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Effect of a multinutrient intervention after ischemic stroke in female C57Bl/6 mice

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Abbreviations used: AD, Alzheimer's disease; AUC, auditory cortex; CBF, cerebral blood flow; CVD, cerebrovascular disease; Cpu+GP, Caudate, Putamen and Globus Pallidus region; DCX, doublecortin; DTI, diffusion tensor imaging; FA, fractional anisotropy; FAIR, flow-sensitive alternating inversion recovery; FC, functional connectivity; GLUT-1, glucose transporter 1; GM, grey matter; HC, hippocampus; IBA-1, ionized calcium-binding adapter molecule 1; MC; motor cortex; MD, mean water diffusivity; OT, optic tract; PPI, prepulse inhibition; ROI, region of interest; rsfMRI, resting state functional MRI; SSC, somatosensory cortex; SYN, synaptophysin; tMCAo, transient middle cerebral artery occlusion; VC, visual cortex; WM, white matter

Abstract

Stroke can affect females very differently from males, and therefore preclinical research on underlying mechanisms and the effects of interventions should not be restricted to male subjects, and treatment strategies for stroke should be tailored to benefit both sexes. Previously, we demonstrated that a multinutrient intervention (Fortasyn) improved impairments after ischemic stroke induction in male C57Bl/6 mice, but the therapeutic potential of this dietary treatment remained to be investigated in females. We now induced a transient middle cerebral artery occlusion (tMCAo) in C57Bl/6 female mice and immediately after surgery switched to either Fortasyn or an isocaloric Control diet. The stroke females performed several behavioral and motor tasks before and after tMCAo and were scanned in an 11.7 Tesla MRI scanner to assess brain perfusion, integrity and functional connectivity. To assess brain plasticity, inflammation and vascular integrity, immunohistochemistry was performed after sacrifice of the mice. We found that the multinutrient intervention had diverse effects on the stroke-induced impairments in females. Similar to previous

observations in male stroke mice, brain integrity, sensorimotor integration and neurogenesis benefitted from Fortasyn, but impairments in activity and motor skills were not improved in female stroke mice. Overall, Fortasyn effects in the stroked females seem more modest in comparison to previously investigated stroked male mice. We suggest that with further optimization of treatment protocols more information on the efficacy of specific interventions in stroked females can be gathered. This in turn will help with the development of (gender-specific) treatment regimens for cerebrovascular diseases such as stroke.

Introduction

Ischemic stroke is the most common type of stroke, accounting for 87% of all strokes (Mozaffarian *et al.* 2016). It may lead to brain lesions with an irreversibly injured core and a peripheral zone (penumbra) containing damaged but potentially salvageable tissue (Astrup *et al.* 1981). Acute symptoms of ischemic stroke can be facial weakness, hemiparesis, hemianopsia, and speech difficulties (Hankey & Blacker 2015), depending on the occluded artery. While patients can (partially) recover from some of these symptoms, demonstrating the capacity of the brain for repair and compensation after injury (Zeiler & Krakauer 2013), up to 40% of stroke patients remain physically disabled (Luengo-Fernandez *et al.* 2013). Ischemic stroke can also result in cognitive and behavioral deficits, as demonstrated by the propensity of stroke patients to develop Alzheimer's disease (AD) (Kalaria 2000). Rather than being the result of damage at the site of the stroke lesion, these cognitive and behavioral deficits seem to be caused by lesions to brain networks (Siegel *et al.* 2016). All of these severe long-term consequences of stroke emphasize the need for effective treatment strategies. Currently, ischemic stroke treatment is mainly aimed at restoring blood flow by thrombolysis (e.g. treatment with tissue plasminogen activator) or thrombectomy and treating motor impairments (Hankey 2017). However, these treatment strategies only have a small therapeutic window to be effective (Lyden 2008) and do not improve the cognitive and behavioral

deficits induced by stroke. Therefore novel treatment strategies, that address both the stroke lesion and affected brain networks, need to be developed.

The food we consume can greatly affect our health. For example, adhering to a so-called Western diet, consisting of large amounts of saturated fatty acids, increases the risk to develop stroke (Foroughi *et al.* 2013) and AD (Hooijmans & Kiliaan 2008). On the other hand, consuming a Mediterranean diet containing, among other things, relatively high amounts of fish, nuts and olive oil is considered beneficial *(Scarmeas et al. 2006)*. As shown in several large double-blind controlled studies like PREDIMED (Estruch *et al.* 2013) and the Northern Manhattan study (Gardener *et al.* 2011), intake of a Mediterranean diet lowers the risk of vascular events such as stroke. Therefore, a diet containing components of the Mediterranean diet such as unsaturated fatty acids, vitamins and polyphenols, may be a valid approach for the prevention and treatment of vascular diseases. A specific multinutrient intervention (Fortasyn) was developed as a therapeutic intervention for early stage AD (van Wijk *et al.* 2014). It consists of several different dietary components facilitating neuronal membrane formation, including fatty acids, phospholipids and vitamins. Indeed, this diet has been shown to protect mouse models with AD-like pathology from neuronal degeneration (Wiesmann *et al.* 2016, Zerbi *et al.* 2014). Interestingly, vascular health also benefited from Fortasyn, as shown by increases in cerebral blood flow (CBF) after treatment. This finding suggests that this specific combination of dietary components may also improve the impairments associated with cerebrovascular diseases (CVD) such as stroke. Recently, we demonstrated the therapeutic potential of the above mentioned multinutrient intervention on ischemic stroke in male C57Bl/6 mice that received a focal ischemic stroke lesion (Wiesmann *et al.* 2017). In these male mice, treatment with Fortasyn improved stroke-induced impairments in functional and structural connectivity, cerebral blood flow and motor function and reduced neuroinflammation. This suggested that this multinutrient intervention may indeed serve as a promising treatment for ischemic stroke patients. However, care has to be taken in generalizing results obtained in males to female subjects, as it is known that responses to treatments can differ between sexes (Soldin & Mattison 2009).

Due to the genetic and biological differences between males and females, stroke impacts women very differently from males (Haast *et al.* 2012). In general, women experience more severe strokes than men. These severe stroke events mostly occur after menopause, suggestive of a protective role of estrogens (Liu *et al.* 2010). Even though traditionally stroke is mostly associated with older age, the number of young adults that experience a stroke event is rising (Kissela *et al.* 2012). In premenopausal women, stroke risk is increased with for example pregnancy, a history of migraines and the use of hormonal contraception (Ekker *et al.* 2016). Personalized treatment strategies that can address stroke both in elderly and young women are therefore required.

In the current study, we aimed to investigate the therapeutic potential of the Fortasyn diet in female stroke mice. We used the same experimental set-up as the previous performed study in male C57Bl/6 mice (Wiesmann et al. 2017). In short, we induced a focal ischemic stroke in 3-4 month old C57Bl/6 female mice by transiently occluding the middle cerebral artery (tMCAo) for 30 minutes and subsequently feeding them either the Fortasyn diet or an isocaloric Control diet. Using state-of-the-art imaging techniques and behavioral tasks, we then investigated the therapeutic effect of the multinutrient intervention on brain structure and function, cerebral blood flow, behavior and motor skills. Gathering more knowledge on the effect of this dietary treatment on stroke in female mice will help with the development of gender-specific treatment regimens for CVD.

