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## Long-term dietary supplementation with plant-derived omega-3 fatty acid improves outcome in experimental ischemic stroke

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**Abstract:** BACKGROUND AND AIMS Early revascularization -the gold standard therapy for ischemic stroke- is often withheld in the elderly population due to high risk of complications. Thus, safe and effective preventive and therapeutic options are needed. The plant-derived omega-3-fatty-acid alpha-linolenic-acid (ALA) has emerged as a novel cardiovascular-protective agent. As of yet, little is known about its potential therapeutic effects on stroke. We hereby aimed to investigate the impact of a clinically relevant long-term dietary intervention with ALA on stroke outcome. METHODS Six month-old C57BL/6 wild-type males were either fed an ALA-rich (high ALA) or a control diet (low ALA) for 12 months. At 18 months, brain ischemia/reperfusion was induced by transient middle cerebral artery occlusion (tMCAO). Stroke size and neurological function were assessed. Functional blood-brain-barrier-(BBB) permeability and protein expression were assessed by immunohistochemistry. Baseline inflammatory markers were measured at 18 months. RESULTS High ALA-fed animals displayed decreased circulating TNF- levels and Neutrophil-to-Lymphocyte Ratios at 18 months. Stroke size and neurological dysfunction were significantly reduced in high ALA-fed animals. Coherently to the reduced stroke size, functional BBB integrity and occludin endothelial expression were maintained by high ALA supplementation. Additionally, ALA reduced endothelial activation and thus recruitment and activation of macrophages and resident microglia. Finally, high ALA diet reduced the expression of BBB-degrading and neurotoxic MMP-3 and MMP-9. CONCLUSIONS We demonstrate the beneficial effects of a clinically relevant and feasible dietary intervention with a safe and readily available compound in the setting of stroke. The protective effects observed with ALA supplementation may relate to blunting of inflammation and might pave the way for novel stroke treatments.

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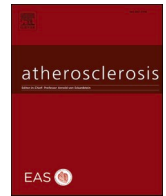
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## Long-term dietary supplementation with plant-derived omega-3 fatty acid improves outcome in experimental ischemic stroke

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### ABSTRACT

**Background and aims:** Early revascularization -the gold standard therapy for ischemic stroke- is often withheld in the elderly population due to high risk of complications. Thus, safe and effective preventive and therapeutic options are needed. The plant-derived omega-3-fatty-acid alpha-linolenic-acid (ALA) has emerged as a novel cardiovascular-protective agent. As of yet, little is known about its potential therapeutic effects on stroke. We hereby aimed to investigate the impact of a clinically relevant long-term dietary intervention with ALA on stroke outcome.

**Methods:** Six month-old C57BL/6 wildtype males were either fed an ALA-rich (high ALA) or a control diet (low ALA) for 12 months. At 18 months, brain ischemia/reperfusion was induced by transient middle cerebral artery occlusion (tMCAO). Stroke size and neurological function were assessed. Functional blood-brain-barrier (BBB) permeability and protein expression were assessed by immunohistochemistry. Baseline inflammatory markers were measured at 18 months.

**Results:** High ALA-fed animals displayed decreased circulating TNF-α levels and Neutrophil-to-Lymphocyte Ratios at 18 months. Stroke size and neurological dysfunction were significantly reduced in high ALA-fed animals. Coherently to the reduced stroke size, functional BBB integrity and occludin endothelial expression were maintained by high ALA supplementation. Additionally, ALA reduced endothelial activation and thus recruitment and activation of macrophages and resident microglia. Finally, high ALA diet reduced the expression of BBB-degrading and neurotoxic MMP-3 and MMP-9.

**Conclusions:** We demonstrate the beneficial effects of a clinically relevant and feasible dietary intervention with a safe and readily available compound in the setting of stroke. The protective effects observed with ALA supplementation may relate to blunting of inflammation and might pave the way for novel stroke treatments.

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## 1. Introduction

Ischemic stroke annually affects more than 15 million people worldwide with massive impact on quality of life and health care expenditures [1]. Yet, its targeted treatment remains an unmet clinical need and re-canalization strategies – the current therapeutic gold standard – are often withheld due to the narrow therapeutic window and numerous contraindications [2]. Such limitations particularly concern the highly susceptible elderly population [3].

The cardio- and cerebrovascular protective effects of fish-derived dietary long-chain omega-3 (n-3) fatty acids eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids have been extensively investigated with often conflicting clinical results [4]. Recently, a large meta-analysis of 40 randomized controlled trials with over 135'000 participants concluded that EPA and DHA dietary supplementation is an effective lifestyle strategy for cardiovascular disease prevention with dose-dependent effects [5]. Nearly simultaneously, Astra-Zeneca announced the early discontinuation of the Outcomes Study to Assess Statin Residual Risk Reduction With Epanova in High CV Risk Patients With Hypertriglyceridemia (STRENGTH) trial investigating EPA + DHA supplementation in a high risk population due to futility [6]. Plant-derived n-3 fatty acids have recently emerged as ecologically sustainable, economically friendly and naturally abundant alternatives to marine-derived supplements [7]. We and others have previously shown the protective effects of the plant-derived  $\alpha$ -linolenic acid (ALA) in different animal models of CV conditions [8]. Specifically, ALA was shown to prevent cardio metabolic diseases [9] and to blunt atherosclerosis [10] and arterial thrombosis [11]. Furthermore, different epidemiological studies inversely associated ALA consumption with the incidence of myocardial infarction and sudden cardiac death [12,13]. Among the variety of mechanisms involved in n-3 fatty acids' biological effects, reduction of inflammation and oxidative stress have been widely described and characterized in human cohorts and experimental models of disease [14,15].

Given the increasing evidence suggesting ALA as a cardio-protective nutritional supplement and in consideration of the deep involvement of inflammation in ischemic stroke pathophysiology [16–18], we hypothesized that plant-derived n-3 fatty acids may not only play a preventive role in stroke predisposition but also directly ameliorate its outcome. Therefore, this study investigated the effects of a long-term dietary intervention with the plant-derived n-3 fatty acid ALA on the outcome of ischemic stroke in old mice and the mechanisms pertaining to the observed effects.

## 2. Materials and methods

### 2.1. Animals

Experiments used male C57BL/6 wild-type (WT) mice (Charles-River Lab, Freiburg im Breisgau, Germany); all rodents were kept in a temperature-controlled animal facility under normal light/dark cycle with free access to food and water. All procedures were approved by the local Ethical Committee for animal research and the Cantonal Veterinary Authority. Animal experiments conformed to the Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes.

