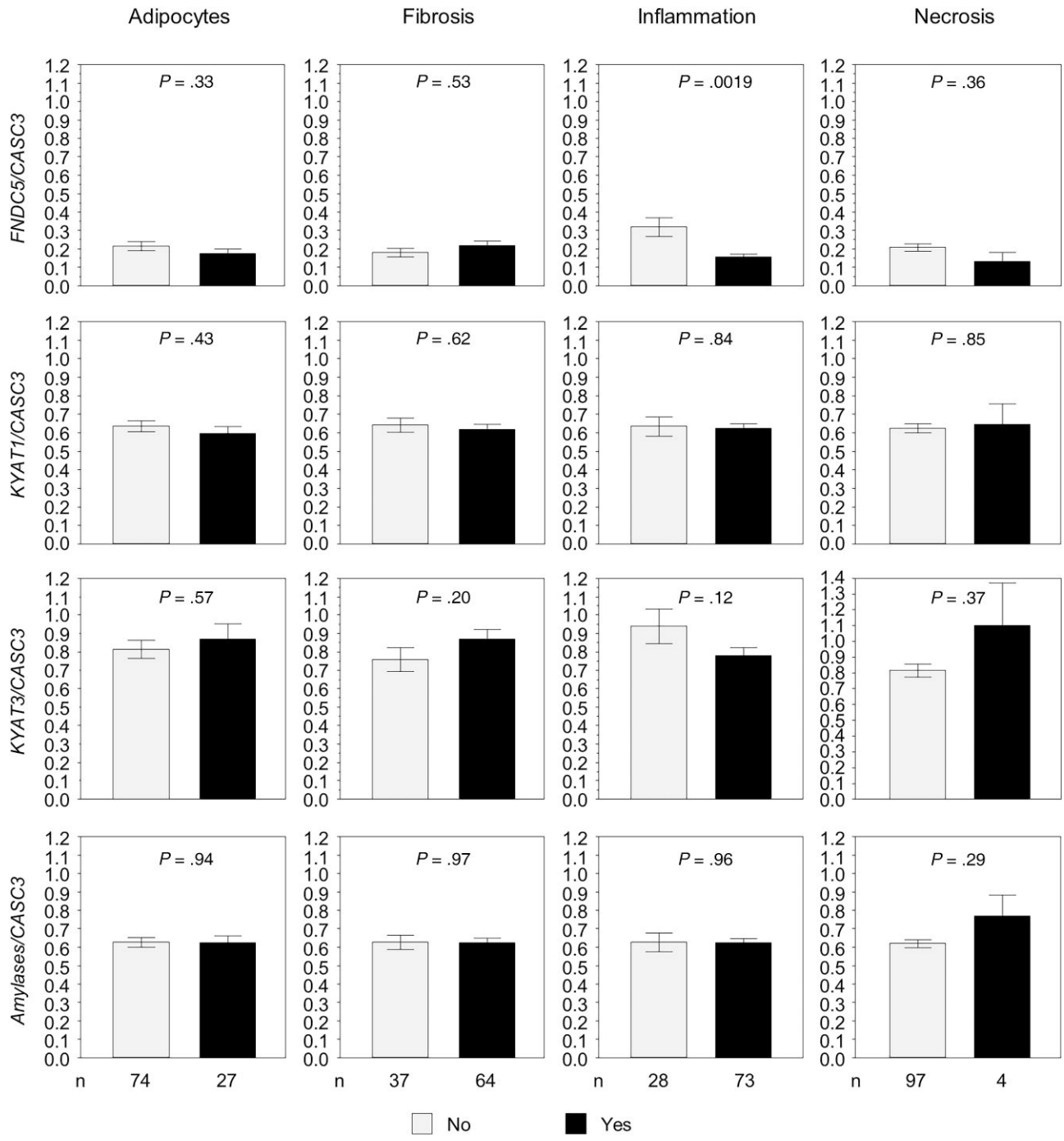

Figure 4. Association of muscle myokine expression with histological abnormalities. Myokine expression is compared for patients showing histological abnormalities in muscle vs those not showing such abnormalities, focusing on the presence of adipocytes, fibrosis/endomysial connective tissue, inflammation, and necrosis. *FNDC5* expression was square root transformed for statistical testing, raw data are shown in the graphs. Data are presented as bar graphs, with bars representing the mean and whiskers representing SEM. SEM: standard error of the mean.

