Catabolic Signaling and Muscle Wasting After Acute Ischemic Stroke in Mice
Indication for a Stroke-Specific Sarcopenia

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Background and Purpose—Muscle wasting is a common complication accompanying stroke. Although it is known to impair poststroke recovery, the mechanisms of subacute catabolism after stroke have not been investigated in detail. The aim of this study is to investigate mechanisms of local and systemic catabolism and muscle wasting (sarcopenia) in a model of ischemic stroke systematically.

Methods—Changes in body composition and catabolic activation in muscle tissue were studied in a mouse model of acute cerebral ischemia (temporal occlusion of the middle cerebral artery). Tissue wasting (nuclear magnetic resonance spectroscopy), tissue catabolism (caspases-3 and -6, myostatin), and proteasome activity were assessed. Food intake, activity levels, and energy expenditure were assessed, and putative mechanisms of postischemic wasting were tested with appropriate interventions.

Results—Severe weight loss in stroke animals (day 3: weight loss, –21.7%) encompassed wasting of muscle (–12%; skeletal and myocardium) and fat tissue (–27%). Catabolic signaling and proteasome activity were higher in stroke animals in the contralateral and in the ipsilateral leg. Cerebral infarct severity correlated with catabolic activity only in the contralateral leg but not in the ipsilateral leg. Lower energy expenditure in stroke animals together with normal food intake and activity levels suggests compensatory mechanisms to regain weight. Interventions (high caloric feeding, β-receptor blockade, and antibiotic treatment) failed to prevent proteolytic activation and muscle wasting.

Conclusions—Catabolic pathways of muscle tissue are activated after stroke. Impaired feeding, sympathetic overactivation, or infection cannot fully explain this catabolic activation. Wasting of the target muscle of the disrupted innervation correlated to severity of brain injury. Our data indicate the presence of a stroke-specific sarcopenia.

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Key Words: cachexia ■ muscles ■ sarcopenia

Stroke is the single greatest cause of physical disability in the adult population, leaving 30% of patients unable to walk without assistance.1 The main effector organ of physical capacity is muscle tissue. The disability after stroke, however, is typically attributed to injury within the brain. By contrast, the structural and metabolic integrity of the muscle tissue itself are poorly addressed. Weight loss is a common observation after stroke, both in experimental2,3 and in clinical studies.4,5 This weight loss involves global tissue wasting, but muscle tissue wasting (ie, sarcopenia) is particularly detrimental because regaining physical capacity largely depends on global muscle function, bulk, and strength. Understanding the mechanisms of muscle wasting and developing interventional concepts to prevent or reverse it are, therefore, of high relevance in an interdisciplinary poststroke rehabilitation concept.
feeding, and immobilization.6 Nutritional deficits are common in patients with stroke,7 and weight loss of ≥3 kg is associated with higher mortality.3 Muscle wasting in acute and subacute stroke may result from direct metabolic, inflammatory, and hormonal signals.4 Systemic sympathomimetic activation and bacterial infections are also common after acute stroke, both carrying catabolic signals. The combined effects result in local and systemic catabolic overactivation and anabolic blunting. Accordingly, a distinct pattern of muscle phenotype change has been observed in stroke, with a combination of atrophy, apoptosis, and remodeling. This stroke-induced sarcopenia shows not only some common characteristics but also differences to other disease-related muscle wasting.9 Sarcopenia, regardless of its cause, is a major complication in hospitalized patients10 and leads to frailty, delayed recovery, and poor long-term health state.11

Few studies have investigated the metabolic and phenotypic changes in muscle tissue after stroke, and most of these investigate the long-term changes at ≥6 months after stroke. Acute and subacute muscle wasting, however, may be even more relevant for recovery and present a significant and to date unexplored therapeutic target in patients with stroke. Notably, metabolic aspects of muscle wasting and sarcopenia are covered neither in European and North American stroke guidelines12,13 nor in rehabilitation-orientated guidelines.14,15 There is an urgent need for mechanistic studies to develop interventional strategies for preventing or reversing muscle wasting and improving rehabilitation success in patients with stroke.

