ADVANCED BIOLOGY
www.advanced-bio.com

Treatment with Cerebrolysin Prolongs Lifespan in a Mouse Model of Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy

*Birgit Kastberger, Stefan Winter, Hemma Brandstätter, Janina Biller, Wolfgang Wagner, and Nikolaus Plesnila**

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a rare familial neurological disorder caused by mutations in the *NOTCH3* gene and characterized by migraine attacks, depressive episodes, lacunar strokes, dementia, and premature death. Since there is no therapy for CADASIL the authors investigate whether the multi-modal neuropeptide drug Cerebrolysin may improve outcome in a murine CADASIL model. Twelve-month-old *NOTCH3*^{R169C} mutant mice (n=176) are treated for nine weeks with Cerebrolysin or Vehicle and histopathological and functional outcomes are evaluated within the subsequent ten months. Cerebrolysin treatment improves spatial memory and overall health, reduces epigenetic aging, and prolongs lifespan, however, CADASIL-specific white matter vacuolization is not affected. On the molecular level Cerebrolysin treatment increases expression of Calcitonin Gene-Related Peptide (CGRP) and Silent Information Regulator Two (Sir2)-like protein 6 (SIRT6), decreases expression of Insulin-like Growth Factor 1 (IGF-1), and normalizes the expression of neurovascular laminin. In summary, Cerebrolysin fosters longevity and healthy aging without specifically affecting CADASIL pathology. Hence, Cerebrolysin may serve a therapeutic option for CADASIL and other disorders characterized by accelerated aging.

1. Introduction

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL, International Classification of Diseases, 11th Revision: 8B22.C0) is a hereditary neurological disorder, first described in the 1950s,[1] with characteristic symptoms emerging in mid-to-late adulthood. These symptoms include migraine, recurrent subcortical strokes, mood disturbances, psychiatric symptoms, cognitive deficits, and eventually dementia, leading to a reduced lifespan.[2]

In 1996, Joutel and colleagues discovered mutations in the *NOTCH3* gene to be the underlying cause of CADASIL.[3] *NOTCH3* mutations trigger misfolding and subsequent aggregation of the extracellular domain (ECD) of the NOTCH3 protein in the extracellular matrix (ECM) between endothelium and pericytes, that are also called vascular smooth muscle cells and pericytes.[4] The resulting NOTCH3^{ECD} aggregates are believed to sequester additional ECM proteins among others also laminin, leading to the accumulation of granular osmiophilic material (GOM) within small- to medium-sized blood vessels, potentially impairing vessel function, most prominently in the brain.[4] Therefore, such accumulations of ECM proteins within GOMs seem to contribute to vasculopathy and vessel function impairment in CADASIL patients.[5]

B. Kastberger, S. Winter, H. Brandstätter
Ever Pharma
Oberburgau 3, Unterach am Attersee 4866, Austria
N. Plesnila
Cluster of Systems Neurology (Synergy)
81377 Munich, Germany
E-mail: nikolaus.plesnila@med.uni-muenchen.de

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adbi.202300439

DOI: 10.1002/adbi.202300439

J. Biller, N. Plesnila
Institute for Stroke and Dementia Research (ISD)
University Hospital
LMU Munich
81377 Munich, Germany
W. Wagner
Institute for Stem Cell Biology
RWTH Aachen University Medical School
52074 Aachen, Germany
W. Wagner
Helmholtz Institute for Biomedical Engineering
RWTH Aachen University
52074 Aachen, Germany
W. Wagner
Cygenia GmbH
52078 Aachen, Germany

Interestingly, NOTCH3 signal transduction is not impaired in CADASIL and mutations of the *NOTCH3* gene are required but not sufficient for disease onset as only one person in 1000 with CADASIL relevant *NOTCH3* mutations is diagnosed with the disease.[6]

Currently, no curative treatment exists for CADASIL. Thus, therapeutic approaches are limited to symptomatic treatments, such as antidepressants to manage mood disturbances and lorazepam or phenytoin for migraine attacks.[7,8] Hence, there is an urgent need to explore new therapeutic options for CADASIL patients.

Cerebrolysin, a neuropeptide-based drug approved for various brain diseases, has shown promise in enhancing neuroplasticity and neurorecovery in clinical and preclinical studies. Cerebrolysin is approved for the treatment of stroke, traumatic brain injury and various forms of dementia, diseases with symptoms also seen in CADASIL. Cerebrolysin is also known as beneficial add-on therapy for endovascular treatment of stroke patients, as Cerebrolysin administration alleviates tPA (Tissue Plasminogen Activator) induced reperfusion injury.[9] Furthermore, it was demonstrated by recent clinical and preclinical trials that Cerebrolysin treatment led to improved recovery from, and limitation of neurological deficits after primary insults, such as ischemic stroke.[10] The efficacy and safety of Cerebrolysin were demonstrated in numerous preclinical and clinical trials and summarized in several meta-analytical studies and systematic-reviews.[10,11]

Cerebrolysin operates through a diverse array of pathways. Current clinical and preclinical evidence indicates that the low molecular weight peptides present in Cerebrolysin stimulate neuroplasticity via prominent signaling pathways, including Phosphoinositide 3-kinase/Protein Kinase B,[12] sonic hedgehog,[13] and various neurotrophins implicated in neuronal recovery, such as Brain-Derived Neurotrophic Factor,[14] Vasular Endothelial Growth Factor,[15] Insulin-like Growth Factor 1 (IGF-1),[16] Ciliary Neurotrophic Factor,[17] and Nerve Growth Factor.[18] These findings align with the observation that Cerebrolysin has neurotrophic activities[18,19] and facilitates functional recovery of neurological deficits.[11,20]

Considering this clinical and preclinical profile, we hypothesized that Cerebrolysin may also have a therapeutic potential for CADASIL. To test this hypothesis, we conducted a study involving 176 12-month-old male and female CADASIL mice (*NOTCH3*^{R169C}) for 9 weeks over a period of 5 months. The mice were treated with either Vehicle control or Cerebrolysin, following a fully randomized and blinded protocol. Cognitive function, general health, epigenetic age, lifespan, histopathology, and molecular markers of aging were carefully evaluated to investigate the potential therapeutic effects of Cerebrolysin in CADASIL.

