Chapter 7

Therapeutic effects of dietary intervention on neuroinflammation and brain metabolism in a rat model of photothrombotic stroke.

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Abstract

Introduction: Stroke is a leading cause of death and disability worldwide. Since effective treatment options are limited, new therapeutic strategies for stroke are urgently needed. One of the possible targets for stroke management is modulation of neuroinflammation to salvage brain tissue at risk. Evidence suggests that food components may exert anti-inflammatory properties. Therefore, we investigated the effects of a diet, supplemented with multiple anti-inflammatory ingredients, on behavior, brain metabolism and astrogliosis in a model of focal ischemic brain damage.

Methods: Focal lesions were induced in the somatosensory cortex of rats by photothrombotic stroke. Rats were fed a control diet or an investigational diet, starting either 2 weeks before or immediately after the induction of ischemia. Rats remained on the diet until termination (either 7 or 21 days post stroke). The effects of ischemia and the investigational diet on brain damage, neuroinflammation, brain glucose metabolism and motor function were assessed with immunohistochemistry, positron emission tomography (PET) and behavior tests.

Results: The induction of ischemia caused transient lateral movement impairment on day 3, which was normalized on day 6 and 20. Photothrombotic stroke induced focal brain damage, surrounded by strong and persistent astrocyte activation. Stroke was accompanied by decreased glucose metabolism in the contralateral hemisphere on day 7, but not on day 21. The investigational diet applied two weeks before the induction of ischemia did not affect astrocyte activation on day 7, but it increased brain glucose metabolism in the ipsilateral hemisphere. When the investigational diet was started immediately after the induction of ischemia, increased astrocyte activation was observed on day 7, while glucose metabolism was not affected. Both treatments with the investigational diet reduced astrocyte activation on day 21 after the induction of ischemia, but did not affect glucose metabolism. Lesion size was not significantly affected by the dietary intervention on day 7, but the post-ischemic dietary intervention prevented the lesion growth between day 7 and 21.

Conclusion: This study reveals a potential beneficial effect of an investigational diet containing elevated amounts of specific anti-inflammatory nutrients on the recovery from ischemic brain damage.
1. Introduction

Stroke occurs when the blood supply to part of brain is interrupted due to obstruction of a vessel by an embolism or thrombus (ischemic stroke) or due to rupture of a weak vessel (hemorrhagic stroke). Current treatments for ischemic stroke consist of thrombolysis with recombinant tissue plasminogen activator and mechanical thrombectomy. However, these treatments need to be applied shortly after the onset of stroke (therapeutic window within 4.5 and 6 h, respectively), which is often not possible [1]. Clearly, there is a need for additional treatments not only focusing on recovery and rehabilitation, but also on prevention, especially in the high-risk population.

Several risk factors for stroke are potentially modifiable, like hypertension, cardiac disease, diabetes, cigarette smoking, alcohol consumption, drug abuse, obesity, lack of physical activity and an unhealthy diet [2,3]. Evidence suggests an important role for diet in the prevention of stroke. For example, the Mediterranean-style diet, characterized by high consumption of vegetables, olive oil, fish, and moderate consumption of meat, milk, and wine, is associated with a lower incidence of stroke [4]. Several studies have demonstrated that consumption of fruit and vegetables [5], dietary fibers [6], multivitamins [7], the microelements magnesium and potassium [8] and cysteine [9] have the potential to reduce the risk of stroke. These data suggest that a proper diet could be a lifestyle intervention that may prevent stroke or ameliorate its consequences [10].

Many of the food components with preventive effects for stroke onset in humans, such as omega-3 fatty acids docosahexaenoid acid (DHA), eicosapenaenoic acid (EPA); vitamins A and D, and fibers, have anti-inflammatory properties [11–15]. Some indirect evidence indeed suggests a positive impact of anti-inflammatory diet on the survival following a stroke [16]. A stroke-induced decrease in cerebral blood flow leads to the interruption of oxygen and glucose supply and disruption of ionic homeostasis. These events can trigger an inflammatory response and cause neuronal death [17]. The inflammatory response is characterized by activation of microglia and astrocytes that produce inflammatory mediators, such as cytokines, reactive oxygen species, chemokines, matrix metalloproteinases and adhesion molecules. Most of these mediators have an ambivalent role as they are involved in both neurotoxicity and neuroprotection [18].
Since inflammation is under investigation as one of the potential targets for new therapeutic approaches in stroke [19,20], we hypothesized that anti-inflammatory food components may have a preventive and possibly a therapeutic effect on stroke. In this study, we induced a photothrombotic stroke in rats [21–24] in order to investigate the effects of a diet containing elevated amounts of specific anti-inflammatory nutrients on the recovery from unilateral cortical ischemia. Dietary intervention was started either two weeks before (prevention), or directly after the induction of ischemia (curative). In particular, we investigated whether dietary intervention is effective in reducing the lesion size by modulating astrocyte activation, since activated astrocytes are believed to play a protective role at the early stages of stroke [25], but may become detrimental if activation persists. Therefore, lesion size and astrocyte activation were measured post-mortem by immunohistochemistry at day 7 and 21 following ischemia. In addition, we investigated the functional parameters: brain metabolism and motor dysfunction. Changes in brain glucose metabolism, which may reflect brain damage, repair or compensatory mechanisms, were investigated non-invasively by positron emission tomography (PET), using 2'-[18F]fluoro-2'-deoxyglucose ([18F]FDG) as the tracer. Motor dysfunction was assessed with the cylinder test [26].

2. Material and methods

2.1 Experimental animals

Animal experiments were performed in accordance with Dutch Regulations for Animal Welfare. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Groningen (protocol DEC 6971A).

Male outbred Sprague Dawley rats (10 weeks of age, n=48, 301.5±2.6 grams) were purchased from Harlan (Horst, The Netherlands) and housed in groups (2-6 animals per cage) in thermo-regulated (21±2 °C) and humidity-controlled rooms under a 12-12 h light-dark cycle (lights on at 6 AM). Food and water were available ad libitum and paper rolls were used as cage enrichment. The rats were allowed to acclimatize for at least 7 days after arrival. All rats were fed with a standard laboratory chow (AIN93-G) from the time of arrival until the start of the experiment [27]. Rats were housed individually after surgery until the end of the experiment.
2.2 Diet

Two diets were used in the study: a control diet (AIN93-G) and an investigational diet. Both diets were iso-caloric and were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands). The composition of the investigational diet is listed in Table 1, and differed from the control diet, as it contains low-glycemic index carbohydrates and is supplemented with vitamins, specific dietary fibers and tryptophan. Furthermore, it has a lipid profile with a high docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) content. The rationale to include these specific nutrients in the dietary intervention is that they have been demonstrated to possess anti-inflammatory properties, acting on diverse anti-inflammatory pathways. The chow was stored at -20 °C prior to use to prevent fatty acid oxidation.