### 2.2. Dietary regimens

Animals were fed with standard chow diet (ALA content 0.123 g%) until the age of 6 months (19 g% protein, 61 g% carbohydrate and 7 g% fat; Research Diets, New Brunswick, NJ, USA). Then, they were randomly assigned by a researcher excluded from endpoint assessment to special diets containing either high ALA dosage (7.3 g%; Research Diets, New Brunswick, NJ) or low ALA dosage (0.03 g%; Research Diets) for next 12 months. Special diets contained 21 g% protein, 50%

carbohydrate and 14% fat; ALA was given as flaxseed oil and cocoa butter was used as the substitute in the control group, as previously described [10,11,19]. Animals had *ad libitum* access to the experimental chow until the age of 18 months. Upon termination of the 12-month long dietary intervention, two different cohorts were set: one for baseline measurement including a total of 18 animals that were sacrificed and harvested without any interventions and another cohort which underwent transient middle cerebral artery occlusion (tMCAO) to induce cerebral ischemia/reperfusion. The cohort size was decided based on our previous experience with tMCAO [18,20–24]. According to our power calculation, the minimal sample size request to detect a 30% decrease in stroke size with a power of 95% and two-sided alpha error of 5% was 12 animals per group. In consideration of the 15% expected mortality, 14 animals per group were employed for tMCAO experiments.

### 2.3. Baseline assessment of inflammatory status

A dedicated set of 18 month-old mice not undergoing tMCAO was employed for baseline neutrophil to lymphocyte ratio (NLR) and plasma tumor necrosis factor (TNF)- $\alpha$  determination. Animals were euthanized with carbon dioxide, blood was collected via intracardiac puncture and immediately mixed with EDTA. Leukocyte count performed using an impedance hematology analyzer (SciVet ABCplus; Horiba, Kyoto, Japan). NLR was calculated by dividing the number of neutrophils by the number of lymphocytes [24]. The EDTA-blood solution was then centrifuged for 15 min at 3000g as previously described [25]. Colorimetric enzyme-linked immunosorbent assay (ELISA) was employed for the quantitative assessment of TNF- $\alpha$  levels in murine EDTA-plasma following the manufacturer instruction (MHSTA50, R&D Systems, Minneapolis, MN). Mean intra- and inter-assay coefficients of variation were <10%, lower level of detection was 0.8 pg/mL.

### 2.4. Transient middle cerebral artery occlusion

Transient middle cerebral artery occlusion (tMCAO) induced ischemia/reperfusion (I/R) brain injury, as previously described [26–28]. Briefly, mice were anaesthetized using isoflurane at 5% and 1.5% for induction and maintenance respectively, while body temperature was tightly maintained at 37 °C. For analgesia, buprenorphine HCl was infiltrated at the incision side (0.1 mg/kg). An experimenter blinded to the group allocation induced ischemia by inserting a 6-0 silicone-coated filament (Doccol Corporation, Sharon, MA, USA) into the common carotid artery until the origin of the left MCA after the dissection of common, internal and external carotid arteries. After 30 min of ischemia, the filament was retracted, and reperfusion allowed for 48 h before animal euthanasia with carbon dioxide. During this time, animals were carefully monitored and received analgesia with buprenorphine-HCL at a dose of 0.1 mg/kg s.c. every 6 h. A score sheet approved by the Cantonal Veterinary Office of the Canton of Zurich monitored the well-being of mice during the experimental period.

### 2.5. Stroke volume

After euthanasia, mice were perfused with 10 mL of phosphate buffered saline (PBS) and relevant organs were excised. Murine brains were cut into 5 equally spaced (2 mm) coronal sections by mean of a steel brain matrice and immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, Chemie GmbH, Buchs, Switzerland) at 37 °C for 20 min [18]. TTC is a redox indicator, which changes its colour from white to red, as it gets reduced to 1,3,5-triphenylformazan in viable tissues. In metabolically inactive tissues lacking cellular respiration, this reduction does not take place, leaving TTC in its unreacted state. Therefore, the stroke area can be distinguished from viable brain tissue by its white appearance.

To measure infarct size, ipsilateral and contralateral hemispheres were quantified using ImageJ software by an experimenter blinded to

the group allocation (Image J, NIH, MD, USA). To correct infarct size measurement for cerebral edema and consequent overestimation, we applied the following formula as previously described [28]: Corrected infarct volume = contralateral hemisphere volume - (ipsilateral hemisphere volume - infarct volume). Infarct size was expressed as volume in mm [3].

## 2.6. Neurological deficit assessment

Baseline and post-infarction neurological status were assessed by a four-point scale neurological score according to Bederson et al. and the RotaRod test as previously described [22,26]. Both composite sensory-motor tests evaluate motor functions, proprioception, spatial orientation and balance. The neurological score test according to Bederson was performed at baseline, 2, 24 and 48 h after reperfusion according to the following scores: grade 0, normal neurological function; grade 1, forelimb and torso flexion on and towards the contralateral side upon lifting of the animal by the tail to 1 m above the work surface; grade 2, circling to the contralateral side; grade 3, leaning to the contralateral side at rest; grade 4, no spontaneous motor activity. The RotaRod test was performed at baseline, 24 and 48 h after reperfusion. Mice were placed on a rotating rod at increasing speed (4–44 rot/min) and the time to fall was measured in seconds. Three consecutive measurements were performed at each time-point for each animal, and the best score was used for statistical analysis.

## 2.7. Immunohistology analyses

Immunohistochemical staining was performed as previously described [24]. Briefly, 48 h after tMCAO mice were euthanized and perfused with PBS (Sigma-Aldrich, Chemie GmbH, Buchs, Switzerland). The brains were removed, then incubated overnight in 4.0% paraformaldehyde (PFA; Sigma-Aldrich, Chemie GmbH, Buchs, Switzerland) at 4 °C and afterward transferred to 30% sucrose in PBS for 36 h. Cryoprotected brains were cut into 100- $\mu$ m thick free-floating sections using a microtome (Leica Jung HN40), pre-treated with proteinase K or 1 M HCl for antigen retrieval and immune-blocked with 10% donkey serum. After these steps, they were incubated with primary antibodies at the following dilutions: ionized calcium binding adaptor molecule (Iba)-1 (1:500; Wako Chemicals, Osaka, Japan), Occludin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), the endothelial marker CD31 (1:50; BD Pharmingen, Allschwil, Switzerland), vascular cell adhesion molecule (VCAM)-1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Intercellular Adhesion Molecule (ICAM)-1 (1:400; BD Pharmingen, Allschwil, Switzerland), P-selectin (1:100; BD Pharmingen, Allschwil, Switzerland), matrix metalloproteinase (MMP)-3 (1:100; Abcam, Cambridge, United Kingdom) and MMP-9 (1:500; Abcam, Cambridge, United Kingdom) at 4 °C overnight, respectively. Secondary antibodies were added at a dilution of 1:750 (Jackson ImmunoResearch, West Grove, PA, USA) for 24 h at 4 °C. Images were acquired using a confocal microscope (Leica SP8; Leica, Wetzlar, Germany). Cells positively stained for the microglial and activated macrophage marker Iba-1, were counted in a pre-specified ipsilateral area (the CA1 hippocampal area) using ImageJ software. Stained ipsilateral areas of occludin, VCAM-1, P-selectin, and ICAM-1 were measured using ImageJ software and co-localized to the area positively stained with the endothelial marker CD31. The area stained for both protein of interest and endothelial marker is expressed as a percentage of the total endothelial surface area. Stained areas of MMP-9 and MMP-3 were assessed using ImageJ and normalized to the total endothelial cell surface area assessed by CD31 staining.