2. Experimental Section

2.1. CADASIL Mouse Model

In the current study Tg*NOTCH3*^{R169C} [21] was used that were developed by Joutel et al.[22] These animals were considered one of the most relevant animal models for CADASIL.[23] The model was developed on an FVB/N background and overexpresses 4–5 times rat *NOTCH3* carrying the human R169C mutation.[22] *NOTCH3*^{R169C} mice boast an early onset of vascular NOTCH3 accumulation and subsequently develop intra-myelinic vacuoles in the white matter. These vacuoles were thought to result from mini-ischemic stroke events which resemble the ones found in CADASIL patients.[23,24] Eighteen breeding pairs were generated, and mice born between October 2018 and February 2019 were genotyped and subsequently used for experiments. Animals were kept at a 12 h/12 h day/night rhythm in a specific pathogen free facility and had free access to water and standard chow (Ssniff V1534, Ssniff, Soest, Germany). No more than five animals were kept in one cage. For histological controls, non-transgenic, wild type (wt) littermates were aged 13 months.

2.2. Study Protocol

Nine experimental groups with n = 20–25 mice each were investigated for up to 34 months (**Figure 1**) in a fully randomized and blinded manner, that is animals were randomly assigned to their respective experimental group and group allocation was concealed until all data was collected and analyzed. Blood samples were collected once per month by check pouch puncture from all mice after the age of four months. All 176 transgenic mice were aged for at least 12 months.

Mice of cohort 1 were treated with Vehicle (n = 21) or Cerebrolysin (n = 23) for three weeks by daily intraperitoneal (i.p.) injections and sacrificed one week after the last treatment at an age of 13 months. Wt littermates were sacrificed in parallel with cohort 1.

Mice of cohort 2 were treated with Vehicle (n = 20) or Cerebrolysin (n = 25) for three weeks by daily i.p. injections. Treatment was repeated after a treatment-free interval of five weeks and animals were sacrificed one week after the last treatment at an age of 15 months.

Mice of cohort 3 were treated with Vehicle (n = 25) or Cerebrolysin (n = 22) for three weeks by daily i.p. injections. Treatment was repeated twice after a treatment-free interval of five weeks each and animals were sacrificed one week after the last treatment at an age of 17 months.

Mice of cohort 4 (Vehicle: n = 20; Cerebrolysin: n = 20) were treated like mice in cohort 3 but were allowed to survive until dying spontaneously or reaching humane endpoints. Mice in this cohort were also tested before and bi-monthly after the initiation of treatment for behavior and memory function by using the Y-maze paradigm.

2.2.1. Treatment Protocol

Cerebrolysin (2.5 ml kg^{-1}) or Vehicle (equivalent amount of 0.9% NaCl) treatment was initiated twelve months after birth. One treatment cycle lasted three weeks and consisted of one i.p. injection per day given Monday to Friday. Each treatment cycle was followed by a treatment-free interval of five weeks.

2.2.2. Blood Sampling

Blood (50–150 µl) was collected monthly starting from 4 months of age by cheek pouch puncture in EDTA-tubes (Sarstedt,

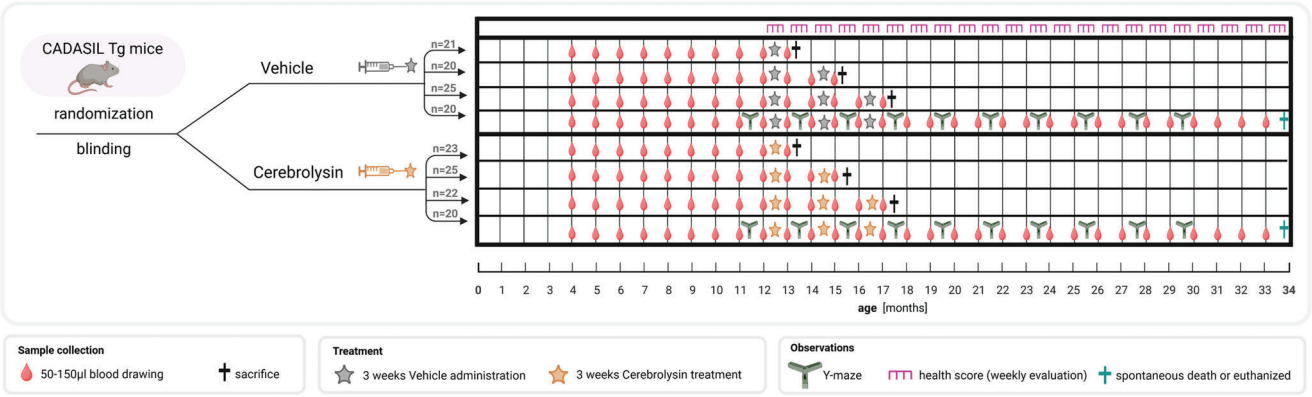

Figure 1. The CADASIL mouse model developed by Joutel et al. 2010 was used for the current study.[22] NOTCH3^{R169C} mutant (CADASIL) animals were aged for 12 months and then subjected to three treatment cycles with Cerebrolysin®. For each cycle animals received Cerebrolysin i.p. Mo-Fr for three weeks. Cycles were separated by five treatment-free weeks. Pre-defined groups were sacrificed after 13, 15, or 17 months or left alive until spontaneous death or reaching humane endpoints. Functional assessment, blood drawing etc. were conducted as outlined. $n = 20–25$ mice per group.

#41.1395.005) and incubated at room temperature for 30 min. The blood was then centrifuged for 10 min at 3000 x g and 4 °C. Plasma and cell pellet were separated and stored until further analysis at −80 °C.

2.2.3. Health Score

Animals were inspected daily by trained staff and their health status was scored using a standardized score sheet including body weight, level of consciousness, general condition (grooming behavior, muscle tone), spontaneous behavior (sleep, social interaction, curiosity), locomotor activity, clinical condition (body temperature, breathing). Depending on their performance in each of these categories mice received 0 (normal) to 4 (burdened) points. A sum of 20 or more points was defined as "humane endpoint" and animals were sacrificed.

2.2.4. Ethics Statement

Mice were bred in the animal facility of the Institute of Stroke and Dementia Research. Experiments were approved by the Ethical Review Board of the Government of Upper Bavaria (Tierversuchsvorhaben Vet_02-19-47).

2.3. Randomization and Blinding

All experiments were performed and analyzed in a strictly randomized and blinded manner. Blinding was terminated only after all animals were sacrificed and all data was analyzed.