2.3 Study design

The study design is depicted in figure 1. Rats were randomly divided in eight groups (n=6 per group). Rats were weighed daily. Photothermbotic stroke and sham-surgeries were performed on experimental day 0. Six groups were subjected to the chemical induction of focal cortical ischemia and to one of three different feeding protocols: SC\textsubscript{7} and SC\textsubscript{21}: the stroke groups fed with control diet until termination on day 7 and 21 after stroke, respectively; SI-post\textsubscript{7} and SI-post\textsubscript{21}: rats subjected to stroke followed by a post-ischemic dietary intervention fed with the investigational diet from the day of the ischemia induction (day 0) until the end of the experiment (day 7 and day 21, respectively); SI-pre\textsubscript{7} and SI-pre\textsubscript{21}: rats subjected to stroke with pre-ischemic dietary intervention that were fed with the investigational diet from 2 weeks before ischemia (day -14) until the end of the experiment (day 7 and day 21, respectively); CC\textsubscript{7} and CC\textsubscript{21}: control groups subjected to sham-surgery fed with the control diet during the entire experiment (until day 7 and 21 after sham-surgery, respectively).

Motor dysfunction was assessed with the cylinder test. The baseline cylinder test was performed in all groups 8 days before the surgery (day -8). Motor deficits following ischemia or sham-surgery were assessed on experimental days 3 and 6 (for groups CC\textsubscript{7}, SC\textsubscript{7}, SI-post\textsubscript{7} and SI-pre\textsubscript{7}) or on experimental days 6 and 20 (for groups CC\textsubscript{21}, SC\textsubscript{21}, SI-post\textsubscript{21} and SI-pre\textsubscript{21}). [\textsuperscript{18}F]FDG PET imaging of brain glucose metabolism was performed in all animals 7 days before the ischemia induction or sham-surgery (day -7). In addition, half of the animals were scanned...
on experimental day 7 (groups CC\textsubscript{7}, SC\textsubscript{7}, SI-post\textsubscript{7} and SI-pre\textsubscript{7}) and the other half of the animals on the experimental day 21 (groups CC\textsubscript{21}, SC\textsubscript{21}, SI-post\textsubscript{21} and SI-pre\textsubscript{21}). After the PET scans on either day 7 or 21, the rats were sacrificed and the brains were collected for immunohistochemistry.

Due to complications with anesthesia two rats did not survive the surgery on day 0 (from the CC\textsubscript{21} group), and two rats did not survive the [\textsuperscript{18}F]FDG PET scan on day 21 (from the SC\textsubscript{21} and SI-post\textsubscript{21} groups).

2.4 Stroke induction and sham surgery

Focal ischemic cortical lesions were induced by photothrombotic stroke using the protocol described previously [22]. Briefly, rats were anesthetized with a mixture of ketamine (Ketalar\textsuperscript{®}, 60 mg/kg; Pfizer, Brussels, Belgium) and medetomidine (Domitor\textsuperscript{®}, 0.4 mg/kg intraperitoneal; Brussels, Belgium, Pfizer). The body temperature of the animals was maintained with heating pads; eye salve was applied onto the eyes to prevent dehydration. The skull was exposed by a lateral incision of the skin. After intravenous injection of the photosensitizer Rose Bengal (20 mg/kg; Sigma-Aldrich, St. Louis, MO), an area of the exposed intact skull was irradiated for 20 min with green light (wavelength 540 nm, bandwidth 80 nm) from a xenon lamp (model L-4887; Hamamatsu Photonics, Hamamatsu City, Japan) with heat-absorbing green filters. The radiation with an intensity of 0.68 W/cm\textsuperscript{2} was directed with a 3-mm optical fiber placed on the skull above the right sensorimotor cortex next to Bregma. Light-induced oxidation of the photosensitizer causes endothelial damage, platelet activation, and consequently vascular occlusion (Carmichael 2005). At the end of surgery, anesthesia was reversed with atipamezol (Antisedan\textsuperscript{®}, 1 mg/kg intraperitoneal; Orion Pharma, Newbury, UK). Finadyne (1 mg/kg) was given prior to and 24 h after surgery in order to reduce pain. In addition, before surgery, 100 µL of xylocaine with 2% adrenaline was applied locally on the skin incision as a local anesthetic and to reduce bleeding. For sham-surgery, the same procedure was performed, except for the application of radiation on the skull. After stroke induction or sham-surgery, rats were housed individually until the end of the experiment.

2.5 Cylinder test

Motor dysfunction was assessed during the light phase and recorded on video for further analysis. The cylinder test (asymmetric paw use test) was used to quantify
forelimb use as described previously [26]. Briefly, rats were placed in a 20-cm wide clear glass cylinder located in front of two mirrors to facilitate the observation of the behavior from each side. A total of 20 contacts with the cylinder wall by each forepaw were scored from the video by a blinded observer. The number of contralateral forelimb (left forelimb) contacts was expressed as a percentage of total forelimb contacts. Normal control rats should score 50% in this test [28].

2.6 PET imaging

$[^{18}\text{F}]$FDG PET scans were performed using a dedicated small animal PET scanner (Focus 220, Siemens Medical Solutions USA, Malvern, PA). The body temperature of the rats was maintained with heating pads, eye salve was applied onto the eyes to prevent dehydration, and heart rate and blood oxygen levels were monitored with a BioVet system (M2M Imaging, Cleveland, OH). Two rats from different experimental groups were scanned simultaneously in each scanning session.