BBB permeability was assessed by quantifying endogenous immunoglobulin G (IgG) extravasation. Sections were incubated with Alexa647-conjugated donkey anti-mouse IgG for 24 h (1:600; Jackson ImmunoResearch, West Grove, USA). IgG-stained area was expressed as a percentage of the contralateral hemisphere.

## 2.8. Plasma fatty acids, lipids, and insulin quantification

Animals were euthanized 48 h after tMCAO with carbon dioxide, blood was collected via intracardiac puncture and immediately mixed with EDTA. Plasma-EDTA samples were stored at  $-80$  °C until analysis. Plasma fatty acids were analyzed using gas-liquid chromatography of fatty acid methyl esters after fractionation of lipid classes by solid-phase extraction [29]. Triglyceride concentrations were determined with the Roche/Hitachi Triglyceride Kit (Rotkreuz, Switzerland; Ref. 11877771 216) according to the manufacturer's instruction. Total plasma cholesterol was measured with the kit (FULFILM Wako Pure Chemicals, Tokyo, Japan; Ref. 294-65801) as recommended by the manufacturer and high density lipoprotein (HDL)-cholesterol levels were measured in ApoB-depleted plasma by PEG precipitation, as previously described [30]. Plasma insulin levels were assessed in duplicates with a mouse insulin ELISA kit (Mercodia AB, Uppsala, Sweden; Ref. 10-1247-10) according to kit instructions. Absorbance was measured with Epoch 2 Microplate Spectrophotometer (BioTek, Winooski, VT, USA). Results were calculated with data reduction of calibrator absorbance versus calibrator concentrations using cubic spline regression. The detection limit of this kit is  $\leq 0.2$   $\mu$ g/L.

## 2.9. Statistical analysis

Data are expressed as mean  $\pm$  SEM. All statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, Inc, La Jolla, CA, USA) and no specific exclusion criteria was defined. Data were analyzed by using one-way analysis of variance with Tukey *post hoc* test for multiple comparisons or unpaired two-tailed Student's *t*-test, as appropriate. For repeated measurements, two-way ANOVA with Sidak *post hoc* test was used. Statistical analysis for survival studies was performed using log-rank (Mantel-Cox) test. A probability value (*p*) below 0.05 was considered as statistically significant.

## 3. Results

### 3.1. Systemic effects of long-term dietary supplementation with ALA

The efficiency of the long-term high ALA diet was assessed by quantifying ALA in plasma-EDTA (Table 1). As expected, mice fed for 12 months with a high-ALA diet in a clinically relevant range showed markedly increased ALA plasma levels ( $0.01\% \pm 0.01$  vs  $0.84\% \pm 0.21$ ,  $p < 0.01$ ) as compared to those receiving low-ALA diet. Also, we assessed the levels of ALA-derived n-3 fatty acids and showed that levels of EPA, docosapentaenoic acid (DPA), and DHA were also increased in high-ALA group suggesting that conversion from ALA to n-3 long chain polyunsaturated fatty acids was taking place ( $0.03 \pm 0.01$  vs  $2.04 \pm 0.43$ ,  $0.12 \pm 0.01$  vs  $0.64 \pm 0.07$  and  $4.37 \pm 0.43$  vs  $11.11 \pm 0.58$ ,  $p < 0.01$  for EPA and  $p < 0.0001$  for DPA and DHA, Table 1).

ALA is thought to exert its cardiovascular protection at least partially through anti-inflammatory effects [14,15]. NLR is widely used as a marker of subclinical inflammation with prognostic value for various medical conditions. In line with the previously reported anti-inflammatory effects of ALA, mice fed with high-ALA diet showed

**Table 1**  
Plasma levels of  $\alpha$ -linolenic acid and long chain n-3 fatty acids.

	Low ALA (n = 8)	High ALA (n = 12)	<i>p</i> -value
% (w/w) FA			
ALA (C18 3n-3)	$0.01 \pm 0.01$	$0.84 \pm 0.21$	$<0.01$
EPA (C20 5n-3)	$0.03 \pm 0.01$	$2.04 \pm 0.43$	$<0.01$
DPA (C22 5n-3)	$0.12 \pm 0.01$	$0.64 \pm 0.07$	$<0.0001$
DHA (C22 6n-3)	$4.37 \pm 0.43$	$11.11 \pm 0.58$	$<0.0001$

FA: fatty acid; ALA:  $\alpha$ -linolenic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid.

reduced baseline NLR as compared to low ALA-fed littermates ( $1.00 \pm 0.11$  vs  $1.56 \pm 0.24$ ,  $p < 0.05$ , Fig. 1A). In humans, dietary ALA supplementation was reported to reduce systemic levels of the primary pro-inflammatory cytokine TNF- $\alpha$  in different cohorts [14,31]. In consideration of the known deleterious role of this mediator in the early phase of ischemic stroke, we assessed its plasma levels in animals fed with high and low ALA diets. Consistent with the NLR results, high ALA animals also showed reduced circulating levels of TNF- $\alpha$  by almost 50% as compared to low ALA controls ( $1.90$  pg/mL  $\pm$   $0.28$  vs  $3.62$  pg/mL  $\pm$   $0.49$ ,  $p < 0.01$ , Fig. 1B).