2.4. Protein Analysis

2.4.1. CGRP

Enzyme-linked immunosorbent assay (ELISA) kits were used to determine the serum levels of Calcitonin Gene-Related Peptide (CGRP, abx054093, Abbexa Ltd, Cambridge, UK). Briefly, standards and samples (1:6,67 dilution) were prepared, 100 µl added into the pre-coated 96-well plate and incubated for 2 h at 37 °C. The liquid was discarded, 100 µl detection reagent A (biotin-conjugated anti-CGRP antibody, 1:100 dilution) added and incubated for 1 h at 37 °C. After washing (3 times with wash buffer, 1:25 dilution), 100 µl detection reagent B (HRP-conjugated avidin, 1:100 dilution) was added and incubated for 1 h at 37 °C. After washing (5 times with wash buffer, 1:25 dilution), 90 µl tetramethylbenzidine substrate were added and incubated for 3 to 5 min at 37 °C, the color development was stopped using 50 µl stop solution and the plates were read at 450 nm using a microplate reader (TECAN Spark). Samples and standards were measured in triplicates.

2.4.2. IGF-1

ELISA kits were used to determine the mouse serum levels of IGF-1 (ab100695, Abcam) following the manufacturers' instructions. Results were assessed by colorimetric detection at 450 nm absorbance using LT-4000 microplate reader (Euroclone).

2.4.3. SIRT6

ELISA kits were used to determine the mouse serum levels of SIRT6 (MBS2885961, MyBioSource, Inc. San Diego, USA) following the manufacturers' instructions. Results were assessed by colorimetric detection at 450 nm absorbance using LT-4000 microplate reader (Euroclone).

2.5. Immunohistochemistry

Mice were deeply anesthetized with medetomidine (0.5 mg kg^{-1}), fentanyl (0.05 mg kg^{-1}), and midazolam (5 mg kg^{-1}) and sacrificed by transcardial perfusion with 0.9% sodium chloride (NaCl) and 4% paraformaldehyde (PFA). Brains were removed and post-fixed overnight in 4% PFA. Free-floating coronal brain sections (50 µm thick) were prepared using a VS1200 vibratome (Leica

Microsystems, Wetzlar, Germany). Sections were either collected in phosphate-buffered saline (PBS) for immediate use or in a cryoprotectant solution for later use. For immunostaining three sections per mouse were blocked with 3% bovine serum albumin (wt/vol) for 60 min and then incubated with the primary antibody diluted in blocking solution overnight at 4 °C. Sections were then washed in PBS twice and incubated with fluorophore-conjugated secondary antibodies for 2 h at room temperature. Sections were then washed and mounted using Fluoromount mounting medium (Sigma, St Louis, MO). The following primary antibodies were used: anti-albumin (1:100, Abcam, ab19194 Mouse) for evaluation of blood brain barrier (BBB) permeability and anti-laminin for visualizing cerebral vessels (1:200; Sigma, L9393). To visualize albumin, sections were incubated with a Cy3-conjugated donkey anti-mouse antibody (1:100, Jackson ImmunoResearch, 715 165 150). For the visualization of laminin an Alexa Fluor 680 goat anti-rabbit IgG (1:100; Invitrogen, Carlsbad, CA; A21109) was used.

2.6. Image Analysis

All tissue sections were imaged using a Zeiss Axiovert 200 M inverted fluorescence microscope and a Leica TCS SP5 II confocal microscope as previously described.[21] Quantitative image analysis was performed by an investigator who was blinded with respect to the genotype and treatment group using ImageJ software (NIH, Bethesda, MD).

To quantify capillary density, maximum projection z stacks were reconstructed. The laminin-positive signal in the blood vessels was subjected to automated threshold processing after background correction, and the signal was quantified using the Area Measurement analysis tool in ImageJ. In each mouse, four Region Of Interests (ROIs) in the cortex were analyzed by randomly selecting four fields. These fields were analyzed in three non-adjacent sections. Six animals per group were analyzed.

To quantify extravascular albumin accumulation, maximum projection z stacks were reconstructed. The albumin- and laminin-positive signal outside blood vessels was subjected to threshold processing after background correction, and the signal was quantified using the Integrated Density analysis tool in ImageJ. In each mouse, four ROIs in the cortex were analyzed by randomly selecting 4 fields. These fields were analyzed in three non-adjacent sections. Six animals per group were analyzed.

2.7. Y-maze Test

Mice of cohort 4 were evaluated by the Y-maze test one week before the initiation of treatment and every second month thereafter. For each measurement two different Y-maze test protocols were used: 1) Spontaneous Agility Test and 2) new object stimulus experiment with protocols as previously described.[25]

For the Spontaneous Agility Test (Figure 3A) each mouse was placed in one of the arms of the Y-maze and allowed to move freely for 5 min in the Y-maze. Agility parameters and correct spontaneous alternation frequencies were recorded by an automated video tracking system. In case the mouse remembered previous visits, it was expected to visit all arms consecutively ("correct alternation"). Test results were expressed as the number of correct alternations. The measurement of agility was used as indicator for frailty. Agility was compared between the Vehicle and Cerebrolysin treated group for mice with 23 months to 29 months of age. The starting-point of 23 months of mouse age was chosen by based on Baumann et al, 2020[26] who demonstrated that physical decline not only correlates with frailty and life-span in mice and humans but furthermore that physical decline becomes statistically evident in mice from the age of 23 months onwards. The last Y-maze measurement was taken at 29 months although some mice survived longer, due to the fact that after 29 months too few animals in the Vehicle group were still alive that could be tested. The period between 23 to 29 months represents a very advanced age for mice.[27]

Spatial memory, mood, and curiosity were assessed using a Y-Maze novelty preference paradigm (Figure 3B). Animals explored a Y-maze for 7–10 min with one blocked arm (exposure training). The next day, animals were returned to the maze with all arms open and monitored for another 5 min. In case the mouse remembered previous visits, it was expected to visit all arms consecutively ("correct alternation"). Test results were expressed as the number of correct alternations.

2.8. Epigenetic Age Analysis

Epigenetic age was estimated based on DNA methylation analysis at three age-associated CG dinucleotides (CpGs) as previously described.[28] Genomic DNA was isolated from 50 µl blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). DNA concentration was quantified by Nanodrop 2000 Spectrophotometers (Thermo Scientific, Wilmington, USA). 200 ng of genomic DNA was subsequently bisulfite-converted with the EZ DNA Methylation Kit (Zymo Research, Irvine, USA) and subjected to PCR amplification within the genes *Prima1*, *Hsf4*, and *Kcns1*.[28] Amplicons were sequenced on PyroMark Q96 ID System (Qiagen, Hilden, Germany) and analyzed with PyroMark Q CpG software (Qiagen). The epigenetic age (in week) was then estimated based on DNA methylation at CpGs in *Prima1*, *Hsf4*, and *Kcns1*, as described before.[28] Epigenetic age acceleration was determined by subtracting the chronological age from the epigenetic age predictions.