First, the rats were anesthetized with isoflurane mixed with oxygen (5% induction and 2% maintenance, 0.8 L/min) and a cannula was inserted into the tail vein to allow the injection of $[^{18}\text{F}]$FDG. Next, the rats were positioned in the camera in a transaxial position with their heads in the center of the field of view. Before each PET acquisition, a transmission scan of 10 min with a $^{57}$Co point source was performed and used during image reconstruction to correct for attenuation, scatter and random coincidences. Next, 18±7 MBq $[^{18}\text{F}]$FDG was injected with a pump over a period of 1 min and a 60-min PET scan was started. There were no statistically significant differences in the injected tracer dose between the groups ($F(7, 39)=0.208$, $p=0.98$). After the baseline scan (day -7), rats were allowed to recover in their home cages. After the scans on day 7 and 21, rats were sacrificed and tissue samples were collected.

2.8 PET image reconstruction and analysis

List-mode data from the 60-min $[^{18}\text{F}]$FDG emission scan was reconstructed into 3 frames (2400 s, 2x600 s). Emission sinograms were normalized and corrected for attenuation and decay of radioactivity and iteratively reconstructed using OSEM2D (4 iterations and 16 subsets) [29]. [Hudson 1994].

A 10-min frame of the $[^{18}\text{F}]$FDG PET scan, starting 50 min post injection, was used to explore the differences in brain glucose metabolism between the groups.
The images were automatically co-registered with a tracer-specific template [30], using Vinci 4.26 software (Max Planck Institute for Neurological Research, Cologne, Germany). Standardized uptake values (SUV) of the tracer in the brain were calculated as follows: 
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\text{SUV} = \frac{\text{tissue activity concentration (MBq/ml) \times bodyweight (g)}}{\text{injected dose (MBq) \times brain tissue density (g/ml)}}.
\]
It is assumed that the brain tissue density is 1 g/ml. Data was analyzed voxel-wise using SPM12 (Statistical Parametric Mapping, Wellcome Trust Centre for Neuroimaging, London, UK) and the SAMIT1.2 toolbox [30]. Voxel-based SPM analysis allows analysis of the data without a prior knowledge or predefined anatomical regions [31]. PET images were smoothed with a 1.2 mm isotropic Gaussian kernel and analyzed with a two samples t-test design. Groups were compared separately for each time point to investigate the effects of stroke induction (CC vs. SC) and investigational diet (SC vs. SI-post and SC vs. SI-pre). \[^{18}\text{F}]FDG PET results from voxel-based analysis are presented as T-maps. For statistical analysis, T-maps were interrogated at a peak voxel level of \(p<0.005\) (uncorrected) and a minimum cluster size threshold of 200 voxels. Only clusters with \(p<0.05\) corrected for family-wise error were considered statistically significant.

2.9 Immunohistochemistry

After the PET scan on day 7 and 21, the rats were sacrificed under deep isoflurane anesthesia (5%) by transcardiac perfusion with saline. Brains were dissected and fixed by immersion in 4% paraformaldehyde for 24 h at room temperature, followed by cryopreservation in 30% sucrose in phosphate-buffered saline (PBS) for 24 h. Brains were stored at -80 °C until further processing. Next, brains were cut in a cryostat into 10 µm thick sagittal sections, which were placed onto adhesive microscope slides and stored at -20 °C until further analysis.

For the analysis of astrocytes, 10 µm brain sections were blocked with 5% bovine serum albumin (BSA, Sigma Aldrich) in PBS for 30 min at room temperature. As primary antibody for astrocytes, mouse anti-Glial Fibrillary Acidic Protein (GFAP) (Sigma Aldrich, G3893) was applied overnight (~16 h) in a 1:400 dilution in PBS containing 1% BSA at 4°C. Next, the sections were washed 3 times with PBS and the secondary antibody, anti-mouse Cy3 (Life Technologies), in a 1:1000 dilution in PBS containing 1% BSA was applied for 1 h. The sections were washed 3 times with PBS and the slides were covered with quick-hardening mounting medium (Eukitt®, Sigma Aldrich) and a microscope coverslip. The sections were analyzed.
with a TissueFAXS system (Tissue Gnostics) and digital images from areas of interest were acquired. Regions of interest were identified based on a stereotaxic atlas [32] (Paxinos and Watson, 2005).

The images were scored by an independent observer, who was blinded to the treatment of the animals (images were coded by numbers without any indication of the group). Morphology of GFAP-stained cells was determined in randomly selected 0.0432 mm² sections of 3-5 brain slices per rat using ImageJ software (NIH, United States). The area surrounding the infarct (located 350 μm from the lesion) and contralateral side were analyzed by integrated density to assess the surface covered by the staining. For data analysis and illustration of the results, the colors on the white/black images have been inverted (white background).

2.10 Statistical analysis

The statistical analysis of bodyweight, behavior and immunohistochemistry data was performed using IBM SPSS software Statistics 22 (SPSS Inc., United States). Results are presented as mean ± standard error of the mean (SEM). Differences in bodyweight between groups at the start of the experiment were analyzed by one-way ANOVA. Bodyweight changes following ischemia induction or sham-surgery were analyzed for differences between time points and between groups with the generalized estimating equations (GEE) model with a Bonferroni post-hoc correction to account for multiple comparisons [33,34]. The exchangeable correlation matrix and the Wald test were used to calculate p-values. Results from the behavioral test and immunohistochemistry were analyzed with one-way ANOVA followed by Bonferroni post-hoc correction to account for multiple comparisons. Differences were considered statistically significant when p<0.05.

3. Results

3.1 Bodyweight

Figure 2 shows the evolution of bodyweight during the experiment for the groups that were sacrificed at day 21. The average bodyweight on day 0 (i.e. before ischemia induction or sham-surgery) was 301.5±2.6 g and was not statistically different between groups (F(3)=2.000, p=0.112), indicating that the diet by itself did
not affect bodyweight. In all groups, a decrease in bodyweight was observed shortly after ischemia induction or sham surgery. This bodyweight loss was accompanied with a tendency towards lower food intake (supplementary data, figure 1). Food intake was not significantly different between the groups. The drop in bodyweight after surgery was larger in the group that switched to the investigational diet immediately after induction of ischemia and bodyweight in this group did not recover to normal before the end of the experiment. The SI-post$_{21}$ group therefore had a significantly lower bodyweight than all other groups (CC$_{21}$, SC$_{21}$, SI-pre$_{21}$; p<0.05) at all time points between stroke induction and termination (day 1-21).