expression, many metabolomic studies have investigated tryptophan/kynurenine metabolism in the context of critical illness. These mostly showed elevated serum concentrations of kynurenine, and some also of kynurenic acid, in patients compared with healthy subjects [51–54]. Elevated serum/plasma kynurenine concentrations were correlated with higher illness severity [51] and were associated with development of chronic critical illness (as opposed to rapid recovery) [55], with duration of invasive mechanical ventilation [56], with development of acute brain dysfunction [57], and with mortality [54, 58, 59]. Although the low muscular *KYAT1* expression that we observed could be consistent with the elevated serum kynurenine concentrations found in critically ill patients, the fact that we did not observe an association of *KYAT* expression with mortality or prolonged need of intensive care may rather suggest that muscle is not the main source of elevated kynurenine in this condition. Interestingly, treatment with exogenous kynurenic acid or synthetic analogs alleviated circulatory and mitochondrial disturbances and exerted neuroprotective effects in experimental sepsis or transient global forebrain ischemia [60–62], and

Table 4. Correlation of myokine expression with myofiber size and type

	% myofibers < 2500 μm^2		% type 1 myofibers	
Gene expression	R	*P*	R	*P*
FNDC5	−0.267	.011	0.013	.90
KYAT1	0.063	.55	0.054	.60
KYAT3	−0.197	.077	0.003	.98
AMY	0.124	.24	0.088	.40

also ameliorated renal ischemia-reperfusion–induced acute kidney injury and thioacetamide-induced liver injury in rats [63–65]. Furthermore, preventing the sepsis-induced rise in kynurenine protected mice from cognitive impairment [66] and reduced mortality [67], whereas exogenous kynurenine administration exacerbated CCl_4-induced acute liver damage [68].

Amylase levels are traditionally measured if diseases of the pancreas or salivary gland are suspected. However, research has indicated that elevated serum amylase levels may occur in up to 80% of intensive care patients [69]. Apart from pancreatic damage or disease, potential mechanisms of elevated amylase in critically ill patients include splanchnic hypoperfusion, bacterial translocation, development of biliary sludge, drug toxicity, and decreased clearance by liver or kidney injury. Elevated serum amylase levels have been associated with increased risk of morbidity and mortality [69–71]. However, recent evidence opened perspectives that also muscular amylase expression and release may signal problems. More specifically, *Drosophila*'s amylase "amyrel" has been identified as myokine released from muscle upon proteasome stress, and amylases may also be produced and secreted by human or mouse nondigestive tissues such as skeletal muscle upon challenged proteostasis [18, 19]. Unlike under these conditions of artificial proteasome stress inflicted by proteasome inhibition, we observed here a (transient) decrease in muscular amylase mRNA expression upon critical illness in patients and mice. On the one hand, this makes a contribution to high circulating amylase levels unlikely. On the other hand, this may suggest that the typical activation of the ubiquitin–proteasome system in response to critical illness [4, 21] is sufficient to avoid proteasome overloading and, as such, hypercatabolism of critical illness may not be perceived as "proteasome stress" by the muscle. The lower muscular amylase expression in critically ill patients was not associated with risk of weakness. This may be consistent with impaired autophagic quality control explaining weakness rather than the critical illness-associated muscle breakdown [21]. Muscular amylase expression did show inconsistent, opposite associations with prolonged need of intensive care (higher risk with lower expression) and mortality (lower risk with lower expression).