2.9. Statistical Methods

Data was tested for normal distribution and based on the results analyzed with Analysis of Varince (ANOVA), t-, Chi square-, or F-test using GraphPad Prism 7.04. Longitudinal data sets were tested against each other as a whole. If significant differences were detected, individual time points were tested pairwise. For the analysis of mortality mice were stratified within four quartile subgroups classified by age of death as previously described.[29]

3. Results

3.1. Cerebrolysin Treatment did not Impact Vacuole Formation but Reduced Load of Laminin Deposits in Cadasil Mice

All animals were healthy at the beginning of the treatment period (Weight: Figure S1, Supporting Information, average health

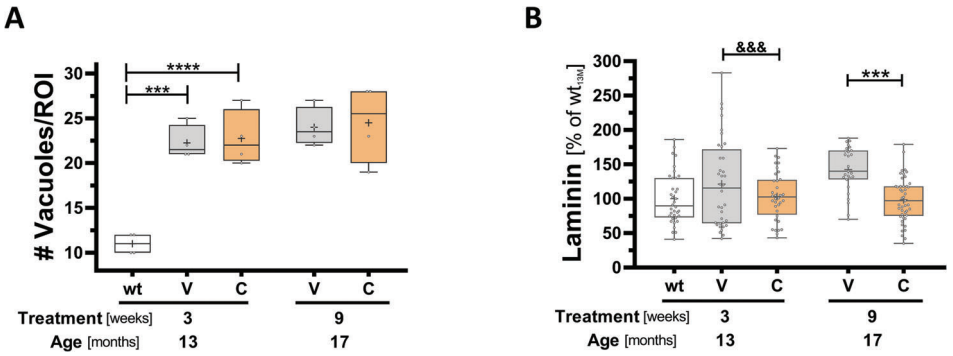

Figure 2. A) Vacuole numbers: No change in vascular density in treated versus untreated CADASIL mice. As in previous studies, wt mice' vacuole numbers at 13 M were significantly lower than the vacuole numbers of both CADASIL treated ($p < 0001$) and untreated ($p = 0.001$) mice. 1-way ANOVA multiple comparisons. Vacuole numbers at 13 M. Average from mice of 13, 15, and 17 months of age. Treatment was administered between the age of 12 months to 17 months. box and whisker blot with max and min borders, "+" symbol indicates the mean value. B) Cerebrolysin reduces the overexpression of laminin in brain vessels in aged CADASIL mice. The analysis was performed at 13 months and 17 months mouse age. Results were normalized to the mean measurement of wildtype (wt) mice, $n = 18$–45 mice per time point. Box and Whisker plot with" +" symbol representing the mean of the distribution. Test of variances F-test: $^{\&\&\&}p = 0.0005$, unpaired two tailed t-test $^{***}p \leq 0.0001$ of distribution statistics.

score at 12 months of age for Cerebrolysin and Vehicle group below 1: data not shown).

To assure that the transgenic animals used in the current study developed the expected pathology,[23,30] we investigated white matter lesions in the corpus callosum by counting vacuoles and blood brain barrier leakage by immunohistochemistry of albumin extravasation at 13, 15, and 17 months of age. Both parameters showed the expected CADASIL-specific changes (vacuoles: **Figure 2**A, albumin: data not shown), demonstrating that all mice used for the current study developed the expected disease phenotype of 1,7 times vacuole number increase and a leaking BBB validated by albumin presence in the mouse brain.

Agglomerated laminin in the CADASIL specific GOMs is considered a biomarker for disease burden. Therefore, we assessed laminin quantity in cerebral vessels by brain tissue staining. By histological quantification, we observed a statistically significant accumulation of laminin in untreated CADASIL mice over time, from age 13 months to the age of 17 months (Figure 2B). This accumulation was not observed in Cerebrolysin treated animals and laminin levels were significantly lower compared to the Vehicle treated controls at 13 and 17 months of age (Figure 2B)

In conclusion, while treatment with Cerebrolysin had no effect on white matter injury or blood-brain barrier permeability at any of the investigated time points; (Figure 2A) Cerebrolysin treated mice showed a significantly reduced amount of laminin deposits after 9 weeks of treatment (17 months of age), relative to Vehicle treated animals, that were comparable to wt animals.

3.2. Improved Agility and Cognition in CADASIL Mice

Using the Y-maze Spontaneous Agility Test we could not detect a change of alternate arm visits, however, the agility of CADASIL mice receiving Cerebrolysin was significantly higher than in the Vehicle treated group (23–29 months of age) (**Figure 3**A). In an environment without fear and minimal stress – as mice could

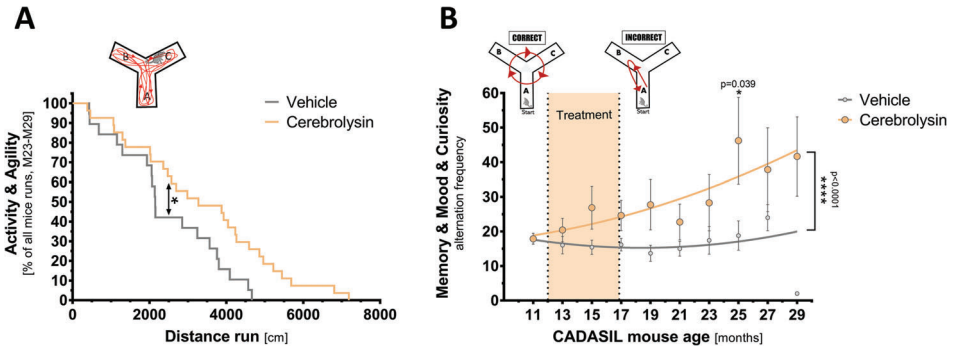

Figure 3. Cerebrolysin increases mobility, curiosity, and spatial memory in CADASIL mice in a Y maze experiment with CADASIL mice with and without Cerebrolysin treatment. A) Spontaneous Agility Test: Mobility measurements of CADASIL mice taken in their last quarter of measured life (23 and 29 months) plotted as Kaplan-Maier-curve.[5,53] Plotted are all mouse run values measured between M23-M29. Chi square comparison: p = 0.02 by Log-rank (Mantel-Cox) test, $n = 19$ Vehicle, $n = 27$ Cerebrolysin mice runs. B) New object stimulus experiment: The spatial memory is measured by the alternation frequency in a new object stimulus experiment. Cerebrolysin increases the spatial memory in aged CADASIL mice, polynomial fit of regression curve, curve comparison F-test, $p < 0.0001$- different curves for each data set. $n = 138$ Vehicle and $n = 156$ Cerebrolysin mice measured.