These findings indicate that (I) body weight was affected by the surgery itself, not the stroke induction; (II) when the investigational diet was started before ischemia, it did not affect post-ischemic body weight; (III) a dietary switch immediately after ischemia induction caused a stronger reduction in bodyweight, which did not normalize before the end of the experiment.

3.2 Infarct size

At day 7 and 21 after ischemia induction, the infarct size was assessed on the isolated brains by measuring the length (alongside with Bregma) and width (perpendicular to Bregma) of the visible scar. As depicted in figure 3, no significant differences between the groups subjected to focal ischemia were observed on day 7 (F(2)=0.043, p=0.958) or 21 (F(2)=1.006, p=0.39). Although the scar tended to be somewhat smaller in the SI-post$_{21}$ group, no statistical significance was reached due to the large between-subject variation (t test: SC$_{21}$: 5.82±1.78 mm vs. SI-post$_{21}$: 3.83±0.59 mm, p=0.94; SC$_{21}$ vs. SI-pre$_{21}$:6.29±1.25 mm, p=0.86).

When we compared the change in average lesion size between day 7 and 21, the lesions in groups SC and SI-pre had increased in this period by about 25% (25.8% and 24.8%, respectively). In contrast, the average lesion size in SI-post group had decreased by 14.8% between day 7 and 21. However, it is important to note that the lesion size on day 7 and 21 was performed on different animals.

3.3 Astrocyte activation

GFAP staining was performed in order to assess the effects of ischemia on astrocytes activation surrounding the lesion (SC vs. CC) and the effect of the investigational diet thereon (SC vs. SI-post and SC vs. SI-pre). GFAP staining
demonstrated significant differences between the groups in astrocyte activation close to the lesion site (350 µm, F(3)=30.84, p<0.0001, fig 4 A and B), with a significant increase of GFAP staining in ischemia-induced animals as compared to sham-surgery controls, both on days 7 (SC7 2.5±0.13, CC7 1.0±0.17, p<0.001) and on day 21 (SC21 1.94±0.20, CC21 1.00±0.08, p<0.0001). Further away from the lesion site (700 µm) no significant differences in GFAP staining between the sham-surgery group (CC7) and the ischemia group on the control diet (SC7) were observed anymore, neither on day 7 nor on day 21 (data not shown).

At day 7, the investigational diet did not have any significant effect on the ischemia-induced activation of astrocytes close to the lesion site, as no significant differences in activated astrocytes were observed for the groups treated with the investigational diet (SI-pre7: 3.03±0.13, p=0.39 and SI-post7: 2.07±0.23, p=0.06) when compared to ischemia controls (SC7). On day 21, the post-ischemic dietary intervention had completely reversed the effect of ischemia on GFAP staining (SI-post21: 0.97±0.04, SI-post21 vs. SC21 p<0.0001, SI-post21 vs. CC21 p=0.86). The pre-ischemic dietary intervention only caused a partial reversal of the astrocyte activation on day 21 (SI-pre21: 1.54±0.06, SI-pre21 vs. SC21 p<0.05, SI-pre21 vs. CC21 p<0.05). Thus, post-ischemic dietary intervention was significantly more potent than pre-ischemic dietary intervention in inhibiting the astrocyte activation on day 21 (SI-post21 vs. SI-pre21: p<0.05).

3.4 Brain metabolism

Brain glucose metabolism was investigated with [18F]FDG PET imaging at baseline (day -7) and either on day 7 (groups CC7, SC7, SI-post7, SI-pre7) or day 21 after surgery (groups CC21, SC21, SI-post21, SI-pre21). The first objective was to investigate whether stroke induction can induce detectable changes in glucose metabolism (SC vs. CC) and the second objective was to assess the impact of the dietary intervention on glucose metabolism following stroke induction (SC vs. SI-post and SC vs. SI-pre).

[18F]FDG PET imaging demonstrated significant differences in brain metabolism between the groups on day 7 (figure 5), but not anymore on day 21. Voxel-wise comparison of the PET images allowed determination of brain regions (clusters of voxels) with a significantly changed brain metabolism between the groups without any a-priori assumptions. The induction of ischemia did not significantly affect glucose metabolism at the lesion site, but caused a significant decrease in brain
metabolism in one big cluster in the hemisphere contralateral to the lesion site (SC\textsubscript{7} vs. CC\textsubscript{7}; cluster peak at Paxinos coordinates 4.5 mm 0.8 mm -2.6 mm). The contralateral decrease in glucose metabolism was mainly observed in the somatosensory cortex and small parts of the insula, motor cortex, caudate putamen and corpus callosum.

Post-ischemic dietary intervention did not reverse the stroke-induced hypometabolism in the contra-lateral hemisphere (SC\textsubscript{7} vs. SI-post\textsubscript{7}), nor did it affect brain glucose metabolism in any other brain region. On the other hand, pre-ischemic dietary intervention increased \(^{18}\text{F}\)FDG uptake in the ipsilateral hemisphere of stroke animals (SC\textsubscript{7} vs. SI-pre\textsubscript{7}), in particular in part of the somatosensory cortex, thalamus and small parts of the hippocampus, visual cortex, motor cortex, and corpus callosum (cluster with a peak at Paxinos coordinates 2.1 mm -3.6 mm -3.4 mm). Increased glucose metabolism was also observed in cerebellum and medulla (cluster of voxels with a peak at Paxinos coordinates 1.9 mm -11.0 mm -6.2 mm). Medulla was the only region equally affected in both hemispheres (regions and voxels are listed in table 2 in the supplementary data). Pre-ischemic dietary intervention did not significantly reverse the stroke-induced hypometabolism observed in the contralateral hemisphere.

Interestingly, the diet by itself also had an effect on brain glucose metabolism. Comparison of the baseline scan between all animals subjected to the investigational diet 2 weeks before the induction of ischemia (SI-pre\textsubscript{7} and SI-pre\textsubscript{21}) with all animals fed with control diet before the baseline scan (CC\textsubscript{7}, CC\textsubscript{21}, SC\textsubscript{7}, SC\textsubscript{21}, SI-post\textsubscript{7} and SI-post\textsubscript{21}) revealed that the investigational diet significantly reduced brain glucose metabolism in healthy animals (supplementary data, figure 2), in particular in part of the cortex, amygdala, caudate putamen, corpus callosum and cerebellum.