The aim of the present study was to systematically investigate muscle wasting and regulation of catabolic activation in muscle tissue in a well-characterized murine model in the acute phase after stroke. We hypothesized that a direct relationship may exist between the severity of the ischemic damage to the brain and the catabolic activation. Systemic catabolic effects were investigated, as well as local changes in the affected target muscle. Mobility level, energy expenditure, and feeding patterns in the acute phase after stroke were taken into account. In addition, putative relevant mechanisms of poststroke wasting were tested by appropriate interventions: hypercaloric feeding to counter reduced food intake, blockade of sympathetic overactivation, and antibiotic treatment to prevent poststroke infections.

Methods

Animal Model
Mice (C57Bl6/N; Charles River, Sulzfeld, Germany; n=92, all male, mean weight, 22.5±1.4 g) were housed in a specific-pathogen-free facility under standard conditions with food and water available ad libitum. Focal cerebral ischemia was induced for 60 minutes by middle cerebral artery occlusion (MCAO) as described previously.16 Sham operation involved all surgical and anesthetic procedures, including application, correct positioning and immediate withdrawal of the monofilament. Control animals received no intervention at all. Metabolic and activity assessments were performed before surgery (baseline) and on day 3 and day 7 after surgery.

Interventions
To investigate the effect of putative pathogenic factors on weight loss after stroke, we targeted in separate groups either sympathetic activation by treatment with a β-blocker (propranolol), infection/inflammation with an antibiotic (enrofloxacin), or impaired food intake with high caloric feeding on top of ad libitum access to food and drinking water. Propranolol (Sigma-Aldrich) was administered at 10 mg/kg dissolved in 0.9% NaCl via intraperitoneal injection immediately before the MCAO and 4 and 8 hours after the procedure. We previously demonstrated that this protocol results in effective inhibition of stroke-induced sympathomimetic activation.17 The antibiotic (gyrase inhibitor, enrofloxacin, Baytril 2.5% oral solution; Bayer, Germany) was added to drinking water (0.35 mg/mL) from day 1, a therapeutic regimen that effectively prevents poststroke infection.18 Poststroke infection (in particular pneumonia) is a common complication of stroke in mouse models,19 as well as in patients,20 and may contribute to wasting. To counter reduced caloric intake by reduced feeding after stroke, 1-mL high caloric fluid nutrition (Peptamen, Nestle Clinical Nutrition Brussels) was administered by gavage twice daily on top of free access to food and water.

Groups sizes were planned according to the sample size calculation (see below) and the following numbers were included in the respective study groups: stroke day 3: 9 animals, stroke day 7: 16 animals,
sham day 3: 10 animals, sham day 7: 8 animals, high caloric feeding: 11 animals, propranolol treatment: 10 animals, antibiotic treatment: 10 animals, and controls: 10 animals.

Animals that after transient occlusion of the MCA did not show the circling behavior indicative of an incomplete arterial occlusion were excluded from the study. The mice were randomly assigned to the treatment groups by an independent person not involved in surgery or in data analysis. The person evaluating functional readouts and postmortem analysis was blinded to the experimental groups. Experiments were reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. All experiments and procedures were approved by the State Office of Health and Social Affairs Berlin (LaGeSo Berlin; G 0226/09).

Body Composition
Body composition (fat and lean body mass) was analyzed by nuclear magnetic resonance spectroscopy using the EchoMRI-100 system (Echo Medical Systems, Houston, TX) as previously described. 20

Energy Expenditure and Locomotor Activity
Energy expenditure in relation with physical activity was measured using a combined indirect calorimetry system (TSE Systems GmbH, Bad Homburg, Germany). The respiratory exchange ratio was calculated as the ratio between CO₂ produced (VCO₂) and O₂ consumed (VO₂). The energy expenditure was normalized to the lean body mass to avoid the possible confounding effects from diverging body weight and lean mass. Mice were housed individually, and spontaneous movement was determined for 24 hours using a 3-dimensional infrared light beam system (Supermex, Muromachi, Tokyo, Japan). The system allows the operator to record physical activity as total ambulatory counts and differentiates between true ambulatory counts and fine movements (rear operator to record physical activity as total ambulatory counts and differentiates between true ambulatory counts and fine movements (rear