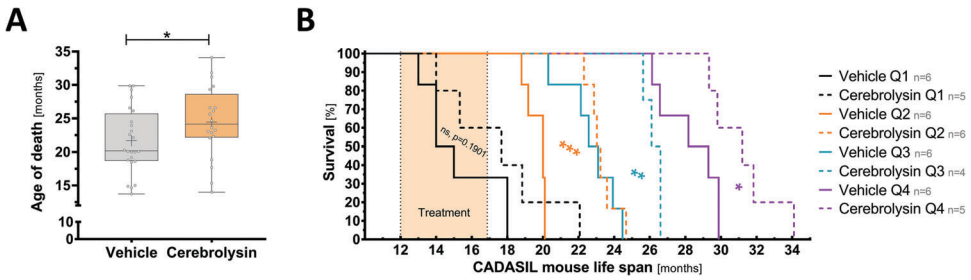

Figure 4. Prolonged survival by Cerebrolysin. A) Cerebrolysin treatment increases CADASIL mouse life span by 3 months or 13%. P = 0.0396, one-tailed unpaired t-test, Cerebrolysin $n = 20$, Vehicle $n = 24$, Box-and-Whisker Plot, dots representing each one mouse's death age, "+" indicates mean of the distribution. B) Cerebrolysin reduces the risk of death and therefore increases the life-expectancy in CADASIL mice, Kaplan-Meier survival curves for CADASIL mouse death events across quartiles: CADASIL mice live significantly longer when treated with Cerebrolysin for 3 months in quartiles Q2, Q3, and Q4. * $p < 0.05$, *** $p < 0.001$, Cerebrolysin $n = 20$, Vehicle $n = 24$ mice.

freely explore the arms of the Y-maze for several minutes – the locomotor activity showed improved agility, or less frail movement capacities of the Cerebrolysin treated CADASIL mice. This Y-maze mobility test, performed in an open Y-maze experiment, revealed that 6 months post Cerebrolysin treatment, CADASIL mice showed increased agility and curiosity compared to Vehicle treated mice.

When using the Novelty Preference test paradigm,[31] we found that treatment with Cerebrolysin significantly increased the spatial and working memory of the CADASIL mice over time (Figure 3B). The increase in spatial memory was not confined to the treatment period but became even more pronounced within the 12 months following the end of treatment (Cerebrolysin M25 vs M17 ***$p = 0.004$/Vehicle M25 vs M17 $p = 0.9$) suggesting that Cerebrolysin treatment may have long-lasting effects on mood and memory function.

3.3. Improved Life-Span and General Health Status in CADASIL Mice upon Treatment with Cerebrolysin

There was a significant difference in the mean life expectancy between the Cerebrolysin and Vehicle treated groups (Vehicle control mean 21.7 ± SEM 1.0 months, $n = 24$, Cerebrolysin mean 24.4 ± SEM 1.2 months, $n = 20$, *$p = 0.04$, **Figure 4**A).To monitor survival over time, animals were stratified into 4 quartiles based on age at death; treatment with Cerebrolysin was associated with a significantly longer life span of 2 to 4 months in all, except the first (youngest) quartile (here only a positive trend was observed) (Figure 4B). The oldest Vehicle treated animal died at an age of 30 months, whereas the oldest Cerebrolysin treated animal died at an age of 34 months (Figure 4B).

In addition to overall lifespan, the health score of the animals was monitored from an age of 11 months until death. Both groups showed a rapid deterioration of the health status within the terminal month of the animals' life (**Figure 5**A). Cerebrolysin treated CADASIL mice showed a significantly improved health status during the terminal two weeks of their life (health score Cerebrolysin mean 2.9 ± SEM 0.7; $n = 34$ vs Vehicle control mean 5.6 ± SEM 1.1; *$p = 0.0401$, $n = 32$, Figure 5A). This was also reflected by analyzing the cause of death; 60% of the animals in the Cerebrolysin group died spontaneously and 40% required euthanasia while in the Vehicle group ≈20% spontaneous deaths and 80% euthanasia were observed (Figure 5B).

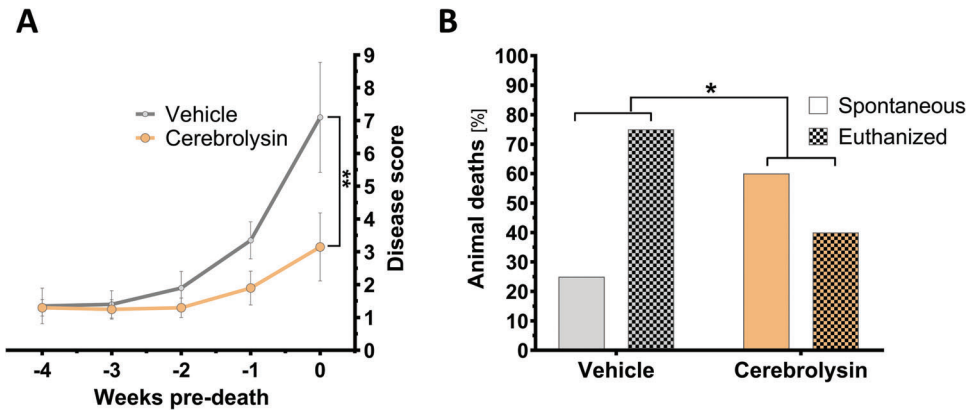

Figure 5. Cerebrolysin prolongs the total health span in CADASIL mice. A) Health span: treated mice present with lower disease scores (average score sum +/- SEM, SEM = Standard Error of Mean) in the last 4 weeks of their life, meaning CADASIL mice live a healthier life till their death, they are less frail during life. Curve comparison with F-test,**$p = 0.0011$, $n = 20$ Cerebrolysin and $n = 20$ Vehicle mice were weekly scored until their death. B) Death cause chart: Cerebrolysin increases the healthiness of CADASIL mice thereby increasing the number of spontaneous age-related deaths and reducing the number of mice which had to be euthanized due to reaching humane end points based on disease burden. *$p = 0.0252$, two-sided Chi square test, mouse number: $n = 20$ for Cerebrolysin and $n = 20$ for Vehicle.