Thus both stroke and the diet affected brain glucose metabolism beyond the site of the ischemic lesion.

3.5 Cylinder test

To investigate the impact of ischemia and the investigational diet on asymmetric paw use, the cylinder test was performed (figure 6). Baseline measurements on day 8 before the surgery did not reveal any preference for the left or right paw and consequently no significant differences between groups (F(3)=0.085, p=0.97). The
cylinder tests on day 3 post surgery demonstrated significant changes in the preferences for the paw contralateral to the lesion (F(3)=8.746, p<0.01), as stroke caused a significant reduction in use of the contralateral paw on day 3 (CC: 60.8±2.7%, SC: 35.0±4.1%, p<0.01). All animals were also subjected to the cylinder tests on day 6 and 21. Only a trend towards a reduced preference to use the contralateral paw was still observed on day 6 (F(3)=2.606, p=0.067), whereas paw preference was completely absent on day 20 (F(3)=0.303, p=0.82).

The investigational diet did not have any effect on stroke-induced asymmetric paw use, as no statistical differences in the cylinder test between dietary regimens were observed 3 days after ischemia induction (SC: 35.0±4.1%, SI-post: 35.0±5.3%, SI-pre 32.5±5.4).

Thus, photothrombotic stroke did induce transient motor dysfunction on day 3 following ischemia, which was virtually resolved at day 6. However, dietary intervention could not prevent or reverse this symptom.

4. Discussion

The aim of this study was to assess the potential of a dietary intervention to moderate neuroinflammation and other effects of focal cortical ischemia in a rat model of photothrombotic stroke. Photothrombotic stroke is a minimally invasive, reproducible method to create a chemically-induced cortical lesion [35]. The severity of the model is relatively low and can be modulated by changing the duration of irradiation. In our study, photothrombotic stroke with mild severity was chosen, because dietary intervention was expected to have subtle effects on the recovery from stroke. These therapeutic effects may remain obscured in a severe stroke model.

We observed that both induction of ischemia and sham-surgery caused up to 10% decrease in bodyweight. This suggests that not the ischemic lesion itself, but the whole surgical procedure caused a transient decrease in bodyweight. This effect of surgery could be ascribed to a reduction in food intake during and early after surgery (Supplemental figure 1). The fact that ischemia did not cause any further decrease in bodyweight is in agreement with the low severity of this stroke model.
All animals subjected to photothrombotic ischemia in this study developed scar tissue at the ischemic site and a robust activation of astrocytes around the lesion, as was measured with tissue staining of GFAP expression. These characteristics are consistent with the previous studies on this model [35]. The neuroinflammatory response following photothrombotic stroke has been extensively studied [21]. Cortical ischemia causes robust tissue damage which is transformed into scar tissue at later stages (within 4 weeks). Massive astrocyte activation in the area surrounding the lesion occurs within hours following stroke onset. Astrocytes contribute to the formation of a glial scar around the lesion. Although a scar can be a barrier preventing innervation of the region, there is also evidence supporting its beneficial role for recovery, as isolating the lesion site can help to prevent further brain damage.

To gain more insight into the effects of stroke on brain function, we applied $^{18}$F-FDG PET imaging to investigate the effect of cortical ischemia on brain metabolism. $^{18}$F-FDG PET has already been successfully used in animal studies on brain damage following chemically induced cortical ischemia [36] and in stroke patients [37]. The objective of this measurement was to investigate (I) hypometabolism resulting from brain damage, and (II) hypermetabolism which may result from increased neuronal activity in the parts of the brain not subjected to the damage as a mechanism to compensate for loss of function due to the lesion or (III) hypermetabolism due to strong activation of immune cells involved in brain damage repair. In our study, each animal was subjected to two PET scans: a baseline scan and a scan at either day 7 or day 21 following ischemia induction or sham-surgery. However, PET imaging at either post-surgery time point did not detect any hypometabolic lesion at the location of the photothrombotic stroke. This lack of sensitivity could be due to the limited resolution of the PET camera (ca. 1.7 mm at 5 cm from the center of the field-of-view), which is in the same range as the size of the lesion, resulting in significant partial volume effects. In addition, the location of the lesion might be slightly different between animals and consequently the effect of the small lesion on brain glucose metabolism will be averaged out over a larger region when group comparisons are made. $^{18}$F-FDG PET on day 21 did not reveal any other significant differences in glucose metabolism between groups either. On day 7, on the other hand, $^{18}$F-FDG PET revealed a reduction in glucose metabolism in the hemisphere contralateral to the lesion. At present it is not clear what could have caused this effect, but it might be related to a general reduction in brain activity due to stroke-induced inactivity.
Additionally, we measured the effect of brain damage on motor function with the cylinder test. We found that introduction of ischemia, but not sham-surgery, led to transient lateral motor dysfunction. Since the severity of the model is relatively low, lateral motor dysfunction was observed only on day 3 after ischemia induction. Motor function had normalized again on day 6 and 20. This is consistent with previous studies demonstrating lateral motor dysfunction shortly after ischemia [26] and suggests that the function of the affected area was quickly restored, possibly as a result of compensation by other brain regions.

The main objective of this study was to investigate the effects of an investigational diet on symptoms observed following photothrombotic cortical ischemia. The diet investigated in this study was designed to target neuroinflammation, as it contains elevated amounts of components, such as vitamins A and D, omega-3 fatty acids and specific amino acids (tryptophan), which all have been described to exert anti-inflammatory effects on immune cells in vitro and in vivo [11,12,38,39]. The indigestible galacto-oligosaccharides and fructo-oligosaccharides have been included in the investigational diet, because they have been shown to modulate the immune system via alteration of gut microbiota and by direct interaction with peripheral immune cells and thus could have an indirect effect on neuroinflammation via the gut-immune-brain axis [15].

Bodyweight loss caused by the surgery (both sham and to induce stroke) was adversely affected by the post-ischemic intervention with the investigational diet, which aggravated the loss in bodyweight. This observation, however, is likely not a cause a pharmacological effect of the ingredient, may be explained by the change of diet early after surgery in this particular group. Apparently, the animals need some time to get used to the new diet, as they hardly consumed any food on the first 2 days after the change of diet (Supplemental figure 1). We observed a similar effect of transient (1-2 days) decrease in food intake in the healthy animals subjected to a change from the control diet to the investigational diet in previous studies (unpublished data).