ALA was previously shown to affect traditional CV risk factors in humans. In our animal cohorts, high ALA-fed mice did not show any weight difference during the treatment, as compared to low ALA group ( $33.71$  g  $\pm$   $0.84$  vs  $36.11$  g  $\pm$   $2$ , Supplementary Fig 1A). Similarly, no statistical difference was shown among the two groups in term of circulating insulin ( $0.27$  ng/mL  $\pm$   $0.05$  vs  $0.89$  ng/mL  $\pm$   $0.26$ , Supplementary Fig 1B). As for the lipid panel, high ALA-treated mice showed reduced total as well as HDL cholesterol levels as compared to those fed with low ALA diet ( $129$  mg/dL  $\pm$   $13$  vs  $205$  mg/dL  $\pm$   $12$  and  $101$  mg/dL  $\pm$   $11$  vs  $160$  mg/dL  $\pm$   $14$  respectively,  $p < 0.05$  for both, Supplementary Fig. 1C and D). On the other hand, LDL cholesterol and triglycerides were not statistically different among the groups ( $28$  mg/dL  $\pm$   $3$  vs  $45$  mg/dL  $\pm$   $12$  and  $44$  mg/dL  $\pm$   $5$  vs  $52$  mg/dL  $\pm$   $7$  respectively, Supplementary Fig. 1E and F).

### 3.2. ALA supplementation decreased brain infarct size and post-stroke neurological deficit

To assess the specific effect of long-term dietary supplementation with ALA on ischemic stroke outcome, WT mice were fed with either low

ALA or high ALA diets for 12 months starting at the age of 6 months before undergoing tMCAO for 30 min followed by 48 h of reperfusion (Fig. 2A). High ALA-fed mice displayed a stroke size two times smaller than that of low ALA littermates, as assessed by TTC staining ( $28.38$  mm<sup>3</sup>  $\pm$   $3.23$  vs  $51.77$  mm<sup>3</sup>  $\pm$   $6.14$ ,  $p < 0.01$ , Fig. 2B). Furthermore, ALA-supplemented animals showed a tendency towards better neurological performance as assessed by Bederson score already at 2 and 24 h after stroke ( $2.08 \pm 0.15$  vs  $2.38 \pm 0.18$  and  $1.67 \pm 0.14$  vs  $2.00 \pm 0.27$ , respectively, Fig. 2C). Such a difference between high and low ALA-fed mice was statistically significant at 48 h ( $1.33 \pm 0.14$  vs  $2.13 \pm 0.30$ ,  $p < 0.01$ , Fig. 2C). However, no difference in neuromotor function was found when assessed by RotaRod test ( $20.67$  s  $\pm$   $7.40$  vs  $14.00$  s  $\pm$   $6.90$  at 24 h and  $18.83$  s  $\pm$   $4.73$  vs  $19.38$  s  $\pm$   $8.30$  at 48 h, Fig. 2D). Of interest, a tendency towards increased survival 48 h after tMCAO was observed for animal fed with high ALA diet which however did not reach statistical significance (85% vs 57%,  $\chi^2$  2.58, Fig. 2E).

### 3.3. High ALA diet associates with reduced blood-brain barrier (BBB) disruption and occludin degradation

BBB damage is a main feature of ischemic stroke pathophysiology and importantly contributes to the determination of its outcome through cerebral edema and haemorrhagic transformation [32]. Immunohistochemical quantification of IgG extravasation is widely used to assess BBB damage. As expected, all experimental groups showed some degree of extravasation in the ischemic hemisphere (Fig. 3A). However, animals fed with high ALA diet displayed blunted BBB disruption upon I/R as compared with control animals ( $26.64\% \pm 3.92$  vs  $51.35\% \pm 4.18$ ,  $p < 0.01$ , Fig. 3A).

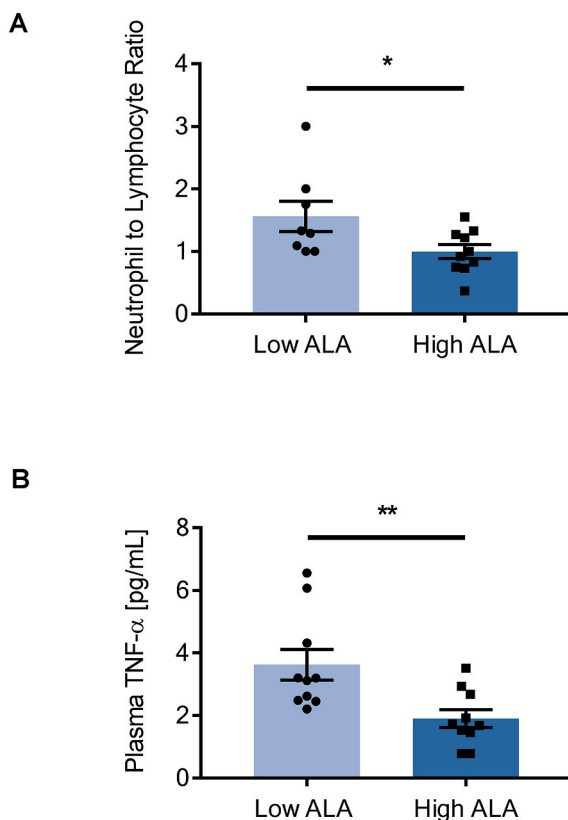
Among regulators of BBB function, tight junction proteins are downregulated after stroke, thereby increasing the paracellular permeability of the endothelial layer [32]. In line with the IgG extravasation data, immunohistochemical analysis of endothelial-specific occludin showed higher expression of this protein in the penumbra area of mice fed with high ALA diet as compared to low ALA ( $52.65\% \pm 6.83$  vs  $26.03\% \pm 3.72$ ,  $p < 0.01$ , Fig. 3B). The representative images show the penumbra area of stroke, the endothelial marker CD31 appears in red and represents the vasculature, while occludin appears in the middle panel in blue. The bottom panel shows an overlay of the junctional protein staining with the total endothelial surface (Fig. 3B).