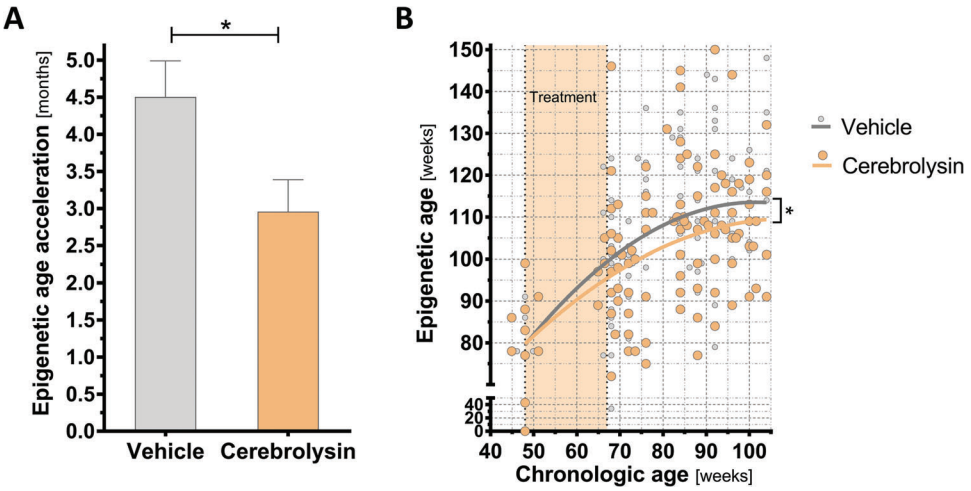

Figure 6. Cerebrolysin treatment reduces epigenetic age acceleration in CADASIL mice. A) The epigenetic age of Cerebrolysin treated CADASIL mice is by 1.55 months younger than in placebo group which is a deceleration by more than 34%. Number of blood samples included: $n = 84$ Vehicle, $n = 100$ Cerebrolysin, t-test two tailed, unpaired, $p = 0.0182$, blood samples drawn at M17, 18, 19, 21, 22, 23, 24, 25, 26 of age in the CADASIL mice. B) Nonlinear curve fitting with second order polynomial function (quadratic) in Prism Graph. The two regression curves of Cerebrolysin and Vehicle are significantly different: F-test $^{*}p = 0.0392$ in 3-month moving average distribution of chronological age. The trendlines of the moving average curves are overlayed with all measured epigenetic measurements, one point represents one mouse sample. Vehicle $n = 95$, Cerebrolysin $n = 111$.

3.4. Epigenetic Age Acceleration is Reduced by Cerebrolysin Treatment

To further corroborate the increased life expectancy and health condition observed for Cerebrolysin treated animals, we included the analysis of the epigenetic age.[32–34] Overall, epigenetic age was accelerated in the CADASIL mice (**Figure 6**A). There was a significant difference in the epigenetic age acceleration between the Vehicle and Cerebrolysin treated groups (mean of epigenetic age acceleration [months]: Vehicle = 4.50 vs Cerebrolysin = 2.95, p = 0.0182, Figure 6A) Chronological and epigenetic age predictions revealed a clear correlation in both Cerebrolysin (Pearson r = 0.899, $R^2 = 0.8046$, p = 0.0004) and Vehicle group (Pearson r = 0.897, $R^2 = 0.8077$, p = 0.0004, Figure 6B) confirming the adequacy and precision of the epigenetic age measurement approach.

3.5. Cerebrolysin Treatment is Correlated with the Modulation of Biomarkers of Disease Burden, Cognition and Longevity

To investigate if the observed effects of Cerebrolysin treatment on life span and frailty are also reflected by the modulation of corresponding biomarkers (besides the epigenetic age) we studied the levels of CGRP, IGF-1, and SIRT6 in mouse plasma.

The three studied plasma molecules, CGRP, IGF-1, and SIRT6, have been described as health and age sensitive biomarkers for neuroplasticity, cognition, and longevity.[35] Cerebrolysin treatment was associated with a significantly increased expression of CGRP starting upon active treatment until 6 months after treatment was terminated (**Figure 7**A). IGF-1 levels decreased in both groups over time, however IGF-1 levels were significantly lower during Cerebrolysin treatment (Figure 7B). SIRT6 levels were also declining over time (Figure 7C). In contrast to IGF-1,

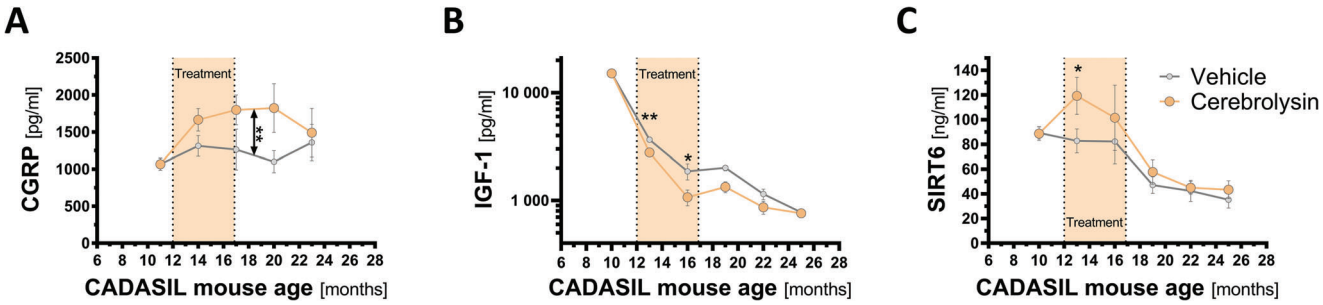

Figure 7. A) Cerebrolysin increases CGRP expression in CADASIL mice by 585 [pg ml^{-1}]. Vehicle and Cerebrolysin expression curves between 11 months and 23 months of age, curve comparison by F-test $^{**}p = 0.0082$, $n = 8$-48 mice per month B) Cerebrolysin decreases IGF-1 expression in CADASIL mice by 747 [pg ml^{-1}], Curve: $n = 6$-40, M13 Cerebrolysin versus Vehicle $^{**}p = 0.0089$, M16 Cerebrolysin versus Vehicle $^{*}p = 0.0375$, C) SIRT6 expression is increased about two-fold with Cerebrolysin treatment in CADASIL mice immediately after treatment start at 13 months of mouse age. M13 Cerebrolysin versus Vehicle $^{*}p = 0.0488$, $n = 6$–40 mice per month.

there was a significant peak increase in SIRT6 levels during the first month of active treatment with Cerebrolysin. Already during further Cerebrolysin treatment, 4 months after treatment start, one month before treatment period ended SIRT6 levels already readjusted to serum levels of untreated animals.