When assessing the effect of the diet on the size of the cortical lesion, we did not observe any between animals subjected to different dietary regimens at day 7. This can be explained by the fact that the diet does not interfere with the mechanisms of induction of the stroke (activation of photosensitive Rose Bengal and the transient blockage of blood supply). The diet is designed to affect neuroinflammatory response following brain ischemia, and therefore aimed to inhibit the further tissue
damage and facilitate tissue recovery. We observed that the lesion size in the ischemic rats on control diet increased with 25.8% between day 7 and day 21. A similar increase was observed in the pre-ischemic dietary intervention group (+24.8%), indicating that this dietary regimen could not prevent the growth in lesion size. In contrast, the group subjected to post-ischemic dietary intervention did not show any increase in lesion size in this time period, but rather a caused reduction in size (-14.8%), suggesting this intervention has therapeutic efficacy.

When investigating the effect of the dietary intervention on the neuroinflammatory response in the tissue surrounding the brain ischemic damage, we observed that the robust increase in astrocyte activation 7 days after ischemia induction was further increased by the investigational diet when started immediately after ischemia, but the concentration of activated astrocytes was not changed when the investigational diet was started two weeks before the ischemia. On day 21, however, the post-ischemic dietary intervention caused complete reversal of the ischemia-induced astrocyte activation to control levels, while the pre-ischemic dietary intervention started 2 weeks before ischemia caused only partial reversal of the astrocyte activation in the area close to the lesion. Astrocytes are believed to act as double-edged sword, by being involved in both neurotoxic and neuroprotective mechanisms [40]. It is believed that the early response of astrocytes has a positive impact on recovery from stroke, while astrogliosis is detrimental for regeneration of the brain in later stages [25]. This would be in agreement with our data on the post-ischemic dietary intervention. The enhancement of the beneficial effect of astrocyte activation in the early response to stroke and complete inhibition of detrimental effect at a later stage, could explain why the lesion did not grow any larger in this particular group.

We considered the fact that the beneficial effects of the post-ischemic dietary intervention might be due caloric restriction, since switching the diet after surgery caused a significant decrease in body weight as a result of reduced food intake. Previous studies have demonstrated that caloric restriction might inhibit the age-related activation of astrocytes [41]. However, this effect of caloric restriction on astrocyte activation was observed only after a long caloric restriction period (24 months). Since the reduction in food intake in this study only lasted for 1-2 days, it seems plausible that the effect observed in this study is caused by the anti-inflammatory components of the dietary intervention rather than by a temporary reduction in caloric intake due to the change of the diet.
Investigation of the effects of the investigational diet on brain glucose metabolism following cortical ischemia by \([^{18}F]FDG\) PET revealed that post-ischemic dietary intervention did not cause any detectable effect on day 7. The pre-ischemic dietary intervention, on the other hand, caused hypermetabolism in the area of the lesion. The increased glucose metabolism in the area of somatosensory cortex and adjacent areas could reflect increased metabolism in cells repairing the ischemia-induced damage in this area, as supported by the enhance astrocyte activation observed by GFAP staining [42]. The increased glucose metabolism in the thalamus could be caused by the fact that this region controls the processing of somatosensory cortex [43]. Hypermetabolism in the SI-pre\(_7\) group on day 7 was also observed in medulla in both hemispheres and in right cerebellum. The latter could be explained by a compensation mechanism to damage in the motor cortex, since cerebellum is involved in motor function [44], although there is no evidence so far that the cerebellum indeed compensates for motor cortex dysfunction. Therefore, this observation requires further investigation.

The cylinder tests that was used in this study for assessment of changes in motor function was not sensitive enough to detect any effects of the intervention after a mild stroke. We did not detect beneficial effects of the investigational diet on the motor dysfunction observed on day 3, probably because the diets did not exert any beneficial effects this shortly after stroke yet. This is in line with the absence of any effect of the diet on lesion size on day 7. At later time points, the effect of the diet on ischemia-induced motor dysfunction could not be assessed anymore as motor function was already restored to normal.

In conclusion, we showed potential beneficial effects of a post-ischemic intervention with an investigational diet containing elevated amounts of specific anti-inflammatory nutrients on chemically-induced cortical ischemia in rats. The beneficial effects mainly concerned modulation of astrocyte activation around the lesion, resulting in a reduction in the expansion of the lesion. This dietary regimen, however, did not significantly affect brain glucose metabolism. In contrast, pre-ischemic dietary intervention did change glucose metabolism in brain regions surrounding the lesion site and regions contralateral to the lesion, but was less effective in modulating astrocyte activation and could not prevent the increase of the lesion size. Taken together, these results warrant further investigation of post-ischemic dietary intervention as new therapeutic option for stroke.
Conflict of interest This study is part of the BrainMenu project and financially supported by the STW-Danone Partnership Program (project number: 11650). J.M. Verkuyl and L.M. Broersen are employees of Nutricia Research and therefore declare potential conflicts of interest. All other authors report no financial interest or potential conflicts of interest.