### 3.4. ALA supplementation reduces post-stroke BBB endothelial activation and adhesion molecule expression

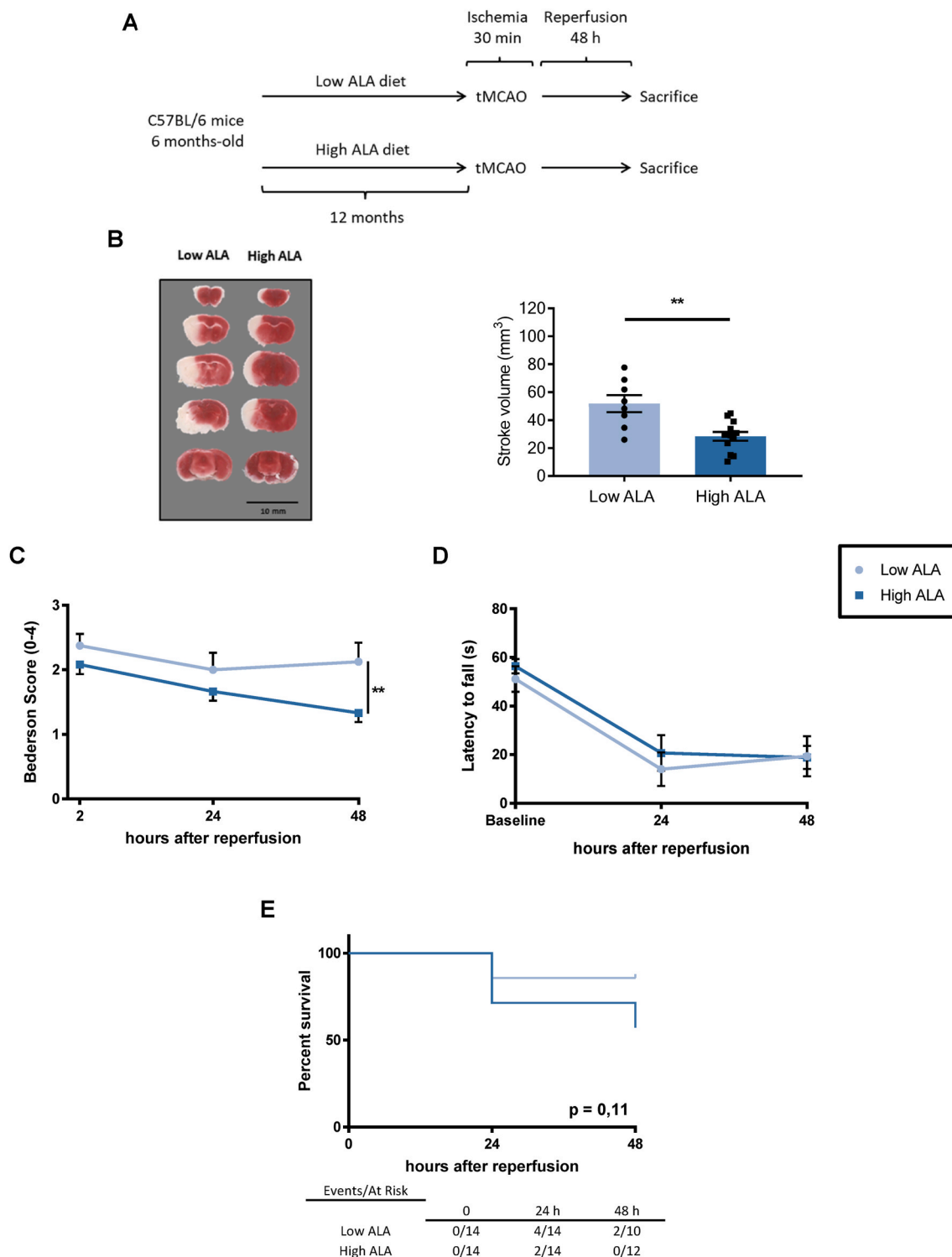
Following the ischemic insult, endothelial cells within the BBB upregulate the expression of adhesion molecules favouring leukocyte recruitment in the penumbra area with deleterious short-term effects on neuronal viability [33,34]. Of interest, mice fed with ALA-supplemented diet showed a reduced endothelial expression of adhesion molecules VCAM-1 and P-selectin in the penumbra area as compared to low ALA controls ( $49.98\% \pm 8.68$  vs  $73.13\% \pm 5.82$  and  $50.60\% \pm 4.41$  vs  $68.73\% \pm 4.33$ ,  $p < 0.05$  for both, Fig. 3C and D, respectively). Meanwhile, post-stroke endothelial expression of ICAM-1 in the penumbra area did not differ among high ALA- and low ALA-fed mice ( $76.14\% \pm 2.47$  vs  $82.07\% \pm 3.79$ , Fig. 3E). The representative immunohistochemistry pictures show the penumbra area of the stroke where vasculature is stained in red for CD31, while the different adhesion molecules are stained in cyan for VCAM-1, yellow for P-selectin and blue for ICAM-1 (Fig. 3C–E).

### 3.5. Long-term dietary ALA supplementation reduces the number of activated macrophages and blunts metalloproteinases expression in the penumbra area

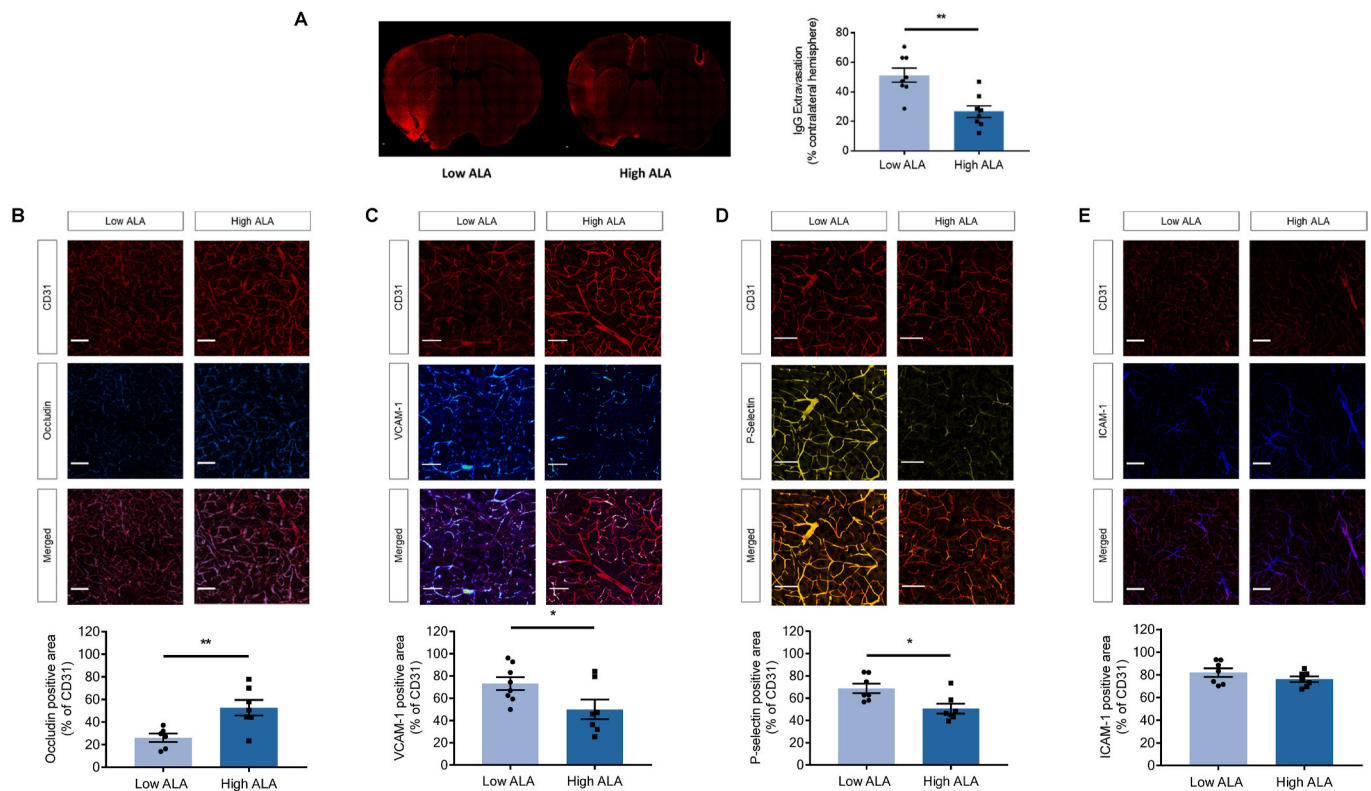
Cerebral ischemia/reperfusion induces the local activation of microglial cells as well as the infiltration of monocytes/macrophages