4. Discussion

4.1. Brief Summary of Findings

Our initial hypothesis that Cerebrolysin may have positive effects on CADASIL specific pathology such as vacuole formation could not be confirmed with our dataset. However, we surprisingly observed a clear therapeutic effect of Cerebrolysin on spatial memory, life span, epigenetic age, and frailty in the CADASIL mutant animals. These effects of Cerebrolysin treatment were associated with changes of biomarkers for longevity and cognitive performance exemplified by CGRP, IGF-1, and SIRT6[35] and markers of vascular pathology such as laminin.[36] Hence, our data suggest that Cerebrolysin improved the outcome in CADASIL mice by ameliorating age-related impairments without directly interfering with CADASIL pathology such as white matter lesions.

The R169C mutation is located in the ECD of NOTCH3 and is one of the most frequent CADASIL mutations in humans.[6,22,23,30] Among all CADASIL mouse models, the R169C NOTCH3 mutation is the only one showing a CADASIL specific early onset of vascular NOTCH3 accumulation with micro-infarcts[24] and subsequent white matter lesions[23] which are associated with disease burden.[37]

Although, CADASIL mice treated with Cerebrolysin displayed the same number of vacuoles as untreated animals, we noted an overall positive effect on the spatial memory performance that persisted even after treatment was discontinued. This suggests that treatment with Cerebrolysin has a positive effect on memory function independent of CADASIL pathology. Further experiments with aged wt mice will need to address this interesting finding in more detail.

A further surprising observation was the significant extension of lifespan in Cerebrolysin treated animals compared to the Vehicle control. Similar observations have been made previously in studies conducted in fruit flies[38] but have not been demonstrated in higher organisms such as rodents before. This long-lasting effect of Cerebrolysin may have been overlooked so far, because long-term follow-up studies like the current one have not been performed.

These observations of overall lifespan extension are corroborated by investigations on epigenetic age. CADASIL mice display accelerated epigenetic ageing when compared to an established reference cohort of wild type mice[28] (Figure 6). This phenotype may be CADASIL specific or may be caused by the different genetic backgrounds of the CADASIL mice and the reference cohort,[39] but in any case Cerebrolysin treatment significantly reduced accelerated aging in CADASIL mice and expanded the overall chronological lifespan of these animals. This is of specific relevance, since epigenetic age was reported to be closely correlated with cognitive function and frailty[33] and to reflect overall biological age and health better than chronological age.[40]

We also recorded the cause of death (spontaneous or euthanized) of the animals and monitored overall health status until death, showing that treatment with Cerebrolysin was significantly associated with higher rates of spontaneous (natural) deaths and lower disease scores in aged mice. Interestingly, the animals which died spontaneously of natural causes were epigenetically two months younger than animals which had to be euthanized because of reaching humane endpoints due to various diseases (Figure S2, Supporting Information). In conclusion, the data on overall life expectancy (chronological age), epigenetic age, and health status indicate that treatment with Cerebrolysin was associated with increased survival rates, reduced epigenetic aging and overall improved health status in the CADASIL mice. Given the established link between epigenetics of aging and multiple neurodegenerative diseases[41] such as Alzheimer's disease or stroke, and the demonstrated clinical efficacy of Cerebrolysin in these indications, it is tempting to speculate that Cerebrolysin may have a more general and novel effect on aging which needs to be investigated in future preclinical and possibly clinical studies.

To investigate if the observed effects of Cerebrolysin treatment on cognitive performance, aging and health status are also underpinned by established biomarkers for these processes, we investigated plasma levels of respective molecules. CGRP has been implicated in the onset of migraine in the general population[42] and migraine is a frequent symptom in early-stage CADASIL. However, it does not seem that CGRP has migraine inducing effects for CADASIL patients compared to the general population.[43] Even more, reduced vasodilation as consequence of reduced CGRP levels, was associated with worse outcome in CADASIL.[44] Interestingly, CGRP was reported to stimulate neurogenesis, angiogenesis, to reduce vascular inflammation and cell death,[45] and to decline (in plasma) during aging.[35,46] These biological effects of CGRP are potentially relevant for the progression of CADASIL and were found to be modified by treatment with Cerebrolysin in previous studies.[10] It is therefore interesting that in the current study Cerebrolysin treatment was associated with a significant increase of plasma CGRP levels and that this effect coincided with a reduced aging phenotype in treated mice. Hence, CGRP may be one of the mediators responsible for the observed positive therapeutic effects of Cerebrolysin.

In addition to CGRP, Cerebrolysin treatment also reduced plasma levels of IGF-1. Reduced IGF-1 levels were demonstrated to correlate with increased longevity,[47] reduced aging, and decreased risk of age-related pathology.[47] Therefore, the observed changes in IGF-1 levels may be another molecular mechanism involved in the aging process which was positively affected by Cerebrolysin treatment.

Low levels of SIRT6 are known to be associated with aging while elevated SIRT6 levels have been related to longevity,[47] enhanced neuroplasticity, and improved cognitive function.[48] Notably, we observed a significant increase of SIRT6 during treatment with Cerebrolysin, which is in line with the observed improvement of cognitive function, the favorable effect on epigenetic ageing and life expectancy.

Changes for the biomarkers IGF-1 and SIRT6 were present during the treatment with Cerebrolysin (between month 13–17) while plasma levels of CGRP remained elevated also several weeks post-treatment. Although IGF-1 and SIRT6 are already clearly linked to life span modulation, it is currently not known if

also time limited and not permanent change of their abundance would nevertheless affect life span. However, studies on short term hormetic stress, that also involves temporal modulation of IGF-1 and SIRT6 levels indicate that even short-term modulation of SIRT6 and IGF-1 levels could impact lifespan duration.[49] We therefore hypothesize, that the temporal changes in SIRT6, IGF-1 and CGRP protein levels, that were observed upon treatment with Cerebrolysin, may also be linked to the observed lifespan prolongation. However, further experiments will be required to decipher if there is a direct link between the temporal modulation of these proteins upon Cerebrolysin treatment or if other pathways are involved.