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Figures and tables

Table 1 The composition of the investigational diet (on the next page)
<table>
<thead>
<tr>
<th>Investigational diet components compared to control diet (per kg diet)</th>
<th>supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carbohydrates</strong></td>
<td>Roquette (Lestrem, France)</td>
</tr>
<tr>
<td>dextrinized corn starch and sucrose substituted by:</td>
<td></td>
</tr>
<tr>
<td>41.5 wt% maltodextrin (DE6)</td>
<td></td>
</tr>
<tr>
<td>15.0 wt% free galactose</td>
<td>Inalco (Milan, Italy)</td>
</tr>
<tr>
<td>42.5 wt% isomaltulose</td>
<td>Beneo-Palatinit (Mannheim, Germany)</td>
</tr>
<tr>
<td>1 wt% fructose</td>
<td>Brenntag (Dordrecht, The Netherlands)</td>
</tr>
<tr>
<td><strong>Fibers</strong></td>
<td>Beneo Orafti (Oreye, Belgium)</td>
</tr>
<tr>
<td>2.8% cellulose substituted by:</td>
<td></td>
</tr>
<tr>
<td>2% rice fiber RemyLiVe200</td>
<td></td>
</tr>
<tr>
<td>0.72% GOS</td>
<td>Friesland Campina (Amersfoort, The Netherlands)</td>
</tr>
<tr>
<td>0.08% Beneo Raftiline HP FOS</td>
<td>Beneo (Leuven, Belgium)</td>
</tr>
<tr>
<td><strong>Proteins</strong></td>
<td></td>
</tr>
<tr>
<td>soy protein isolate 770LN substituted by:</td>
<td></td>
</tr>
<tr>
<td>1:1 soy protein isolate 770LN</td>
<td>Solae company (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>α-lac enhanced whey</td>
<td>Arla Food ingredients (Wageningen, The Netherlands)</td>
</tr>
<tr>
<td>addition of: 2.3 g tryptophan</td>
<td></td>
</tr>
<tr>
<td><strong>Lipids</strong></td>
<td>Nippon Suisan Kaisha (Tokyo, Japan)</td>
</tr>
<tr>
<td>to obtain 0.53% DHA and 0.92% EPA, part of lipid fraction substituted by:</td>
<td></td>
</tr>
<tr>
<td>27.5 g Nissui anchovy oil</td>
<td></td>
</tr>
<tr>
<td>6.5 g Biopure DHA IF tuna oil</td>
<td>Bioriginal (Den Bommel, The Netherlands)</td>
</tr>
<tr>
<td>7.6 g soy lecithin Emulpur</td>
<td>Cargill (Mechelen, Belgium)</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
</tr>
<tr>
<td>extra vitamins (reaching 200 % value as compared to the control diet): vitamin A, B6, B12, D2, folic acid</td>
<td></td>
</tr>
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</table>
Table 2  The main effects of ischemia and its modulation by the investigational diet.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>The effects of ischemia</th>
<th>The effects of the investigational diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC vs. CC</td>
<td>SI-post vs. SC</td>
</tr>
<tr>
<td>Bodyweight (day 1 – 21)</td>
<td>~</td>
<td>↓</td>
</tr>
<tr>
<td>Motor activity (cylinder test, day 3)</td>
<td>↓</td>
<td>~</td>
</tr>
<tr>
<td>Infact size growth (day 7 → 21)</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Contralateral glucose metabolism (FDG PET, day 7)</td>
<td>↓</td>
<td>~</td>
</tr>
<tr>
<td>Ipsilateral glucose metabolism (FDG PET, day 7)</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>Astrocyte activation (GFAP staining, day 7)</td>
<td>↑</td>
<td>~</td>
</tr>
<tr>
<td>Astrocyte activation (GFAP staining, day 21)</td>
<td>↑</td>
<td>↓↓</td>
</tr>
</tbody>
</table>
**Figure 1** Study design. Eight experimental groups with three different dietary regimens were subjected to focal cortical ischemia or sham-surgery at experimental day 0 and sacrificed on day 7 (short protocol, A) or day 21 (long protocol, B). The stroke control groups (SC7 and SC21) were fed with the control diet for the whole experiment; the stroke groups receiving pre-ischemic treatment with the investigational diet (SI-post7 and SI-pre21) were fed with the investigational diet from the day of the ischemia (day 0) until the end of the experiment (day 7 or 21, respectively); and the stroke groups receiving pre-ischemic treatment with the investigational diet (SI-pre7 and SI-pre21) were fed with the investigational diet from two weeks before ischemia (day -14) until the end of the experiment (day 7 or 21, respectively). The control groups (CC7 and CC21) were subjected to sham surgery at day 0 and fed with the control diet for the whole experiment. Behavioral tests were performed 8 days before (day -8, baseline) and 3, 6 and 21 days after ischemia or sham-surgery. PET scans were performed 7 days before (day -7, baseline) stroke induction or sham-surgery and on day 7 (groups CC7, SC7, SI-post7 and SI-pre7) or 21 (groups CC21, SC21, SI-post21 and SI-pre21) after stroke or sham-surgery. (on the next page)
Chapter 7

A Short protocol

1. [¹⁸F]FDG PET scan (baseline)
2. Stroke induction/sham surgery
3. [¹⁸F]FDG PET scan
4. Tissue analysis

CC₇
SC₇
SI-post₇
SI-pre₇

Investigational diet

B Long protocol

1. [¹⁸F]FDG PET scan (baseline)
2. Stroke induction/sham surgery
3. [¹⁸F]FDG PET scan
4. Tissue analysis