Histology
On day 7, brains were snap frozen and stored at –20°C until analysis. Coronal cryosections (30 μm) were produced using a cryostat. Sections of 5 brain slices at defined distances (eg, interaural 6.6 mm, 5.34 mm, 3.94 mm, 1.86 mm, and –0.08 mm) were mounted on 1 superfrost plus glass slide, air dried, and frozen at –20°C until further use. The lesion was visualized in all 5 sections (see above, 1 glass slide per animal) by hematoxyline staining. The slide was scanned and lesion size calculated using ImageJ (National Institutes of Health, Bethesda, MD) as previously described. 21

Proteasome Activity
Muscle proteasome activity was determined by evaluating the cleavage of specific fluorogenic substrates for all 3 major catalytic β-subunits of the proteasome as described previously. 21 Gastrocnemius muscle tissue from the contra- and ipsilateral legs was homogenized in ice-cold lysis buffer and 40-μg protein incubated with the fluorogenic substrates (benzyloxy-carbonyl-Leu-Leu-Glu-7-amido-4-methylcoumarin [Z-LLE-AMC] for trypsin-like activity, succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin [LLVY-AMC] for chymotrypsin-like activity, and benzoyl-Val-Gly-Arg-7-amidocoumarin [Bz-VGR-AMC; Biomol, Hamburg, Germany] for peptidyl-glutamyl-protein-hydrolyzing [PGPH] activity). The change in fluorescence intensity over time was measured with a fluorometer (Twinkle LB 970; Berthold, Bad Wildbad, Germany) for peptidyl-glutamyl-protein-hydrolyzing [PGPH] activity). The change in fluorescence intensity over time was measured with a fluorometer (Twinkle LB 970; Berthold, Bad Wildbad, Germany) at 360 nm excitation and 469 nm emission in 5-minute intervals for 1 hour. The activity, expressed as nmol/mg protein per minute, was calculated using free amidomethylcoumarin (AMC) as the working standard.

Laboratory Analyses
Caspase-3 and caspase-6 activity were measured in the gastrocnemius muscle from the contralateral and the and ipsilateral legs as described previously. 22 The fluorogenic substrates (50 μmol/L) Ac-DEVD-AMC or Ac-VEID-AMC were added, respectively. Assay conditions were identical to the proteasome assay. Mysostatin in skeletal muscle tissue was assessed by Western blotting using primary antibodies (AF788; R&D Systems), as well as appropriate secondary antibodies according to standard protocols. Interleukin (IL)-1α and β, IL-6, and insulin-like growth factor-1 were assessed using a multiplex-assy as described by the manufacturer.

Figure 2. A. Activity of caspase-3 and caspase-6 of the contralateral (right leg) and the ipsilateral (left leg), day 7. *P<0.05, ***P<0.001 vs control; #P<0.05, ###P<0.01 vs sham. B. Association of infarct size and caspase activity in the contralateral (right leg) and the ipsilateral (left leg), day 7. GC indicates gastrocnemius muscle.
Statistics
Using G*Power 3.1 software (Heinrich-Heine-Universität, Düsseldorf, Germany; http://www.gpower.hhu.de/), a sample size of n=9 per group was calculated a priori for the primary end point weight change, a fixed effect, omnibus, 1-way ANOVA with 8 groups, and an effect size $f=0.5$, at $\alpha=0.05$ and $\beta=0.2$. Consequently, group sizes $\geq 9$ (range, 9–16) were used.

GraphPad PRISM 5.0 (GraphPad Software, Inc, La Jolla, CA) was used for statistical analyses. Results are shown as mean±SD for descriptive purposes and as mean±SEM for weight change over time. Data were tested for normal distribution using the Kolmogorov–Smirnov test. Data with normal distribution were analyzed with ANOVA followed by Tukey tests for data with normal distribution, whereas data without normal distribution were analyzed by Kruskal–Wallis and Dunns tests. Correlation analysis was performed using Pearson test. A 2-sided $P$ value $<0.05$ was considered significant.

Results
Eight animals could not be evaluated and were excluded from the analyses because of death during or after the surgery (n=3) and ineffective arterial occlusion (no circling behavior, n=5) and were quantified on a Luminex IS 100 system.