Material and methods

Animals

A randomized double-blind controlled study using 24 female C57BL/6JRj mice (Harlan Laboratories Inc., Horst, the Netherlands; 2-3 months old) was conducted at the preclinical imaging center (PRIME) of the Radboud university medical center (Radboudumc) (Nijmegen, the Netherlands). Before tMCAo, the animals were group-housed (four animals per cage) in enriched, individually ventilated cages. Standard food pellets (Ssniff rm/h V1534-000, Bio Services, Uden, The Netherlands)

and autoclaved water were available *ad libitum*. In the room where the mice were kept, there was an artificial 12 hour light-dark cycle (lights on at 7 a.m.), humidity control and background music. Room temperature was kept constant at 21 ± 1°C. After tMCAo, the mice were housed separately to monitor intake of the experimental diets by each individual mouse. Experiments were performed according to Dutch federal regulations for animal protection and the European Union Directive of 22 September 2010 (2010/63/EU). They were approved and pre-registered by the Animal Ethics Committee (called the Dierexperimentencommissie or DEC, RU-DEC 2014-171) of the Radboudumc (Nijmegen, The Netherlands). Furthermore, our experiments were performed according to the (updated) recommendations made by the Stroke Therapy Academic Industry Roundtable (STAIR) for the preclinical development of therapies for ischemic stroke (Fisher *et al.* 2009, Stroke Therapy Academic Industry *et al.* 1999) and ARRIVE guidelines (Kilkenny *et al.* 2010). All applicable international, national, and institutional guidelines for the care and use of animals were followed. Our study was also in concurrence with the European regulations on ethics and responsible conduct regarding scientific communication. All behavioural and MRI experiments were performed in the Preclinical Imaging Centre (PRIME) of the Radboudumc between 8 a.m. and 6 p.m..

Transient Middle Cerebral Artery Occlusion (tMCAo)

At 3-4 months of age, all mice underwent transient (30 minutes) occlusion of the right middle cerebral artery (tMCAo), thus mimicking one of the most common types of ischemic stroke in patients (Endres & Dirnagl 2002, Engel *et al.* 2011). The intraluminal occlusion model was performed as described elsewhere with minor modifications (Engel et al. 2011, Zinnhardt *et al.* 2015). To ensure comparability to our previous study (Wiesmann et al. 2017), the exact same procedures were followed and all surgeries were performed by the same experienced researcher that performed the surgeries on the male mice (co-author D.R.). In short, mice were anesthetized with 1.5% isoflurane (Abbott Animal Health, Abbott Park, IL, USA) in a 2:1 air and oxygen mixture and were kept under

anesthesia during the total time of the surgery. Next, a 7-0 monofilament (tip diameter 190 to 200 µm, coating length 2 to 3 mm, 70SPRePK5, Doccol Corp., Sharon, MA, USA) was inserted in the right common carotid artery and positioned at the point where the middle cerebral artery branches out. A Laser Doppler probe (moorVMS-LDF2, Moor Instruments, UK) placed on the skull of the mice monitored CBF to assess the efficacy of the occlusion (≥80% loss of CBF). The middle cerebral artery was occluded for 30 minutes before retracting the filament allowing for reperfusion. After surgery, mice were carefully monitored to assess pain or other discomforts. Mice were weighed at least once a week. Exclusion criteria were decreased motor activity (<50% of the baseline measurements combined from the baseline values of each behavioral test) or extreme weight loss (>20% within three consecutive days). Lesion size was comparable in all animals and no dietary effect on lesion size could be detected as measured with a T2-weighted RARE sequence (data not shown). None of the mice died during or after tMCAo or reached the aforementioned exclusion criteria.

Group allocation and diets

Immediately after tMCAo, using a random sequence generator mice were randomly divided in two experimental groups, with one group being fed the Fortasyn diet (n=12) and the other group receiving an isocaloric Control diet (n=12). Group sizes were calculated based on the effect sizes (Type I error: 0.05, statistical power: 0.80), exclusion and mortality rates determined in our previous study (Wiesmann et al. 2017).

Both the Fortasyn and the Control diet were based on AIN-93M (Reeves *et al.* 1993) with 5% fat, but differed with respect to their fatty acid composition and some additional nutrients. The Fortasyn diet contained 0.1% coconut oil, 1.9% corn oil and 3.0% fish oil, while the Control diet contained 1.9% soy oil, 0.9% coconut oil and 2.2% corn oil. Furthermore, the Fortasyn diet contained a specific multi-nutrient composition comprising uridine, omega-3 PUFAs, choline, B vitamins,

phospholipids and antioxidants (the specific composition is specified in (Wiesmann et al. 2017). Both diets were manufactured and pelleted by Ssniff (Soest, Germany) and stored at −20°C until use. In the current study, the same batches of the diets as given to the mice in our previous study (Wiesmann et al. 2017) were used. Food intake was measured two times a week to calculate the average daily food intake per mouse.

Behavior, cognition, and motor tasks

All imaging and behavioral procedures and all data analyses were performed by researchers that were blinded to the treatment conditions. One mouse of the Fortasyn group was excluded from all of the experiments described below due to an enlarged kidney, leaving n=12 in the Control group and n=11 in the Fortasyn group. During the behavioral training/ acclimatization before the tMCAo all mice were trained on, and acclimatized to the testing equipment of both the grip strength test and the pole test. Resembling the actual experimental post-stroke situation all mice performed before the tMCAo only five consecutive trials for the grip strength test (5 for trapeze/ forepaws + 5 for grid/ all four paws) and also five consecutive trials for the pole test. The design of this study is illustrated in Figure 1.

Open field

Locomotion and explorative behavior were evaluated for 10 minutes in a square open field (45 x 45 x 30 cm) prior to the tMCAo and diet switch (pre-stroke) and 3 and 21 days post-stroke as previously described. Using EthoVision XT10.1 (Noldus, Wageningen, The Netherlands), locomotion was automatically recorded. The floor of the arena was divided into center, periphery, and corners. The frequency of entering these zones was measured automatically. In addition, exploratory behavior was manually scored (walking, sitting, wall leaning, rearing and grooming) and analyzed as

previously described (Streijger *et al.* 2005). One mouse of the Fortasyn group was considered an outlier in the baseline measurements (i.e. hyperactive mouse) and was therefore excluded from the open field analyses.

Grip strength test

The grip strength test was performed prior to the tMCAo and diet switch (pre-stroke) and 14 and 27 days post-stroke using a grip strength meter (Grip-Strength Meter, 47200, Ugo Basile, Italy) as previously described (Wiesmann et al. 2017). Muscle strength in the forelimbs (trapeze) and in all four limbs (grid) was determined. Trials were the mouse grasped the trapeze with one forelimb or grasped the grid with less than four limbs were excluded from the analyses. One mouse of the Fortasyn group was excluded from the analyses as pre-stroke it did not demonstrate a trapeze pull with both forelimbs. For both the trapeze and the grid, the maximum value of the peak force (in gf) was averaged per experimental group.