CC₂₁
SC₂₁
SI-post₂₁
SI-pre₂₁

Investigational diet
**Figure 2** Changes in body weight in animals sacrificed on day 21 after ischemia induction or sham-surgery. Body weight is displayed as percentage of the body weight on the day of ischemia induction or sham-surgery (day 0). CC$_{21}$=control group (n=4), SC$_{21}$=stroke + control diet (n=5), SI-post$_{21}$=stroke + post-ischemic dietary intervention (n=5), SI-pre$_{21}$= stroke + pre-ischemic dietary intervention (n=6). The body weight of the stroke group treated with the investigational diet after stroke induction (SI-post$_{21}$) was significantly lower than all the other groups (CC$_{21}$, SC$_{21}$, SI-pre$_{21}$) at all time points after stroke induction (day 1-21). No significant differences between the CC$_{21}$, SC$_{21}$ and SI-pre$_{21}$ groups were observed. *p<0.05 for SI-post$_{21}$, when compared to CC$_{21}$, SC$_{21}$, SI-pre$_{21}$ (GEE model).
Figure 3 Infarct size on day 7 and 21 after focal ischemia induction. The infarct size was assessed on the isolated brains by measuring the length (alongside Bregma) and width (perpendicularly to Bregma) of the visible scar. Data are displayed as length*width in millimeters. SC₇, SC₂₁=stroke + control diet (n=6, n=5), SI-post₇, SI-post₂₁=stroke + post-ischemic dietary intervention (n=6, n=5), SI-pre₇, SI-pre₂₁=stroke + pre-ischemic dietary intervention (n=6, n=6).
Figure 4 Astrocyte activation. An example of GFAP staining in the cortex surrounding the stroke lesion on day 7 (A) and day 21 (B). Quantification of GFAP staining of astrocyte activation 350 µm from the lesion for groups sacrificed on day 7 (C) and day 21 (D). Astrocyte activation was assessed by the area covered by GFAP staining in the region of interest. The data are displayed as the ratio between the lesion and the contralateral hemisphere, normalized to the values of the corresponding control group (CC7 or CC21). CC7, CC21=control group (n=6, n=4), SC7, SC21=stroke + control diet (n=6, n=5), SI-post7, SI-post21=stroke + post-ischemic dietary intervention (n=6, n=5), SI-pre7, SI-pre21=stroke + pre-ischemic dietary intervention (n=6, n=6). Statistically significant differences are indicated as * p<0.05, ** p<0.01, *** p<0.001.
Figure 5 Differences in brain metabolism caused by ischemia and by the investigational diet in animals sacrificed on day 7. (A): Sagittal, coronal and transverse projections of the brain regions with voxels representing a significant decrease in $[^{18}F]$FDG uptake in animals from SC$_7$ group as compared to the control animals (B), a difference in $[^{18}F]$FDG uptake in animals from SI-post$_7$ group as compared to the SC$_7$ group and (C) an increase in $[^{18}F]$FDG uptake in animals from SI-pre$_7$ group as compared to the SC$_7$. Letters R and L indicate right (the side of ischemia) and left (contralateral to ischemia) hemisphere. CC$_7$=control group (n=6), SC$_7$=stroke + control diet (n=6), SI-post$_7$=stroke + post-ischemic dietary intervention (n=6), SI-pre$_7$= stroke + pre-ischemic dietary intervention (n=6).
Figure 6 Behavioral changes. Rats were subjected to cylinder tests one week before the stroke induction or sham surgery (baseline) and on days 3, 6 and 20 following the ischemia or sham surgery. The graphs represent the percentage usage of the left paw, contralateral to the stroke lesion. CC7, CC21 = control group (n=6, n=4), SC7, SC21 = stroke + control diet (n=6, n=5), SI-post7, SI-post21 = stroke + post-ischemic dietary intervention (n=6, n=5), SI-pre7, SI-pre21 = stroke + pre-ischemic dietary intervention (n=6, n=6). CC = all control animals (12), SC = all surgery + control diet (n=11), SI-post = all stroke + post-ischemic dietary intervention (n=11), SI-pre = all stroke + pre-ischemic dietary intervention (n=12). Significant differences between experimental groups and the CC7 group are indicated by **p<0.01
**Supplementary data**

**Table 1** The clusters with significantly increased (left) or decreased (right) $[^{18}\text{F}]$FDG uptake in the brains of rats subjected to ischemia induction and sacrificed on day 7 (SC$_7$) as compared to the control animals (CC$_7$) or rats that underwent ischemia combined with dietary intervention (SI-post$_7$ and SI-pre$_7$) as compared to rats that received ischemia alone (SC$_7$). The voxels are organized in clusters, which may be spread over (parts of) multiple brain regions (the hemisphere is indicated in brackets). The peak of each cluster (coordinates with highest significant difference) are listed next to the cluster number.

<table>
<thead>
<tr>
<th></th>
<th>INCREASE</th>
<th></th>
<th>DECREASE</th>
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<tbody>
<tr>
<td><strong>Brain regions</strong></td>
<td><strong>Number of voxels</strong></td>
<td><strong>T</strong></td>
<td><strong>Brain regions</strong></td>
<td><strong>Number of voxels</strong></td>
</tr>
<tr>
<td>SC$_7$ versus CC$_7$</td>
<td></td>
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</tr>
<tr>
<td>Cluster 1 (-4.5 0.8 -2.6)</td>
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<tr>
<td>No clusters detected</td>
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<tr>
<td>SC$_7$ versus SI-post$_7$</td>
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<tr>
<td>No clusters detected</td>
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<tr>
<td>SC$_7$ versus SI-pre$_7$</td>
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<tr>
<td>Cluster 1 (2.1 -3.6 -3.4)</td>
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<tr>
<td>Cortex</td>
<td>1462</td>
<td>3.8899</td>
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<tr>
<td>Somatosensory (right)</td>
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<td>±0.4537</td>
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<td>Thalamus (right)</td>
<td>1086</td>
<td>3.9778</td>
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<tr>
<td></td>
<td>238</td>
<td>3.5219</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>±0.6824</td>
<td>±0.1731</td>
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<tr>
<td>Hippocampus (right)</td>
<td>901</td>
<td>4.0447</td>
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<tr>
<td></td>
<td>±0.6333</td>
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<tr>
<td>Corpus callosum (right)</td>
<td>559</td>
<td>4.1271</td>
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<tr>
<td></td>
<td>±0.5715</td>
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<tr>
<td>Visual cortex (right)</td>
<td>305</td>
<td>3.6908</td>
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<tr>
<td></td>
<td>±0.3942</td>
<td></td>
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<tr>
<td>Motor</td>
<td>221</td>
<td>3.5693</td>
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<tr>
<td>Cortex (right)</td>
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<td>±0.2485</td>
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<tr>
<td>Cluster 2 (1.9 –11.0 -6.2)</td>
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<tr>
<td>Medulla (right)</td>
<td>1078</td>
<td>3.7379</td>
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<tr>
<td></td>
<td>±0.3668</td>
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<td>Medulla (left)</td>
<td>1076</td>
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<tr>
<td></td>
<td>±0.2900</td>
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<td>Cerebellum (right)</td>
<td>891</td>
<td>3.7775</td>
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</tr>
<tr>
<td></td>
<td>±0.4588</td>
<td></td>
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</tbody>
</table>
**Supplementary Figure 1** Changes in food intake in animals sacrificed on day 21 after ischemia induction or sham surgery. Food intake is displayed in gram. No statistical differences between the groups were observed (GEE model).
**Supplementary Figure 2** The effect of the investigational diet on $[^{18}\text{F}]$FDG uptake in healthy animals. Sagittal, coronal and transverse projections of the brain regions with voxels representing a significant decrease in $[^{18}\text{F}]$FDG uptake at day -7 in animals fed with investigational diet from day -14 (SI-pre$_7$ and SI-pre$_{21}$ group) versus the animals fed with the control diet (CC$_7$, CC$_{21}$, SC$_7$, SC$_{21}$, SI-post$_7$, SI-post$_{21}$). CC=control group (n=12), SC=stroke + control diet (n=12), SI-post=stroke + post-ischemic dietary intervention (n=12), SI-pre= stroke + pre-ischemic dietary intervention (n=12).

Reference:


Diet intervention in stroke