**Fig. 1.** Immunomodulatory effects of long-term dietary ALA supplementation. (A) Distribution of neutrophil to lymphocyte ratio in high ALA- and low ALA-fed mice at baseline. (B) Circulating plasma levels of the primary pro-inflammatory cytokine TNF- $\alpha$  in the two study groups at baseline.  $n = 8$ – $10$ . ALA =  $\alpha$  linolenic acid; TNF = tumor necrosis factor.



**Fig. 2.** Dietary ALA supplementation reduces infarct volume and post-stroke neurological deficit. (A) Schematic of the experimental study design. (B) Stroke volume distribution among mice receiving low ALA or high ALA dietary supplementation 48 h after tMCAO as assessed by TTC staining. In TTC stained coronal sections, the white colour distinguishes the stroke area from viable tissue (representative staining on the left, quantification on the right). (C) Assessment of post-stroke neurological impairment by Bederson scale at 2, 24 and 48 h after tMCAO. (D) Assessment of post-stroke neuromotor deficit by RotaRod test 24 and 48 h after tMCAO. (E) Overall survival 48 h after tMCAO in the two study groups (Kaplan-Meier curve and summarizing table). n = 8–14. ALA =  $\alpha$  linolenic acid, tMCAO = transient middle cerebral artery occlusion. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 3.** ALA supplementation reduces post-stroke BBB impairment and endothelial activation.

(A) Representative images and quantification of endogenous IgG extravasation (red) into the brain parenchyma 48 h after tMCAO in the two study groups. (B) Representative pictures of immunostaining for occludin (blue) and the endothelial marker CD31 (red) in the penumbra area 48 h after tMCAO in the study groups. Quantification refers to occludin/CD31 co-localization. (C) Representative pictures of immunostaining for VCAM-1 (cyan) and the endothelial marker CD31 (red) in the penumbra area 48 h after tMCAO in the study groups. Quantification refers to VCAM-1/CD31 co-localization. (D) Representative pictures of immunostaining for P-selectin (yellow) and the endothelial marker CD31 (red) in the penumbra area 48 h after tMCAO in the study groups. Quantification refers to P-selectin/CD31 co-localization. (E) Representative pictures of immunostaining for ICAM-1 (blue) and the endothelial marker CD31 (red) in the penumbra area 48 h after tMCAO in the study groups. Quantification refers to ICAM-1/CD31 co-localization.  $n = 6-8$ . ALA =  $\alpha$  linolenic acid, BBB = blood-brain barrier, ICAM = intracellular adhesion molecule, IgG = immunoglobulin G, tMCAO = transient middle cerebral artery occlusion, VCAM = vascular cell adhesion molecule, VE-cadherin = vascular endothelium cadherin. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with increased release of inflammatory mediators including matrix metalloproteinases (MMPs) [35–37]. Ionized calcium binding adaptor molecule (Iba)-1 is widely accepted as a marker of microglia/macrophage activation. Immunohistochemical counting of Iba1+ cells showed animals fed with high ALA diet to have reduced numbers of activated macrophages in the penumbra area as compared to low ALA littermates ( $314 \pm 30$  vs  $422 \pm 33$ ,  $p < 0.05$ , Fig. 4A; Iba1+ cells in red). Accordingly, 48 h after tMCAO, levels of MMP-9 and MMP-3 were higher in the penumbra area of animals with low ALA supplementation as compared to treated ones ( $0.58 \pm 0.09$  vs  $0.98 \pm 0.09$  and  $0.49 \pm 0.07$  vs  $0.94 \pm 0.09$ , respectively,  $p < 0.05$  for both, Fig. 4B and C, respectively). The representative images depict a specified area in the ipsilateral hemisphere, where the vasculature appears in red in the top panel (for the endothelial marker CD31) and the respective MMPs in magenta (MMP-9) or yellow (MMP-3) in the middle panel. The bottom panel shows an overlay of MMP immunoreactivity with the total endothelial surface, where a reduction of said enzymes in high ALA mice becomes apparent.