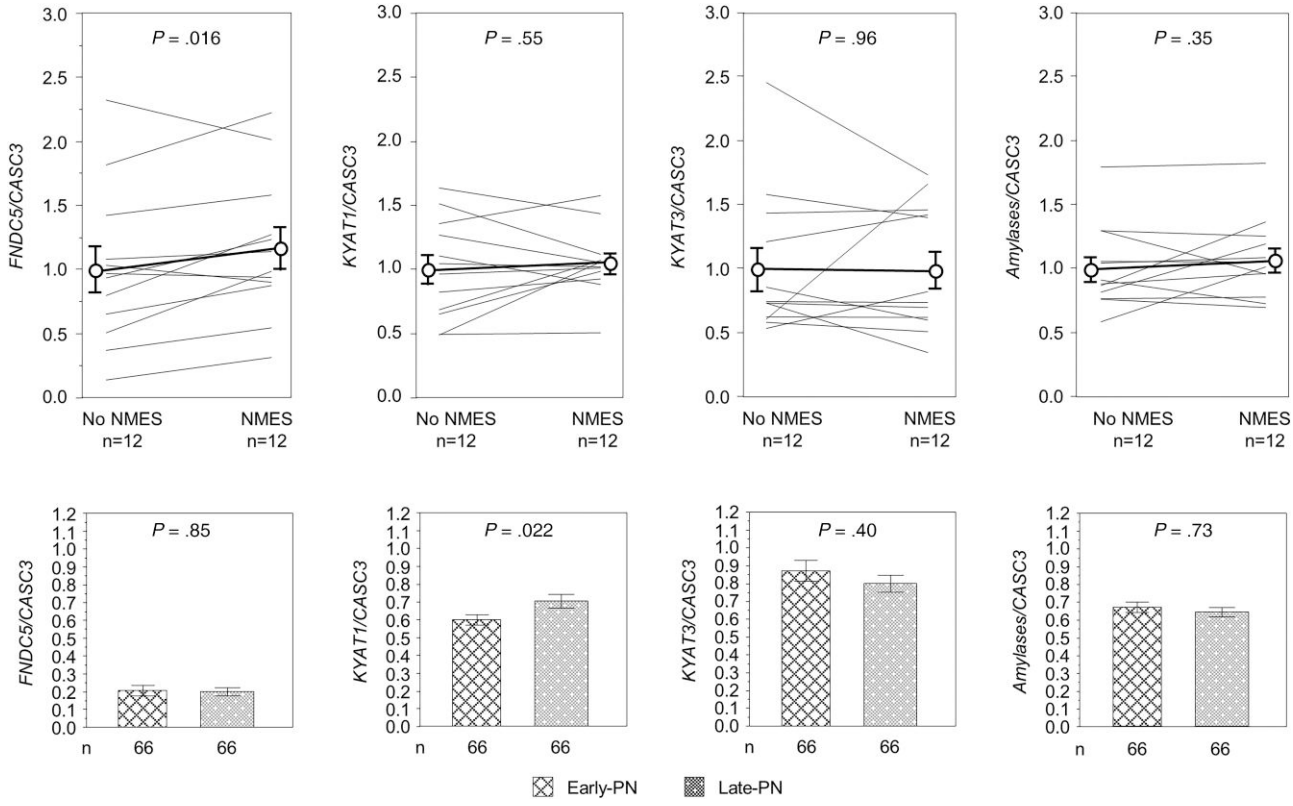

Figure 5. Impact of interventions that attenuate weakness or loss of muscle mass on myokine expression. The upper panel shows myokine expression for the stimulated and nonstimulated muscle per patient (thin lines) and for all patients as a group (thick line, with circles and whiskers representing mean and SEM). The lower panel compares myokine expression in patients randomized to early PN or late PN. *FNDC5* expression was square root transformed for statistical testing; raw data are shown in the graphs. Data are presented as bar graphs, with bars representing the mean and whiskers representing SEM. SEM, standard error of the mean.