Experimental Stroke Induces Acute Wasting and Sarcopenia
Weight loss was observed after MCAO but not in sham-operated animals or in controls (Figure 1A). Weight loss peaked at day 3 ($-21.7\pm2.2\%$) and recovery of body weight (starting at day 4) remained incomplete until the end of the study ($-13.9\pm2.9\%$). Reduction of body weight at day 7 was the result of loss of both fat tissue ($-27.3\pm9.0\%$) and lean tissue ($-12.5\pm2.8\%$; Figure 1B). Muscle tissue loss was observed in skeletal muscle of both the ipsilateral leg and the contralateral leg. Heart muscle mass was reduced after MCAO when compared with controls and sham animals (Figure 1B, bottom).

Stroke Induces Intensified Apoptotic and Proteolytic Activity in Skeletal Muscle
Caspase-3 and caspase-6 are effector caspases of apoptosis and increased caspase activity in musculus gastrocnemius indicates enhanced proteolysis of skeletal muscle. After MCAO, a 2- to 3-fold increase in activity of caspase-3 and -6 was observed in the leg muscle contralateral and ipsilateral to the brain lesion. No such changes were seen in sham animals (Figure 2A).

![Graph showing protease activity](image-url)
A significant correlation was observed between infarct size in the brain and caspase activities in the muscle of the contralateral but not of the ipsilateral leg (Figure 2B).

Increased proteasome activity was observed in skeletal muscle after MCAO in both the ipsilateral and the contralateral leg (Figure 3A). A significant inverse correlation between cerebral infarct size and proteolytic activation in the muscle was observed for trypsin-like and for PGPH activity and showed a tendency for chymotrypsin-like activity. Again, the correlation between infarct size and proteasome activity was particularly strong in the contralateral leg but was weaker or nonsignificant in the ipsilateral leg (Figure 3B). Myostatin, a key regulator of muscle growth, was elevated after MCAO when compared with controls (Figure 4A).

**Food Intake and Locomotor Activity**

In animal models, physical activity and food intake are considered to be indicators of well-being or disease burden, respectively. At baseline, spontaneous activity was similar in all groups (all $P>0.2$). No change in 24-hour food intake was observed in controls or in sham animals during the observation period. A reduced food intake was seen on day 3 after MCAO, which correlated with infarct volume (Figure 4B and 4C), and on day 7 after MCAO a tendency toward increased food intake was observed. No correlation was observed between food intake and insulin-like growth factor-1 levels or food intake and caspase-3 levels in stroke animals (Figures I and II in the online-only Data Supplement). Water intake was monitored but was not reduced in sham and stroke animals (data not shown).

Locomotor activity was assessed for 24 hours in all groups (Figure 5, top). The day-and-night pattern of physical activity was maintained on day 3 and day 7 after stroke. Locomotor activity pattern was similar in control and in sham animals. There was a significant increase in locomotor movement after MCAO on day 3 both at night (activity phase) and at daytime (resting phase) but this returned to normal activity levels on day 7. The increased activity after MCAO on day 3 can be attributed to an increase in true physical activity because both fine movements and ambulatory activity were increased in these animals (data not shown). The inflammatory factors IL-6, IL-1α and IL-1β were assessed. No obvious and statistically significant signal was observed in stroke animals in our study. However, minor changes may have been missed because of the inherent variance of such measurements (Figure III in the online-only Data Supplement).

The respiratory quotient was elevated after stroke at day 7 (but not at day 3), particularly during the activity hours at night (representing postprandial metabolism; Figure 5, middle) and was partially elevated during daytime resting periods (representing basal metabolism). The RQ showed stronger variability in 24-hour monitoring after MCAO when compared with controls or sham. Total energy expenditure did not differ between control and sham animals but was reduced after MCAO on day 3, and further reduced but with a sustained day/night pattern on day 7 in 24-hour monitoring in both resting and active periods (Figure 5, bottom).

**Targeting Potential Pathogenic Factors Did Not Ameliorate Wasting or Sarcopenia**

Weight loss and wasting of muscle or fat tissue were not affected by high caloric gavage, sympathetic blockade with propranolol, or preventive antibiotic treatment with enrofloxacin (Figure 6A; except for reduced loss of fat mass...
In addition to this systemic effect, a direct and local catabolic activation in skeletal muscle tissue secondary to cerebral ischemic injury. This catabolic overactivity is foremost a systemic effect, with propranolol treatment). Nor did these treatments prevent wasting of the gastrocnemius muscle and heart muscle mass or attenuate muscle apoptotic and proteolytic activity (Figure 6B).