#### 4. Discussion

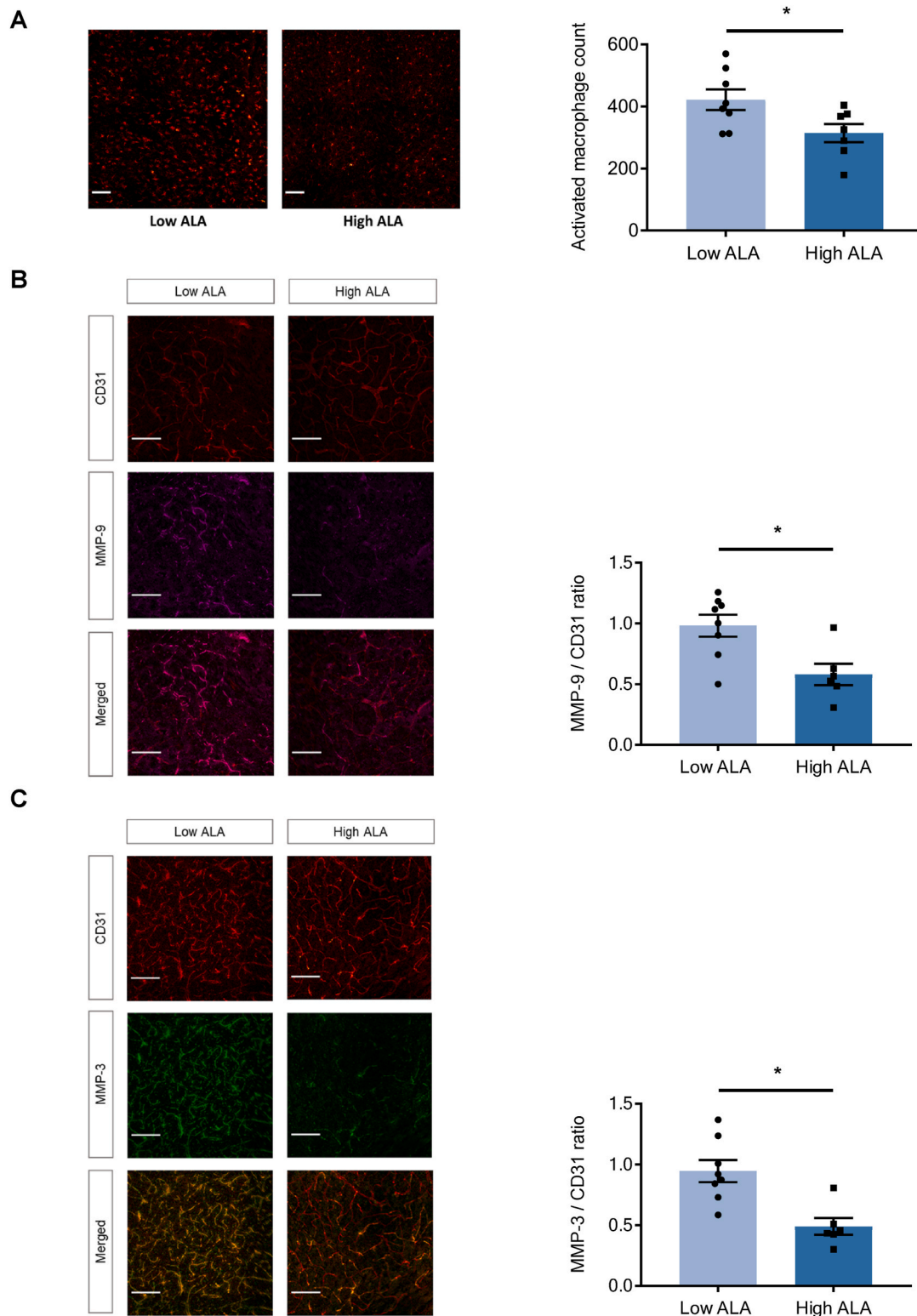
Ischemic stroke prevention and treatment remain longstanding unmet clinical challenges. The very promising basic research on neuroprotective drugs in this setting unfortunately failed the “bench to bedside” translation and to date, none of these agents entered clinical practice [16]. By acting on various pathophysiological processes, dietary

ALA supplementation has shown the ability to positively modulate different cardiovascular conditions [5,8–11]. However, it remains unknown whether dietary ALA supplementation affects outcome after ischemic stroke.

In this study, we demonstrated that: (i) long-term dietary ALA supplementation at clinically relevant doses exerts systemic anti-inflammatory effects reducing NLR and circulating levels of the pro-inflammatory cytokine TNF- $\alpha$ ; (ii) long-term high ALA diet associates in mice with modest changes in term of metabolic profile mainly due to reduced total and HDL cholesterol with no important effects on weight, insulin, LDL cholesterol and triglycerides; (iii) ALA supplementation reduces cerebral infarct size after tMCAO and ameliorates post-stroke neurological deficit; (iv) improved stroke outcome in ALA-supplemented animals associates with reduced BBB functional impairment and endothelial activation; (v) dietary ALA-supplementation associates with reduced activation of mononuclear phagocytes in the penumbra area of the stroke and lowers levels of neurotoxic MMPs.

ALA is an essential n-3 fatty acid that is naturally contained in vegetable oils (e.g. flaxseed). Thus, ALA is thought to represent an eco-friendly, widely available, low cost and more sustainable alternative to the widely studied, marine-derived n-3 fatty acids EPA and DHA, whose use is restricted by limited availability, high costs, fishing restrictions and water pollution [38,39]. In humans, ALA consumption is associated with reduced primary and secondary cardiovascular events [40]. Of interest, in a large cohort consisting of 20 069 generally healthy subjects ranging from 20 to 65 years old, low ALA intake was a risk factor for





**Fig. 4.** Dietary supplementation blunts I/R-induced mononuclear phagocyte activation and penumbral MMP content. (A) Representative pictures and quantification of immunostaining for the activated microglia/macrophage marker Iba1 (red) in the penumbra area of the brain 48 h after tMCAO in the two study groups. (B) Representative pictures of immunostaining for MMP-9 (magenta) and the endothelial marker CD31 (red) in the penumbra area 48 h after tMCAO in the study groups. Quantification of MMP-9 was normalized to total endothelial surface. (C) Representative pictures of immunostaining for MMP-3 (green) and the endothelial marker CD31 (red) in the penumbra area 48 h after tMCAO in the study groups. Quantification of MMP-3 was normalized to total endothelial surface. n = 6–8. I/R = ischemia/reperfusion, MMP = matrix metalloproteinases; tMCAO = transient middle cerebral artery occlusion. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

incident stroke, while the higher quintile of consumption associated to a 35–50% lower risk [41]. Although the molecular mechanisms of its protective effects are not completely characterized, several experimental and clinical observations point towards reduced inflammation as a possible mediator [42]. In a very recent systematic review and meta-analysis of randomized control trials including 32 different studies, flaxseed intake was significantly associated with a reduction in circulating levels of C-reactive protein and TNF- $\alpha$  [43]. Accordingly, in our experimental setup, animals fed with high ALA diet showed reduced baseline NLR and circulating TNF- $\alpha$  compared to low ALA-fed littermates, meanwhile showing similar body weights and modest reductions of total and HDL cholesterol levels. Among the different anti-inflammatory mechanisms of ALA, this mediator can be converted to DHA and then resolvins or protectins. Among them, neuroprotection D1 (PD1) is synthesized by microglia and peripheral blood monocyte to suppress inflammation and promote T-cell apoptosis. PD1 is protective in experimental models of ischemic stroke and could play a role in the observed anti-inflammatory ALA effect [44]. In humans, baseline NLR can predict ischemic stroke occurrence alongside its clinical outcome [45]. Similarly, circulating and cerebrospinal fluid TNF- $\alpha$  levels are increased in patients after ischemic stroke and correlate with its severity [46].