Pole test

The pole test was performed prior to the tMCAo and diet switch (pre-stroke) and 15 days post-stroke as previously described (Wiesmann et al. 2017). The time needed to turn completely downward was measured manually, while the time to reach the floor was determined with EthoVision XT10.1 (Noldus, Wageningen, The Netherlands) and used to determine downward velocity (cm/s). The first trial of each pole test was excluded from the statistical analyses (acclimatization). Furthermore, trials where a mouse was turning on the pole before being released by the researcher were excluded and each mouse had to have at least three successful trials. Based on these criteria, three mice of the Control group and one mouse of the Fortasyn group were excluded from the pole test analyses. Furthermore, the turning time of four additional mice (2 of the

Control group and 2 of the Fortasyn group) could not be determined (e.g. the mouse walked over the top of the pole). These four mice were included in the downward velocity analyses.

Prepulse inhibition (PPI)

To examine sensorimotor integration, PPI was examined 16 days after stroke induction as previously described (Janssen *et al.* 2015). The startle reactivity of the mice was measured in the SR-LAB startle response system (San Diego Instruments, San Diego, CA, USA). PPI of the mouse was calculated during the second block of startle pulses (PPI (%) = 100x (response to startle pulse – response to startle pulse after a prepulse) / response to startle pulse). Furthermore, habituation to 120 dB startle pulses (given without prepulses) was examined by comparing the responses to startle pulses (in arbitrary units) of block 1 to block 3 (Streijger et al. 2005). Two mice of the Control group were considered statistical outliers (i.e. high response) in the no stimulus and 120dB startle pulse trials respectively and were therefore excluded from the PPI analyses.

Novel object recognition test (ORT)

All mice performed the ORT to measure short term memory 22 days (first acquisition day; 30 min delay) and 23 (second acquisition day; 60 min delay) days post-stroke as previously described (supplemental (Wiesmann et al. 2017). Using EthoVision XT10.1 (Noldus, Wageningen, The Netherlands), a recognition index (i.e. the time spent examining the novel object (N) minus the time spent examining the familiar object (F) in the test trial) was determined to assess recognition memory. Mice were excluded from the analyses when they either demonstrated a preference for one of the objects in the familiarization trials (>75% of time spend on one object) or did not explore either N or F in the test trial. One mouse of the Fortasyn group was considered an outlier in the baseline measurements (i.e. hyperactive mouse) and was therefore excluded from the ORT analyses.

MRI protocol

MRI measurements were performed at 7 and 35 days post-stroke on a 11.7 T BioSpec Avance III small animal MR system (Bruker BioSpin, Ettlingen, Germany) operating on Paravision 6.0.1 software platform (Bruker, Karlsruhe, Germany) as previously described (Wiesmann et al. 2017, Zerbi et al. 2014). In short, mice were anesthetized with isoflurane (Abbott Animal Health; 3.5% for induction and 1.8% for maintenance) in a 2:1 oxygen and N_2O mixture and placed in a stereotactic holder. Body temperature was maintained at 37˚C and respiration was monitored. Mice were excluded from MRI analyses if their scans showed motion and/or echo planar imaging artifacts. Supplementary table 1 contains a list of parameters used in each MRI scan.

Cerebral blood flow (CBF)

To assess CBF, MR perfusion data were acquired under resting conditions using a flow-sensitive alternating inversion recovery (FAIR) technique as previously described (Wiesmann et al. 2017, Zerbi et al. 2014). In short, three regions of interest (ROI; cerebral cortex, hippocampus, and thalamus according to the atlas of Paxinos and Franklin (Paxinos & Franklin 2008)) were analyzed by two researchers that were blinded to the treatment conditions. CBF was then analyzed for each ROI in both the unaffected (contralateral/ left) and affected (ipsilateral/ right) hemisphere separately for each dietary group. The loss of CBF in the affected ROI was calculated as the difference in CBF between right and left hemispheric ROI relative to left hemispheric ROI. One mouse of the Control group and also one mouse of the Fortasyn group were excluded from analysis.

Diffusion tensor imaging (DTI)

To assess the integrity of white matter (WM) and grey matter (GM), DTI was performed as previously described (Zerbi et al. 2014, Wiesmann et al. 2017). Mean water diffusivity (MD) and fractional anisotropy (FA) were derived from the tensor estimation following a protocol as described elsewhere (Zerbi *et al.* 2013). MD is considered an inverse measure of membrane density and is sensitive to changes in GM, while FA is an estimate of myelination and fiber density in WM (Le Bihan *et al.* 2001, Alexander *et al.* 2011, Feldman *et al.* 2010). MD and FA values were measured in several WM and GM areas that were manually selected based on an anatomical atlas (Paxinos & Franklin 2008). No mice were excluded from analysis.

Resting state functional MRI (rsfMRI)

To assess patterns of functional connectivity, rsfMRI was determined as previously described (Wiesmann et al. 2017), see the supplementary material for a detailed description. Based on a previous functional connectivity study in mice (Jonckers *et al.* 2011) seven brain areas (bilateral) were selected for analysis. These areas were: the dorsal hippocampus, the ventral hippocampus, the auditory cortex, the motor cortex, the somatosensory cortex and the visual cortex. Three mice of the Control group and also three mice of the Fortasyn group were excluded from analysis.

Immunohistochemistry

Immediately following the MRI measurements performed 35 days after the stroke, the mice were sacrificed by transcardial perfusion using 4% paraformaldehyde (PFA) solution as previously described (Wiesmann et al. 2017). The brains were collected and separated into two parts. The frontal part of the brain (Bregma: -0.10 to 0.98) was post-fixed in 4% PFA, embedded in paraffin and

sliced into 5 µm sections. To measure presynaptic density, a staining for synaptophysin (SYN) was performed using monoclonal rabbit anti-synaptophysin clone EP1098Y (1:250; Abcam Inc., Cambridge, UK). The secondary antibody used was donkey anti-rabbit biotin (1:200; Jackson ImmunoResearch). Quantification of selected stained sections (Bregma: 0.02 to 0.62) was performed as previously described (Wiesmann et al. 2017).

After a 24 hour incubation in a 30% sucrose solution, the other part of the brain (Bregma: -0.7 to -4.36) was cut in 30 µm frontal sections using a sliding microtome (Microm HC 440, Walldorf, Germany) equipped with an object table for freeze-sectioning at -60°C. These free-floating sections were used to assess a) vascular integrity, measured with an antibody against glucose transporter-1 (GLUT-1), b) neuroinflammation measured with an antibody against ionized calcium-binding adapter molecule 1 (IBA-1) and c) immature neurons (i.e. neurogenesis) measured with an antibody against doublecortin (DCX). For GLUT-1, a polyclonal rabbit anti-GLUT-1 primary antibody (1:80.000, Chemicon AB 1340, Chemicon International, Inc., Temecula, CA, USA) and a donkey anti-rabbit biotin secondary antibody (1:1500 Jackson ImmunoResearch, West Grove,PA, USA) was used. The primary antibodies used for IBA-1 and DCX were: polyclonal goat anti-IBA-1 (1:3000; Abcam) and polyclonal goat anti-DCX (1:8000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). For both these proteins, donkey anti-goat biotin (1:1500; Jackson ImmunoResearch, West Grove, PA, USA) was used as a secondary antibody. Quantification of selected stained sections (Bregma: -1.46 to -2.30) was performed as previously described (Wiesmann et al. 2017). In short, the percentage of stained area (relative to unstained tissue) was determined using ImageJ software (National Institute of Health, Bethesda, MD, USA). Additionally, the number of DCX+ and IBA1+ cells and the number of GLUT-1+ blood vessels was manually counted in selected brain areas. All mice were included for all IHC procedures.