In healthy subjects, physical exercise exerts physiological benefits at least in part through production and secretion of myokines. Interestingly, aerobic exercise, which is known to increase serum irisin, protected against LPS-induced lung injury in rodents [72]. However, critically ill patients are at least partially immobilized. NMES emerged as a safe and effective physical exercise substitute for myokine induction in individuals unable to engage in optimal exercise [73]. Several studies have investigated NMES in critically ill patients, with some showing benefit and others a neutral outcome [24, 74]. However, the impact of NMES on myokine expression remained unclear. We showed here that NMES did not affect KYAT or amylase but increased *FNDC5* (irisin) expression in muscle of critically ill patients. Unlike NMES, accepting an early macronutrient deficit under the late PN nutritional strategy did not affect *FNDC5* expression in these patients. This may be consistent with the idea that exercise is the major driver of induced irisin expression rather than caloric restriction [75]. Nevertheless, nutritional intervention may have a certain impact that requires further investigation as most data on impact of nutrition on irisin are obscured by simultaneous exercise [53, 76]. Patients of the late PN group did show higher *KYAT1* expression than early PN patients, which theoretically may result in lowering of kynurenine by increased conversion to kynurenic acid. This may be consistent with the lowering of serum kynurenine with a (very) low-calorie diet in overweight adults (serum) [77] and lowering of kynurenine in plasma and the hippocampus with calorie restriction or a ketogenic diet in rats [78], although such an effect may more likely be explained by lower tryptophan intake. We previously observed that late PN decreased the risk of ICU-acquired weakness compared with early PN [21]. This effect does not appear to be mediated via an effect on *KYAT1* expression as we found here a lower *KYAT1* expression to be associated with a lower risk of weakness.

Our study has some limitations. First, we focused on a selected number of myokines, based on proteins (most) abundantly expressed in muscle and identified in the literature as (plausible) myokines, but not or hardly explored in muscle during critical illness (unlike myostatin, myogenin, and certain interleukins, among others). Second, human muscle was harvested and studied at a single time point, whereas the mouse studies pointed to time-dependent changes. Finally, our study was limited to quantification of muscular gene expression. We did not investigate protein expression in muscle, because the protein level in muscle may not adequately reflect the muscle's synthetic response as myokines per definition may be secreted from the muscle, a process that may theoretically be disturbed. We also did not study secretion from muscle into the circulation, where measuring myokine levels in blood samples may also reflect other sources apart from muscle, nor did we study correlation of gene expression with distant organ function. As all myokines we have studied here may have a link with distant effects on the brain, particularly association with delirium as marker of brain dysfunction would have been interesting. Unfortunately, however, this complication was not part of the outcomes of the original study protocols.

In conclusion, skeletal muscle expression of irisin precursor *FNDC5*, KYAT, and amylase mRNA was found to be reduced in critical illness and was associated with several clinical outcomes. In particular, low *FNDC5* expression was associated with adverse outcome, including a higher risk of death and of acquiring weakness in the ICU. NMES and withholding of early supplemental parenteral nutrition, interventions that have previously shown to attenuate muscle wasting or weakness, had limited effects on the studied myokines. Whereas several studies already demonstrated improved outcome with exogenous administration of irisin or manipulation of the kynurenine/kynurenic acid ratio in animal models, it remains to be investigated whether critically ill patients may benefit from such intervention.

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Disclosure

The authors declare that they have no conflicts of interest.

Data Availability

Restrictions apply to the availability of some or all data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

Clinical Trial Information

Early Parenteral Nutrition Completing Enteral Nutrition in Adult Critically Ill Patients (EPaNIC) trial: ClinicalTrials.gov-NCT00512122, registered on July 31, 2007; Neuromuscular Electrical Stimulation in Critically Ill Patients (NESCI) trial: ClinicalTrials.gov-NCT02133300, registered on May 8, 2014.

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