In line with ALA's immunomodulatory effects and the known deleterious role of inflammation in the early ischemic stroke pathophysiology, long-term dietary ALA supplementation blunted stroke size and neurological deficit as assessed by Bederson score in our experimental setting. Of interest, high ALA-fed animals also showed a tendency towards an increased survival 48 h after the ischemic events which, however, did not reach statistical significance. Our findings are in line with previous reports showing ALA as a neuroprotective agent against focal and global ischemia in rodent models [47–50]. However, our report holds additional novel value relating to the duration of the treatment as well as its route of administration and dosage. First, we used for the first time a 12-months dietary intervention, since data on such long-term exposure are as of yet lacking in the experimental setting and in humans are often subject to relevant confounders, such as co-medication, co-morbidities and general lifestyle preferences. Such an experimental set-up allowed us to perform tMCAO in 18 months-old mice that are generally considered as old animals. Therefore, our findings might have translational value in the highly susceptible and affected elderly population, which is paradoxically under-represented in clinical trials. Of interest, in both rodents and humans, aging associates to a chronic low-grade activation of the innate immune system in absence of appropriate immunogenic stimuli [51]. Such a dysfunctional process is termed « inflamm-aging » and thought to play a major role in several age-associated CV diseases including ischemic stroke [52]. From this point of view, the reduction of NLR and TNF- $\alpha$  shown by the high ALA group might suggest - for the first time - that long-term dietary supplementation of this compound could effectively blunt the inflamm-aging process. Further studies specifically investigating this aspect and assessing the inflammatory status in a more comprehensive way are needed to confirm this first observation. Additionally, we selected a dietary intervention rather than an intravenous treatment, as it is more translatable and relevant to the human situation in a primary preventive setting. Importantly, the ALA concentrations herein employed are of clinical relevance as they have been shown to associate with plasma levels of ALA ranging from 17 to 19  $\mu\text{mol/L}$ , whereas daily supplementation of 3 g of ALA in humans leads to ALA plasma levels of  $32 \pm 17 \mu\text{mol/L}$  and is well tolerated [11,53].

Previous studies identified direct neuroprotective effects of n-3 fatty acids through potassium channel rectification and reduction of glutamate-mediated excitotoxicity [50,54,55]. Here, we found that ALA reduces post-stroke BBB damage and endothelial activation by preserving occludin levels and reducing endothelial expression of VCAM-1 and P-selectin, but not ICAM-1. BBB function deeply impacts on ischemic stroke outcomes; accordingly, imaging studies in human associated

increased BBB disruption with hemorrhagic transformation, worsened Rankin scores and impaired recovery at time of discharge [56]. Of interest, both post-stroke junctional protein disassembly and BBB endothelial activation are inflammation-dependent mechanisms highly regulated by TNF- $\alpha$ /NF- $\kappa\text{B}$  signaling pathways [27,57,58]. Alongside the effects on TNF- $\alpha$  circulating levels that we showed in the current report, ALA was previously shown to reduce NF- $\kappa\text{B}$  signaling in different settings [47,59]. Endothelial expression of adhesion molecules together with the increased BBB permeability increase infiltration of circulating monocytes towards the penumbra area where they get activated and release different deleterious mediators including MMPs [16]. By reducing occludin degradation and blunting endothelial expression of adhesion molecules, high ALA diet could reduce the surge of cellular inflammation in the penumbra area as outlined by reduced counts of activated mononuclear phagocytes. This beneficial effect likely leads to reduced levels of MMP-9 and MMP-3 - known mediators of brain damage through increased junctional protein degradation and direct neurotoxic effects [60–62]. Of interest, TNF- $\alpha$  is a well-recognized inducer of MMP synthesis and release by microglia/macrophages and ALA was previously shown to dampen the inflammatory phenotype of classically-activated M1 macrophages by a specific set of mechanisms distinct from those described for EPA and DHA [60,63].

Noteworthy, this study only employed male mice to exclude a possible role for sex hormones in the observed effects, nevertheless this represents a limitation that will be overcome by follow-up projects.

In conclusion, we demonstrate that long-term dietary supplementation with the plant-derived ALA significantly ameliorates ischemic stroke outcome in an experimental model. Through its protective anti-inflammatory effect, ALA reduces mononuclear phagocyte activation in the penumbra area of the stroke, MMP production, junctional protein degradation, BBB damage and dysfunctional endothelial activation. In consideration of the limited therapeutic strategies available for stroke treatment (particularly in elderly patients), there is great need for randomized controlled trials to confirm the beneficial effects observed in the experimental setting in patients suffering ischemic stroke.

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#### CRedit authorship contribution statement

**Nicole R. Bonetti:** conceived and planned the study, performed experiments, Formal analysis, Writing – original draft, analyzed the data and drafted the manuscript. **Luca Liberale:** conceived and planned the study, performed experiments, Formal analysis, Writing – original draft, analyzed the data and drafted the manuscript. **Alexander Akhmedov:** provided critical technical support. **Lisa Pasterk:** performed experiments. **Sara Gobatto:** performed experiments. **Yustina M. Puspitasari:** performed experiments. **Ana Vukolic:** performed experiments. **SS:** performed experiments. **Bernd Coester:** performed experiments, Formal analysis, Writing – original draft, analyzed the data and drafted the manuscript. **Carla Horvath:** performed experiments, Formal analysis, Writing – original draft, analyzed the data and drafted the manuscript. **Elena Osto:** performed experiments, Formal analysis, Writing – original draft, analyzed the data and drafted the manuscript. **SK:** performed experiments. **Thomas F. Lüscher:** conceived

and planned the study. **Jürg H. Beer:** conceived and planned the study. **Giovanni G. Camici:** conceived and planned the study, Formal analysis, Writing – original draft, analysed the data and drafted the manuscript, All authors revised the article for important intellectual content and approved the article.

#### Author contributions

LL, NRB, TFL, JHB and GGC conceived and planned the study. LL, NRB, LP, SG, YMP, AV, SS, BC, CH, EO, SK performed experiments. AA provided critical technical support. LL, NRB, BC, CH, EO, and GGC analysed the data and drafted the manuscript. All authors revised the article for important intellectual content and approved the article.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: LL and GGC are coinventors on an international patent that was approved in April 2020. The patent relates to the use of antibodies which specifically bind IL-1 $\alpha$  to reduce various sequelae of ischemia-reperfusion injury to the central nervous system. All other authors report no conflict of interest.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.atherosclerosis.2021.04.005>.

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