Statistical analysis

For the statistical analysis of outliers and data, IBM SPSS 22 software (IBM Corporation, New York, NY, USA) was used. A multivariate ANOVA, univariate ANOVA or repeated measures ANOVA with Bonferroni corrections was conducted with between-group-factor diet and, if necessary, time and left-to-right-hemisphere-differences to analyze possible differences. Statistical significance was set at $p<0.05$, with $p<0.08$ considered a trend. Degrees of freedoms, F-values and p-values are given for each statistical analysis. All data are expressed as mean ± SEM.

Results

Body weight, diet intake, and estrous cycle

To assess the well-being of the mice and monitor intake of the diets, body weight and diet intake were measured every week. Before stroke and subsequent diet switch, the average body weight of the mice that were randomly allocated to the Control diet group (21.3 ± 0.30 gr) was lower ($F(1,21=4.7$, $p=0.042$) than the body weight of the mice that were going to receive the Fortasyn diet (22.2 ± 0.28 gr; *data not shown*). To correct for this baseline difference, body weight differences after stroke and diet switch were all determined relative to pre-stroke weight (*Supplementary Figure 1A*). Compared to baseline, the body weight of the mice decreased in the first week after stroke induction and diet switch (-16.1 ± 1.6%; $F(1,21)=108.4$, $p<0.001$). In the next two weeks, body weight of all mice increased with 9.22 ± 0.87 % ($F(1,21)=113.5$, $p<0.001$) relative to their weight in the first week after stroke and diet switch. In weeks 3 to 5 after the stroke, the body weight of all mice stabilized. Five weeks after the stroke and diet switch, body weight was 5.89 ± 1.3% lower than pre-stroke body weight ($F(1,21)=19.9$, $p<0.001$). There was no diet effect on body weight.

Food intake was monitored after stroke and diet switch (*Supplementary Figure 1B*). In the first week after stroke and diet switch, mice on Fortasyn diet had a higher average daily food intake than the mice on control diet (3.14 ± 0.09 vs. 2.52 ± 0.08 gr/day; $F(1,21)=5.3$, $p=0.032$). Average daily food intake of both diet groups increased in the following week ($F(1,21)=16.4$, $p=0.001$), followed by a decrease in week 3 ($F(1,21)=21.4$, $p<0.001$). In weeks 3 to 5 after the stroke, average daily food intake of all mice stabilized. There were no differences in average daily food intake between Control diet and Fortasyn diet after the first week.

35 days after the stroke the stage of the estrous cycle of each individual mouse was evaluated by visual observation based on (Byers et al. 2012). A digital image of each mouse was taken. Each experimental mouse was in the diestrus stage of the estrous cycle.

Behavior, cognition and motor tasks

Open field

To measure activity, anxiety and explorative behavior, a 10 minute open field test was performed before stroke (pre-stroke baseline) and 3 and 21 days after the stroke and subsequent diet switch (*Figure 2*). Compared to baseline, mice on both diets moved a shorter distance 3 days after stroke (*Figure 2A*: $F(1,20)=46.8$, $p<0.001$) and had a lower velocity ($F(1,20)=46.7$, $p<0.001$; *data not shown*). Both parameters remained decreased 21 days after the stroke (distance: $F(1,20)=0.920$, $p=0.349$; velocity: $F(1,20)=0.924$, $p=0.348$). There was no diet effect on these parameters at 3 or 21 days after the stroke.

The frequency of performing different types of (exploratory) behavior was manually scored (*Figure 2B*). Three days after the stroke and subsequent diet switch, the frequencies of walking ($F(1,20)=23.0$, $p<0.001$), wall leaning ($F(1,20)=33.2$, $p<0.001$; *data not shown*) and rearing ($F(1,20)=27.1$, $p<0.001$) decreased versus pre-stroke, while the frequency of sitting increased

(F(1,20)=96.3, p<0.001). Grooming frequency was initially unchanged (F(1,20)=2.59, p=0.123; *data not shown*), but decreased from 3 to 21 days after stroke (F(1,20)=5.66, p=0.029; *data not shown*). While between 3 and 21 days after stroke there was no change in sitting frequency (F(1,20)=1.11, p=0.305) or wall leaning frequency (F(1,20)=0.011, p=0.918; *data not shown*), walking frequency (F(1,20)=6.48, p=0.019) and rearing frequency (F(1,20)=40.0, p<0.001) decreased further. Three days after stroke and diet switch, mice on Fortasyn diet reared more frequent than mice on control diet (F(1,20)=7.1, p=0.015). There were no other effects of diet within these parameters.

To assess anxiety and exploration, the position of the mice in the open field (center, corners, periphery) was determined (*Figure 2C and 2D*). Three days after the stroke, all mice visited the corners (F(1,20)=13.0, p=0.002), periphery (F(1,20)=26.0, p<0.001) and center (F(1,20)=19.1, p<0.001) of the open field less frequently than before the stroke (*Figure 2C*). Time spent in the center and periphery decreased (*Figure 2D:* F(1,20)=7.33, p=0.014 and F(1,20)=12.9, p=0.002 respectively) for all mice compared to pre-stroke (while time spent in the corners increased (F(1,20)=20.7, p<0.001). Three weeks (21 days) after stroke, the number of visits to the center decreased further (*Figure 2C*: F(1,20)=6.4, p=0.020). There was no change in the number of visits to the corners (F(1,20)=0.10, p= 0.920) or periphery (F(1,20)=1.22, p=0.282) compared to 3 days after the stroke. The time spent in the corners did increase further for all mice (*Figure 2D:* F(1,20)=11.7, p=0.003) compared to 3 days after stroke, while time spent in the periphery decreased further (F(1,20)=8.49, p=0.009). Time spent in the center slightly tended to decrease (F(1,20)=3.56, p=0.074). There was no effect of diet on the positions within the open field at either time point after stroke. Before stroke induction, the mice that would receive Fortasyn spend less time in the corners than the mice that would receive Control diet (*Figure 2D:* F(1,20)=4.57, p=0.045).

Grip strength test

The grip strength of the mice was tested before stroke induction (pre-stroke baseline) and 14 and 27 days after stroke and subsequent diet switch (*Figure 3A*). Mice had to repeatedly pull either a trapeze with their forepaws or a grid with all four paws and the maximum grip strength (in gram-force) was recorded. Two weeks after the stroke, maximum limb strength on the grid of all mice was lower than at baseline ($F(1,20)=7.71$, $p=0.012$), while maximum strength on the trapeze was unaltered ($F(1,20)=0.977$, $p=0.335$). There was no change in strength when comparing 14 days and 27 days after the stroke (trapeze: $F(1,20)=0.04$, $p=0.851$; grid: $F(1,20)=0.71$, $p=0.411$) nor was there any diet effect.

Pole test

Motor coordination was determined 15 days after the stroke and subsequent diet switch (*Figure 3B*). Mice were repeatedly placed on a pole facing upwards and the time needed to turn around its body axis and the speed of the mouse while descending the pole were measured. Diet had no effect on either parameter.

Prepulse inhibition (PPI)

To assess sensorimotor integration, a PPI test with 4 different, randomly administered prepulses (PP2, PP4, PP8 and PP16) was performed 16 days after stroke and subsequent diet switch (*Figure 3C and 3D*). In this test, mice that received the Fortasyn diet tended to have a higher PPI than Control animals when a prepulse that was 4dB louder than background noise was given (*Figure 3C:* PP4: 40.7 ± 5.0% and 23.2 ± 7.2% respectively; $F(1,19)=4.1$, $p=0.056$). There was no other diet effect on PPI.