**Discussion**

This is the first study to investigate muscle wasting and increased catabolic activation in muscle tissue in the acute phase after stroke systematically. We show that the severe weight loss (>20%) results from tissue wasting of both muscle and fat tissue. Muscle tissue wasting was observed for both skeletal muscle and the myocardium. Catabolic activation in skeletal muscle resulted from increased apoptotic activation (caspase signals), as well as proteolytic breakdown of muscle tissue (increased proteasome activity of all 3 major proteasome catalytic domains). Myostatin levels were higher after MCAO than in the control groups.

These findings suggest an upregulation of catabolic pathways in skeletal muscle tissue secondary to cerebral ischemic injury. This catabolic overactivity is foremost a systemic effect because both the contralateral and the ipsilateral leg, as well as fat tissue and the myocardium, showed tissue wasting. In addition to this systemic effect, a direct and local catabolic signal may be emitted from the brain damage to the target area of the disrupted muscular innervation. This is suggested by the correlation between the severity of the brain infarction and many proteolytic and apoptotic signals, particularly in the contralateral, but not in the ipsilateral leg.

This study provides novel insights into the metabolic and structural changes of muscle tissue after stroke. Although muscle is the main effector organ of physical ability, relatively little is known about the acute metabolic alterations in muscle tissue itself secondary to the neuromuscular disruption after a stroke. Physical disability after stroke has traditionally been attributed to brain and upper neuron injury and was not thought to result in muscular atrophy. This concept has been challenged by recent studies that have revealed specific phenotypic alterations in muscle tissue after stroke. Along with muscle atrophy,4 these changes include reduced capillarization and impaired glucose use,25 proinflammatory cytokine activation,26 fiber type changes,2 and endothelium dysfunction.27 Stroke-induced local damage of inhibitory pre-ganglionic pathways of the sympathetic nervous system may result in massive sympathetic overflow, particularly in the target tissues, with subsequent metabolic and inflammatory overactivation.28

Atrophic changes in muscle tissue in hemiplegic patients are well known. However, the early changes and (mal-)adaptive responses of muscle tissue in the acute and subacute phase after stroke have not been studied extensively. Indeed, a recent systematic review searching 17,042 related publications reported a mere 14 clinical studies with a total of 490 patient participants, which investigated stroke-induced skeletal muscle changes.29 This survey included both cross-sectional and longitudinal studies. Notably, none of these studies investigated the early (<6 months) changes in muscle function and structure after stroke. The dynamics of adaptive and maladaptive phenotype changes of muscle tissue may be most pronounced, however, in the early phase after stroke. Our study adds novel and crucial information on this early and vulnerable phase of muscle change. Indeed, targeting muscle metabolism early in the early poststroke phase may be of particular importance for preventing or reversing muscle wasting and for supporting rehabilitation efforts.

**Food Intake, Activity Levels, and Energy Expenditure**

Obvious factors confounding weight loss in stroke are impaired feeding behavior, diminished food or fluid intake, and changes in activity levels. These factors have been carefully addressed in our model. We observed a temporal decrease in food intake after MCAO that normalized with a tendency of over compensation on day 7. Fluid intake was not reduced after stroke. Underfeeding leads to a reduction of insulin-like growth factor-1 which, in turn, may via IRS-1-PI3K/Akt signaling account for an activation of caspase-3. In line with this physiological signal, in the control group, a significant correlation was indeed observed between the amount of food intake and insulin-like growth factor-1 levels. This correlation, however, was not found in sham and stroke animals, and insulin-like growth factor-1 levels were not lower in stroke animals in our study (Figure I in the online-only Data Supplement).
Hence, the insulin-like growth factor-1 signal is unlikely to be the underlying mechanism of increased caspase activity in the stroke animals.

For activity level, a temporal increase in locomotor activity with maintained regular day/night pattern was observed on day 3 after MCAO; locomotor activity returned to normal on day 7. These findings show that the weight changes observed are not merely the result of stroke-induced immobilization and subsequent inability to feed sufficiently. Moreover, in 1 experimental group, we targeted a potential caloric deficit after MCAO by high caloric feeding. This intervention did not diminish weight loss or proteolytic activity in muscle after MCAO. Other mechanisms of impaired feeding could be involved and cannot be fully excluded here, such as central regulation of appetite, intestinal absorption, or substrate use. To investigate these mechanisms, however, was beyond the scope of this study and should be explored in subsequent studies.