Fortasyn mice, but not Control mice (F(1,9)=0.55, *p*=0.477), habituated to the 120 dB pulses, as demonstrated by a reduction in the startle response triggered by these pulses from the beginning (12.5 ± 1.4) to the end (9.9 ± 0.9) of the test (*Figure 3D:* F(1,10)=5.5, *p*=0.04).

Novel object recognition test

To assess cognitive function, 22 and 23 days after stroke induction and diet switch a novel object recognition test was performed with a 30 minute or 60 minute intertrial interval respectively. There was no effect of diet on recognition memory (*data not shown*).

MRI measurements

Cerebral blood flow (CBF)

To assess stroke and diet effects on brain perfusion, CBF was measured in three brain regions (in both left and right hemisphere) 7 and 35 days after the stroke and subsequent diet switch (*Figure 4*). Seven days after the stroke, CBF in the right cortex (Control: F(1,20)=4.4, *p*=0.050; Fortasyn: F(1,18)=15.6, *p*=0.001), right hippocampus (Control: F(1,20)=43.2, *p*<0.001; Fortasyn: F(1,18)=83.3, *p*<0.001), and right thalamus (Fortasyn: F(1,18)=11.5, *p*=0.003) was lower than in the corresponding left ROI (*Figure 4A*). There was no difference between the diets at this time point.

Thirty-five days post-stroke (*Figure 4A*), mice on both diets demonstrated lower CBF in the right hippocampus (Control: F(1,20)=30.4, *p*<0.001); Fortasyn: F(1,18)=34.5, p<0.001) compared to the left hippocampus. Additionally, mice on Fortasyn had a lower CBF in the right cortex (F(1,18)=4.9, p=0.041) and in the right thalamus (F(1,18)=8.4, p=0.010) compared to the corresponding left ROI. Fortasyn mice tended to have a higher CBF (243.8 ± 11.5) than Control mice (211.2 ± 11.0) in the left thalamus (F(1,19)=4.2, *p*=0.05).

Over time (35 days vs. 7 days post-stroke), mice on Fortasyn diet showed an increase in CBF in the right hippocampus (F(1,9)=9.9, p=0.012) and in the right cortex (F(1,9)=4.2, p=0.072) (*Figure 4A*). These effects were not seen in mice on Control diet. In both diet groups, the CBF difference between the right and left hippocampus, i.e. loss of CBF, decreased (*Figure 4C:* Control, F(1,10)=8.7, p=0.015; Fortasyn, F(1,9)=37.0, p<0.001) over time. A similar decrease over time was also revealed in the thalamus of Fortasyn mice (F(1,9)=17.2, p=0.002), but not significant in the cortex of Control mice (F(1,10)=3.8, p=0.080).

Diffusion tensor imaging (DTI)

To assess brain integrity, quantitative assessment of diffusion tensor derived indices (fractional anisotropy, FA, *Figure 5A*; and mean diffusivity, MD, *Figure 5B*) was performed for nine white and gray matter regions 7 and 35 days after stroke and diet switch.

Fractional anisotropy (FA)

Seven days after stroke (*Figure 5A*), mice had a lower FA in the right caudate putamen and globus pallidus region (Cpu+GP; Control: F(1,22)=4.7, p=0.042; Fortasyn: F(1,20)=7.9, p=0.011), right hippocampus (HC; Control: F(1,22)=32.0, p<0.001; Fortasyn: F(1,20)=22.5, p<0.001), and right motor cortex (MC; Control: F(1,22)=4.9, p=0.038; Fortasyn: F(1,20)=5.0, p=0.038) than in the corresponding left ROI. Furthermore, at this time point Fortasyn mice tended to have a lower FA in the right visual cortex (VC; F(1,20)=4.3, p<0.051) compared to the left VC. At this time point, there were no effects of diet in either the left or right hemisphere.

Thirty-five days after the stroke (*Figure 5A*), mice had a lower FA in the right Cpu+GP (Control: F(1,22)=23.1, p<0.001; Fortasyn: F(1,20)=7.6, p=0.012) and right HC (Control: F(1,22)=37.6, p<0.001; Fortasyn: F(1,20)=5.5, p=0.030) than in the corresponding left ROI. Additionally, Control mice demonstrated a trend towards a lower FA in the right VC (F(1,22)=3.5, p=0.074) compared to left VC. At this time point after stroke, Fortasyn mice had a higher FA in the right Cpu+GP (F(1,21)=4.7, p=0.041) and in the right HC (F(1,21)=7.0, p=0.015) compared to Control mice. Furthermore, Fortasyn mice tended to have a higher FA in the left Cpu+GP (F(1,21)=3.5, p=0.077) and in the left HC (F(1,21)=3.4, p=0.079) than Control mice. There were no other differences between the right and left ROI in any of the other regions examined.

We also investigated FA differences over time from 7 days to 35 days post-stroke (*Figure 5A*). Control mice showed a decrease in FA over time in both the left (F(1,11)=39.6, p<.001) and right (F(1,11)=57.1, p<0.001) auditory cortex (AUC), left (F(1,11)=34.2, p<0.001) and right HC (F(1,11)=4.9, p=0.05), left MC (F(1,11)=4.1, p=0.068), the left (F(1,11)=16.9, p=0.002) and right somatosensory cortex (SSC; F(1,11)=11.5, p=0.006) and in the left (F(1,11)=10.0, p=0.009) and right VC (F(1,11)=12.5, p=0.005). Furthermore, Control mice demonstrated an increase in FA over time on both the left (F(1,11)=71.0, p<0.001) and right optic tract (OT; F(1,11)=40.8, p<0.001). Except for a tendency to an increase in FA in the left OT (F(1,10)=4.0, p=0.075), Fortasyn mice showed no FA changes over time.

Mean diffusivity (MD)

Seven days after stroke (*Figure 5B*), mice had a higher MD in the right OT (Control: F(1,22)=6.6, p=0.018; Fortasyn: F(1,20)=9.1, p=0.007) compared to the left OT. Furthermore, in Control mice, but not Fortasyn mice, MD in the right HC was higher than in the left HC (F(1,22)=5.0, p=0.036). There were no other left-right differences at this time point nor were there diet effects present.

Thirty-five days after the stroke (*Figure 5B*), MD of mice on Control diet was higher in the right AUC ($F(1,22)=10.1$, $p=0.004$), in the right HC ($F(1,22)=4.4$, $p=0.047$), and tended to be higher in the right Cpu+GP ($F(1,22)=3.5$, $p=0.074$) and compared to the corresponding left ROI. At this time point, mice on Fortasyn diet did not show any differences in MD between any of the left and right ROI. The Fortasyn mice did exhibit a decreased MD in the left HC ($F(1,21)=5.0$, $p=0.037$) and right OT ($F(1,21)=4.8$, $p=0.040$) compared to Control mice.

We also investigated MD differences over time from 7 to 35 days post-stroke (*Figure 5B*). Control mice showed an increase in MD over time in the fornix ($F(1,11)=8.5$, $p=0.014$), left HC ($F(1,11)=8.7$, $p=0.013$), and both left ($F(1,11)=24.8$, $p<0.001$) and right OT ($F(1,11)=9.6$, $p=0.010$). Fortasyn mice only demonstrated an increase over time in the MD of the left OT ($F(1,10)=14.4$, $p=0.003$).

Resting state fMRI (rsfMRI)

To compare functional connectivity (FC) patterns, 7 and 35 days after stroke and diet switch total and partial rsfMRI correlations were determined for six brain regions. With total correlations, no significant diet nor time effects could be detected (*data not shown*). Partial correlation analysis (*Figure 5C*), accentuating the direct connectivity between two ROI while regressing the temporal BOLD signal from all other ROI, revealed significant time effects in mice on both diets (*Figure 5E*). Mice on Control diet demonstrated lower FC with time between left VC and left ventral HC ($F(1,20)=7.6$, $p=0.012$) and between right VC and right SSC ($F(1,20)=4.9$, $p=0.039$). Furthermore, in these Control mice FC between right dorsal HC and right ventral HC ($F(1,20)=9.3$, $p=0.006$), between right dorsal HC and left AUC ($F(1,20)=5.1$, $p=0.035$), and between left MC and right MC ($F(1,20)=12.8$, $p=0.002$) was increased over time. On Fortasyn diet, FC was decreased with time between left SSC and left ventral HC ($F(1,18)=15.1$, $p=0.001$) and between right SSC and right ventral HC ($F(1,18)=5.0$,

p=0.039). Fortasyn mice had a higher FC between left and right MC (F(1,18)=6.2, *p*=0.023), between left dorsal HC and left MC (F(1,18)=5.4, *p*=0.031), and between left AUC and right MC (F(1,18)=5.0, *p*=0.039) with time. Overall, FC showed a similar amount of increases and decreases with time in both diets. Fortasyn mice did show a higher FC between right AUC and left MC (F(1,15)=17.2, *p*=0.001) than Control mice 35 days after stroke and diet switch (*Figure 5D*).

Immunohistochemistry

After sacrifice of the mice, immunohistochemical stainings were performed on selected brain areas to assess neurogenesis (DCX), vascular density (GLUT-1), neuroinflammation (IBA-1) and presynaptic density (SYN). No left-right differences were detected in any of the stainings (*Figure 6*).

Compared to Control mice, Fortasyn mice had a larger DCX+ area in the subventricular zone (*Figure 6A:* F(1,42)=4.1, *p*=0.050) while the number of DCX+ neurons in the hippocampus did not differ (*data not shown*). Fortasyn fed mice had both a larger relative IBA-1+ area (*Figure 6E:* F(1,42)=6.1, *p*=0.018) and more IBA-1+ cells than Control fed mice (*Figure 6D:* F(1,42)=4.2, *p*=0.046) in the cortex. In the hippocampus and thalamus, no diet effects on these parameters were detected (*data not shown*). In the hippocampus, Fortasyn mice furthermore exhibited a lowered vascular density (i.e. number of Glut-1+ vessels) compared to Control fed mice (*Figure 6H:* F(1,42)=6.5, *p*=0.014). No diet effects were found for the number of Glut-1+ vessels in the cortex and thalamus or the relative Glut-1+ area in any of the assessed ROI (*data not shown*). Finally, relative SYN+ area did not differ between the diet groups (*data not shown*).

Discussion

In search for much-needed novel therapies for stroke, interventions with dietary components appear promising (Aquilani *et al.* 2011, Belayev *et al.* 2011, Schwarzkopf *et al.* 2015). Indeed, previously we determined that a multinutrient intervention (Fortasyn) first developed for use in early AD could improve brain perfusion, brain connectivity and motor function after the induction of a transient experimental focal ischemic stroke in male C57Bl/6 mice (Wiesmann et al. 2017). As it may be expected that female mice respond differently to treatment than males (Soldin & Mattison 2009), in the current study we further examined the therapeutic potential of this multinutrient intervention in female mice. As far as we know, we are the first to treat stroke females with Fortasyn. In fact, only a small percentage of preclinical studies investigating mechanisms or interventions of stroke have been performed using female rodents (Beery & Zucker 2011) and, with few exceptions (e.g. (Janickova *et al.* 2015, Broersen *et al.* 2013)), most of the preclinical studies tested the efficacy of Fortasyn in males (Cansev *et al.* 2015, de Wilde *et al.* 2011, Jansen *et al.* 2013, Jansen *et al.* 2014, Wiesmann *et al.* 2013, Wiesmann et al. 2016, Wiesmann et al. 2017, Zerbi et al. 2014). In our stroke females, the induction of a focal ischemic lesion by tMCAo impaired the activity and motor skills of the mice and affected the perfusion, integrity and connectivity of the brain. The effects of Fortasyn on stroke-induced impairments in the females were diverse. Fortasyn improved brain integrity and neurogenesis and, to some extent, CBF and brain connectivity, but did not affect deficits in activity or motor skills. Increased inflammation and decreased vascular density in the Fortasyn-treated stroke females need further study for a proper interpretation. Sensorimotor integration, as assessed in the PPI test, however did seem to benefit from Fortasyn in these females, while no effects were previously observed in males (Wiesmann et al. 2017).

Cerebral hemodynamics

In the male stroke mice, Fortasyn improved cerebral perfusion (Wiesmann et al. 2017), which is in line with the beneficial effects of Fortasyn on CBF seen in our previous studies using mouse models for AD (Wiesmann et al. 2016, Zerbi et al. 2014). In line with the previous studies, Fortasyn did also increase CBF in the stroke females with time in individual brain areas such as the right cortex and right hippocampus. However, different from the Fortasyn stroke males (Wiesmann et al. 2017) left-right differences in CBF in the female Fortasyn mice did not totally recover with time. Therefore, it seems as though the cerebral blood flow in stroked females did not benefit from Fortasyn to the same extent as it did in the male stroke mice and it remains to be investigated why the stroke females on Fortasyn did not show at least a similar recovery. Future studies should therefore investigate also the impact of such a multinutrient intervention on possible mechanisms (e.g. smooth muscle cell behavior) being involved in the cerebral vasoreactivity.

Brain integrity, sensorimotor integration, and functional connectivity

Stroke effects on WM and GM integrity are clearly present in the females. Indeed, these stroke effects seem more extensive than the stroke effects on brain integrity seen in the males (Wiesmann et al. 2017), i.e. a greater number of brain areas demonstrate ipsilateral WM and GM integrity impairments in the females. This finding seems in contrast to the well-known fact that specifically in women estrogens should be able to reduce stroke-induced brain damage (Liu et al. 2010). A study simultaneously examining stroke effects in both male and female mice may resolve this discrepancy.