Notably, decreased energy expenditure after MCAO was observed both in resting and in activity periods and was even more pronounced on day 7 than on day 3. Given the similar (and even temporarily higher) activity levels observed after MCAO, the lower energy expenditure may reflect an adaptive
energy-saving response to the catabolic overactivation and a countermeasure against further weight loss. Accordingly, partial recovery of body weight was observed between day 3 and day 7, despite unchanged food intake. The increased respiratory quotient particularly during postprandial periods (nighttime) suggests a compensatory effect toward increased triglyceride synthesis as described previously. This effect corresponds with the observed partial recovery of body weight.

Inflammation and Sympathetic Activation as Confounding Factors

Several confounding factors that would also be involved in postaggression metabolism (such as inflammation, sympathetic overactivity, problems of feeding, and intestinal absorption) may contribute to the global tissue wasting effects seen in stroke. A range of factors may account for sympathetic activation, such as physical and emotional stress, pain, spasms, and disruption of inhibitory preganglionic control in the autonomic nerve system. The latter is known to result in an organ-specific and systemic sympathetic overactivation with subsequent hypertensive episodes, dysrhythmias, and elevated body temperature. Sympathetic overactivation contributes to immunosuppression, susceptibility to infections, and fever. Infection, in particular pneumonia, is the most common serious medical complication in patients with stroke and affects skeletal muscle, as well as myocardium and fat tissue. Increased local proteolysis in the target muscle of the disrupted innervation is directly related to the severity of the cerebral injury. This suggests that in addition to the systemic catabolic effects, a direct and local proteolytic signal may occur after cerebral infarction. Common confounders, such as impaired feeding, sympathetic overactivation, immobility, and infection, cannot fully explain this catabolic activation. Our data support the concept of a stroke-specific sarcopenia in the acute phase after stroke. Additional studies need to identify the underlying mechanisms and target them therapeutically. Preventing muscle wasting after stroke with targeted metabolic intervention may be an important principle to improve rehabilitation and outcome after stroke.

Conclusions

Increased catabolism and global tissue wasting occurs after stroke and affects skeletal muscle, as well as myocardium and fat tissue. Increased local proteolysis in the target muscle of the disrupted innervation is directly related to the severity of the cerebral injury. This suggests that in addition to the systemic catabolic effects, a direct and local proteolytic signal may occur after cerebral infarction. Common confounders, such as impaired feeding, sympathetic overactivation, immobility, and infection, cannot fully explain this catabolic activation. Our data support the concept of a stroke-specific sarcopenia in the acute phase after stroke. Additional studies need to identify the underlying mechanisms and target them therapeutically. Preventing muscle wasting after stroke with targeted metabolic intervention may be an important principle to improve rehabilitation and outcome after stroke.

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Disclosures

Dr Dinnagl and Meisel are owners of a patent application on anti-infective agents and immunomodulators used for preventive anti-bacterial therapy after stroke filed to the European Patent Office (PCT/EP03/02246). Dr Anker reports consultancy relationships with Psioxus and Aveo Oncology. Dr Haehling reports honoraria from Pfizer, Solarium Dietsics, Professional Dietsics.

References

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SUPPLEMENTAL MATERIAL

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**Suppl Figure 1:** IGF-1 plasma levels in animals on day 3 (upper panel), association of food intake and IGF-1 levels on day 3 (middle) and on day 7 (lower panel). *p<0.05, **p<0.01 vs sham.

**IGF-1 [pg/mL]**

![Bar chart showing IGF-1 levels with significance markers.](chart1)

**Day 3**

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**Day 7**

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<td>Stroke</td>
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Suppl Figure II: Association of food intake and caspase-3 activity on day 3 (upper panel) and on day 7 (lower panel).
Supplementary Figure III: Inflammatory mediators in stroke animals, sham and controls on day 3 (left panel) and on day 7 (right panel). *p<0.05 vs controls, ND: not detectable