Fortasyn seems to inhibit the progressive impairment of WM and GM microstructure that was demonstrated in the female stroke mice fed the Control diet. Furthermore, in several brain areas, WM and GM integrity of the Fortasyn mice was higher than in the Control mice. These beneficial effects of Fortasyn are in line with previous studies testing the effect of (components of) this multinutrient intervention on brain integrity (Wiesmann et al. 2016, Jiang *et al.* 2016) and they

are furthermore supportive of the membrane synthesizing potential of this particular multinutrient intervention (van Wijk et al. 2014). Up to now, mechanisms are still unknown how tissue integrity could be influenced by dietary components like omega-3 fatty acids. Notably, investigating T2-values to detect changes in brain membrane composition, Hirashima et al. demonstrated in women with bipolar disorder that omega-3 fatty acid supplementation for 4 weeks lowered T2 values indicating a treatment-induced increase in membrane fluidity (Hirashima et al. 2004). This result could help us to understand the beneficial also treatment-induced effects on WM and GM microstructure being found in our study. As stroke-induced WM impairments can cause sensorimotor impairments (Wang *et al.* 2016), the improved performance in the PPI test by Fortasyn-treated mice may be linked to the rescue of WM integrity by this diet. Indeed, in mice treated with n-3 polyunsaturated fatty acids, improvements in WM integrity were positively correlated to sensorimotor performance in the cylinder test (Jiang et al. 2016). The beneficial effect of Fortasyn on sensorimotor integration in this study may be indicative of the efficacy of multinutrient interventions in psychiatric disorders with extensive sensorimotor impairments, such as schizophrenia and Gilles de la Tourette's syndrome (Kohl *et al.* 2013, Geyer 2006).

Our MRI data revealed that the enhancement of brain integrity by Fortasyn was accompanied by a Fortasyn-specific improvement of functional connectivity in stroke females; comparably to stroke males (Wiesmann et al. 2017), Fortasyn increased FC between the right AUC and the left MC. However, while the stroke males showed several beneficial effects of Fortasyn on FC, in the females this effect was limited to this one improved connection. An explanation for this modest effect of Fortasyn in the females may be that the duration of treatment (i.e. 35 days) was insufficient to elicit a substantial beneficial effect of Fortasyn on stroke-induced impairments of functional connectivity present in our stroke females. Indeed, previously when Fortasyn improved functional connectivity, treatment periods could last up to 16 months (Wiesmann et al. 2016). The relatively short duration of our treatment may also explain the lack of a (beneficial) effect of Fortasyn on the synaptic marker SYN in both female and male mice (Wiesmann et al. 2017). In

ApoE4 mice, levels of a postsynaptic marker, PSD-95, were unaffected with 10 months of treatment with Fortasyn diet, but finally increased after 16 months of treatment (Wiesmann et al. 2016).

Neurogenesis and inflammation

Stroke can trigger neurogenesis both in rodent and human brain (Lindvall & Kokaia 2015), presumably in an effort to repair the ischemic damage, but this neurogenesis is insufficient to induce lasting, functional recovery. While we did not investigate effect of tMCAo on neurogenesis, in the current study we did find that Fortasyn can enhance neurogenesis in both the ipsi- and contralateral subventricular zones. It seems that Fortasyn is capable of enhancing the production of novel cells after stroke, which is in line with the effect of Fortasyn on neurogenesis seen in the stroke males (Wiesmann et al. 2017) and in male APP/PS1 mice (Jansen et al. 2013). It remains to be investigated whether these novel cells can develop into mature and functional cells capable of repairing stroke damage.

Functional integration of novel cells into damaged areas in part relies upon the presence of a beneficial inflammatory environment. An ischemic event in the brain triggers an immediate inflammatory response, with recruitment and activation of microglia particularly in the affected ischemic brain area (Taylor & Sansing 2013). In this study, there was no difference between the affected hemisphere and the unaffected hemisphere regarding Iba-1 expressing inflammatory cells, perhaps because the initial inflammatory response occurring immediately after stroke induction had already vanished 35 days after the stroke. However, compared to the mice fed the Control diet, the Fortasyn mice demonstrated more IBA-1+ cells in the cortex, suggesting that Fortasyn can modulate inflammation. As IBA-1 expressing microglia are capable of being both harmful and protective (Taylor & Sansing 2013), discovering the exact nature of a Fortasyn-induced inflammatory response in both females and males is necessary to establish if Fortasyn has a beneficial or detrimental effect on inflammation.

Motor function, behavior, and cognition

tMCAo is known to result in impairments of motor skills and activity (Balkaya *et al.* 2013), and indeed the stroke females demonstrated functional impairments. Stroke males on the Fortasyn diet showed improvements in motor strength, motor coordination and activity compared to Control mice (Wiesmann et al. 2017), but none of these effects could be replicated in the stroke females. Three days after stroke induction, Fortasyn-fed females did demonstrate increased rearing frequency compared to Control females. Finally, the ORT revealed that Fortasyn did not affect cognition in the stroke females, probably because the ORT was measured from 22 to 24 days post-stroke. Another study demonstrated a significantly reduced preference at 7 and 14 days following stroke, but also normal values between 21 and 28 days post-stroke suggesting recovery of cognition after 3 to 4 weeks (Wang *et al.* 2012). Therefore in future research, ORT or other cognition tests should be performed earlier after the induction of the experimental stroke to detect subtle deficits and dietary effects. Nevertheless, MCAo leads to a range of cognitive changes, relying upon gender, strain, and the occlusion time (Jiwa et al. 2010). Moreover, in some studies impaired learning with the MWM was found (Gibson & Murphy 2004), while other studies could not replicate this result (Bouët et al. 2007) indicating possible differences in cerebral vasculature between murine strains. Thereby cognitive decline in patients is influenced by co-morbidities and pre-existing conditions that affect the entire brain (and pre-dispose stroke) and these conditions are hard to model in the otherwise healthy rodent with only a restricted unilateral injury.

Conclusion

As previously demonstrated, treatment with the Fortasyn multinutrient intervention has multiple beneficial effects on impairments seen in AD (van Wijk et al. 2014) and ischemic stroke (Wiesmann et al. 2017). In the current preclinical stroke study, as far as we know one of the first to use females, the beneficial effects of the Fortasyn diet were less pronounced compared to those found in male

mice (Wiesmann et al. 2017), perhaps because the stroke seemed to impact the females more severe than the males. This suggests that indeed, as previously observed (Soldin & Mattison 2009), the outcome of treatment in males and females can differ and emphasizes the necessity to include both sexes in preclinical studies, preferably simultaneously. Rodents like mice and especially the female mice may be affected by the dissimilar amount of phytoestrogens in the two diets. This difference may also explain that in this recent study less beneficial effects of the Fortasyn diet were found in female stroke mice than in male stroke mice compared to the Control diet. However, very small amounts of phytoestrogens are present in soy oil and even smaller amounts will be present in the control diet containing 3% soy oil which will in all probability not provide ample amounts to evoke a physiological response in mice. Nevertheless, in future research this should be taken in consideration when composing the diets. In our opinion, the fact that the effects of Fortasyn in the current study were diverse does not negate the efficacy of multinutrient intervention treatments in the recovery of female stroke patients. As discussed, stroke females may benefit from extended treatment durations. Furthermore, testing multinutrient interventions in female mice modeling the changes occurring with menopause, e.g. an ovariectomized female mouse (Diaz Brinton 2012), may be more appropriate to assess the effects of interventions in stroke females. In our opinion, these future studies, together with the current study, will help create effective dietary treatment regimens for CVD such as stroke in both females and males. Nevertheless, a longer multinutrient intervention period would possibly have a much stronger effect on cardio- and cerebrovascular health, and potentially have some benefit to prevent dementia via increasing cognitive reserve. Future studies should take this into account and study the effect of longer dietary treatments. Multinutrient interventions seem to be a beneficial add-on to the already existing ways of therapy (thrombolysis and/ or thrombectomy) to enhance recovery of (female) stroke patients. Therefore, future studies are needed to investigate the efficacy of the treatment combination of the traditional stroke therapies with such a multinutrient intervention.

--Human subjects --

ARRIVE guidelines have been followed:

Yes

=> if No or if it is a Review or Editorial, skip complete sentence => if Yes, insert "All experiments were conducted in compliance with the ARRIVE guidelines." unless it is a Review or Editorial

Acknowledgments/Conflict of interest disclosure

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Figure legends

Figure 1. Study design. Before ischemic stroke induction (tMCAo on day 0), female C57Bl/6 mice were allowed to acclimatize after which they underwent several prestroke behavioral tests (baseline measurements). Immediately after stroke induction, the females were given either the Fortasyn multinutrient diet (n=11) or an isocaloric Control diet (n=12). After the stroke, several behavioral tasks were (again) performed. Furthermore, MRI scanning was performed twice after stroke induction. Ppi, prepulse inhibition; tMCAo, transient middle cerebral artery occlusion; ORT, novel object recognition test

Figure 2. Activity, anxiety and (explorative) behavior was measured in the open field pre-stroke (pre) and 3 days (3d) and 21 days (21d) after stroke induction and diet switch. (A) Compared to pre-stroke, 3 days after the stroke all mice moved less distance. The distance moved remained decreased 21 days after the stroke. (B) Compared to pre-stroke, the frequency of walking and rearing decreased 3 days after the stroke, while the frequency of sitting increased. Rearing frequency decreased further 21 days after stroke compared to 3 days after stroke. Mice fed the Fortasyn diet reared more frequently 3 days after the stroke induction and diet switch. (C) Compared to pre-stroke, the frequency of entering the center, corners and periphery decreased 3 days after the stroke. (D) The time spend in the center and periphery decreased compared to pre-stroke, while the time spend in the corners increased. The time spend in the periphery had decreased further 21 days after stroke induction and the time spend in the corners increased further. Values represent mean ± SEM. Control: n=12, Fortasyn: n=10. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Figure 3. Motor strength (A), motor coordination (B) and sensorimotor integration of female stroke mice measured before (pre) and after (grip test: 14 and 27 days; pole test: 15 days; prepulse inhibition: 16 days) stroke induction and diet switch. (A) On the trapeze of the grip test (Control: n=12, Fortasyn: n=11) no differences over time or with diet were demonstrated. On the grid, stroke females had a decreased maximum strength 14 days after stroke induction. (B) In the pole test (Control: n=6, Fortasyn: n=8), diet did not affect rotation time of the females 15 days after stroke induction and diet switch. (C) Fortasyn stroke females (n=11) tended to have a higher PPI (%) than Control stroke females (n=10) when a prepulse of 4dB was given (PP4). There was no effect of diet with any of the other prepulses. (D) Fortasyn stroke females (n=11) habituated to the 120 dB startle pulses as demonstrated by a reduction in startle response from the beginning to the end of the PPI test. Values represent mean ± SEM. Abbreviations: g-f, gram-force; PP, prepulse (of either 2, 4, 8 or

16 dB above background noise); PPI, prepulse inhibition; a.u., arbitrary units. # p<0.08, * p<0.05, ** p<0.01.

Figure 4 Cerebral blood flow (CBF) determined with flow-sensitive alternating inversion recovery (FAIR) MRI 7 and 35 days after stroke induction and diet switch in cortex, hippocampus and thalamus. (A) In general, at both time points CBF was lower in the right brain hemisphere, where the middle cerebral artery was occluded, than in the left brain hemisphere. Only in the cortex of female Control mice (35 days after stroke induction) and in the thalamus of female Control mice (7 and 35 days after stroke induction) did CBF not differ between hemispheres. In the cortex and hippocampus of the Fortasyn females, CBF tended to increase with time. In the left thalamus, Fortasyn females tended to have a higher CBF than Control females. (B) Representative high-resolution voxel-wise analyzed CBF images at 7 and 35 after stroke induction and diet switch. (C) The CBF difference (Δ%CBF normalized to CBF in the left brain area) between the left and right cortex and between the left and right hippocampus decreased with time in female Control mice. In stroke females on Fortasyn diet, the CBF difference between the left and right hippocampus and the left and right thalamus decreased with time. Values represent mean ± SEM. Control: n=11, Fortasyn: n=10. # p<0.08, * p<0.05, ** p<0.01, *** p<0.001

Figure 5. White matter (WM) and grey matter (GM) integrity as measured by quantitatively assessed diffusion tensor derived indices (A+B) and resting-state functional connectivity (FC) based on partial correlation analyses (C-E) measured 7 and 35 days after stroke induction and diet switch. (A) In several brain areas of both Control and Fortasyn stroke females, FA (a measure of WM integrity) was lower in the right hemisphere than in the left hemisphere. In the Control stroke females, FA became lower with time in the AUC, HC, SSC and VC. FA in the OT increased with time in both the Control and Fortasyn stroke females. Thirty-five days after stroke induction and diet switch,

the Fortasyn stroke females had an increased FA compared to Control stroke females in both the left and right HC and Cpu+GP. (B) In several brain areas of particularly Control stroke females, MD (a measure of GM integrity) was higher in the right hemisphere than in the left hemisphere. In the Control stroke females, MD became higher with time in the F, HC and OT. In the Fortasyn stroke females, MD became higher with time in the left OT. Thirty-five days after stroke induction and diet switch, the Fortasyn stroke females had a decreased MD compared to Control stroke females in the left HC and right OT. Abbreviations: FA= fractional anisotropy, MD=mean diffusivity, AUC=auditory cortex, CC=corpus callosum, F=fornix, HC=hippocampus, MC=motor cortex, OT=optic tract, SSC=somatosensory cortex, VC=visual cortex, Cpu+GP= caudate putamen+globus pallidus. Control: n=11, Fortasyn: n=10. Values represent mean ± SEM. # $p<0.08$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (C-E) Thirty-five days after tMCAo, the partial correlations demonstrated that Fortasyn stroke females had an increased FC between right AUC and left MC. No other diet effects were found. Over time, the same amount of FC improvements (+) and impairments (-) were found for Fortasyn and Control stroke females. Abbreviations: DH= dorsal hippocampus, VH=ventral hippocampus, AUC=auditory cortex, MC=motor cortex, SSC=somatosensory cortex, VC=visual cortex. Control: n=12, Fortasyn: n=11. Values in (C) represent mean z-scores.

Figure 6. Neurogenesis (A-C), inflammation (D-G) and vascular density (H-J) markers were assessed in postmortem brains of the females that were sacrificed 35 days after stroke induction and diet switch. (A) The Fortasyn stroke females (n=11) had a larger DCX+ area in the SVZ than Control stroke females (n=12). (B) and (C) are representative images of the DCX staining in the SVZ of respectively Control and Fortasyn stroke females. The Fortasyn stroke females (n=11) had a higher number of IBA-1+ cells (D) and a larger IBA-1+ area (E) in the cortex than Control stroke females (n=12). (F) and (G) are representative images of the IBA-1 staining in the cortex of respectively Control and Fortasyn stroke females. (H) The Fortasyn stroke females (n=11) had a decreased number of GLUT-1+ cells

(per mm2) in the hippocampus compared to Control stroke females (n=12). (I) and (J) are representative images of the GLUT-1 staining in the hippocampus of respectively Control and Fortasyn stroke females. Values represent mean ± SEM. * p<0.05, ** p<0.01

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