We also observed that Cerebrolysin treatment reduced accumulation of laminin in cerebral vessels of CADASIL animals. Laminins are collagen-like glycoproteins located in the ECM,[6] one of the main vascular structures affected in CADASIL.[50] As pointed out above, CADASIL is characterized by accumulation of NOTCH3 ECD in the vascular ECM,[51] a process which causes co-aggregation of other ECM proteins, e.g. laminin.[50,52] Since we did not compare aged 17 months-old-wt mice with aged 17 months-old-CADASIL mice, it remains unclear whether laminin is a specific feature of CADASIL or only associated with the aging process. In any case, 9 weeks Cerebrolysin treatment significantly reduced vascular laminin in 13 as well as aged (17-month-old) CADASIL mice suggesting that the observed positive effects of Cerebrolysin may be caused by reduced vascular pathology. Further research needs to clarify which by Cerebrolysin induced molecular mechanisms are exactly responsible for the observed alleviation of disease burden in the CADASIL mice.

In summary, treating one year old CADASIL mice for nine weeks within a period of five months with Cerebrolysin did not alter CADASIL specific pathology such as vacuole formation despite the fact, that vascular laminin burden was reduced. However, Cerebrolysin treatment was associated with a significant life-span extension, reduced epigenetic (biological) aging and at the same time improved general health and reduced frailty as compared to untreated controls. Thus, the current study provides the first in vivo evidence that Cerebrolysin may have beneficial effects for CADASIL patients. This is highly relevant as there is a tremendous unmet need for effective treatments for CADASIL.

It is currently not clear, if the anti-aging effect is specific for the CADASIL mutant background or may represent a more general effect of Cerebrolysin. Therefore, our findings may pave the way for further research on Cerebrolysin's potential impact on aging as the observed anti-aging effect of Cerebrolysin may also be relevant in other neurodegenerative disorders such as Alzheimer's or Parkinson's disease where accelerated aging represents a significant feature of disease pathology. We suggest including corresponding biomarkers in future studies with Cerebrolysin in these indications.

Furthermore, the current study suggests that Cerebrolysin, which has previously been shown to exert pleiotropic effects, can modulate the expression levels of CGRP, IGF-1 and SIRT6 and may impact live-span, biological aging, and frailty in CADASIL mice through these pathways. Further studies are needed to confirm the exact mechanisms involved.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

F.F.G., Grant: Basisprogramm, Award Number: 875 188.
Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

B.K., H.B. and S.W. are employees of EVER Neuro Pharma. EVER Pharma has financed the work by a research grant but was not involved in the execution of the study. W.W., is CEO of Cygenia and Professor of Stem Cell Biology and Cellular Engineering at the Medical Faculty of RWTH Aachen University. W.W. has provided the epigenetic data measurements which EVER Pharma financed but was not involved in the data analysis. All experiments and analysis were performed under randomized and blinded conditions.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

aging, CADASIL, Cerebrolysin, healthspan, lifespan

Received: August 22, 2023
Published online:

[1] L. Caeiro, J. Ferro, *J. Neurol. Neurosurg. Psychiatry* **2006**, *77*, 144.
[2] a) C. Opherk, N. Peters, J. Herzog, R. Luedtke, M. Dichgans, *Brain* **2004**, *127*, 2533; b) M. H. Taylor, G. A. Doody, *Adv. Psychiatr. Treat.* **2008**, *14*, 350.
[3] A. Joutel, C. Corpechot, A. Ducros, K. Vahedi, H. Chabriat, P. Mouton, S. Alamowitch, V. Domenga, M. Cécillion, E. Maréchal, J. Maciazek, C. Vayssière, C. Cruaud, E.-A. Cabanis, M. M. Ruchoux, J. Weissenbach, J. F. Bach, M. G. Bousser, E. Tournier-Lasserve, *Nature* **1996**, *383*, 707.
[4] A. Zellner, E. Scharrer, T. Arzberger, C. Oka, V. Domenga-Denier, A. Joutel, S. F. Lichtenthaler, S. A. Müller, M. Dichgans, C. Haffner, *Acta Neuropathol.* **2018**, *136*, 111.
[5] K. Z. Young, G. Xu, S. G. Keep, J. Borjigin, M. M. Wang, Overlapping Protein Accumulation Profiles of CADASIL and CAA. The American Journal of Pathology **2021**, *191*, 1871.
[6] a) E. Cognat, C. Baron-Menguy, V. Domenga-Denier, S. Cleophax, C. Fouillade, M. Monet-Leprêtre, M. Dewerchin, A. Joutel, *Stroke* **2014**, *45*, 842; b) B. P. H. Cho, S. Nannoni, E. L. Harshfield, D. Tozer, S. Gräf, S. Bell, H. S. Markus, *J. Neurol. Neurosurg. Psychiatry* **2021**, *92*, 694; c) E. Muiño, I. Fernández-Cadenas, A. Arboix, *Int. J. Mol. Sci.* **2021**, *22*, 7357.
[7] T. Akhvlediani, A. Henning, P. S. Sándor, P. Boesiger, H. H. Jung, *J. Neurol.* **2010**, *257*, 171.
[8] H. Chabriat, *Stroke* **1995**, *26*, 1729.
[9] a) A. Kulesh, *Neurol., Neuropsychiatry, Psychosomatics* **2021**, *13*, 94; b) Z. Poljakovic, S. Supe, J. Ljevak, K. Starcevic, I. Peric, N. Blazevic, M.

Krbot-Skoric, I. Jovanovic, D. Ozretic, *Clin. Neurol. Neurosurg.* **2021**, *207*, 106767; c) W. Lang, C. H. Stadler, Z. Poljakovic, D. Fleet, *Int. J. Stroke* **2013**, *8*, 95; d) H. Teng, C. Li, Y.i Zhang, M. Lu, M. Chopp, Z. G. Zhang, M. Melcher-Mourgas, B. Fleckenstein, *Neuroreport* **2021**, *32*, 359.

[10] a) M. Brainin, *Expert Rev. Neurother.* **2018**, *18*, 681; b) C. M. Stinear, C. E. Lang, S. Zeiler, W. D. Byblow, *Lancet Neurol.* **2020**, *19*, 348.

[11] a) S. Gauthier, J. V. Proaño, J. Jia, L. Froelich, J. C. Vester, E. Doppler, *Dementia Geriatr. Cognit. Disord.* **2015**, *39*, 332; b) N. M. Bornstein, A. Guekht, J. Vester, W.-D. Heiss, E. Gusev, V. Hömberg, V. W. Rahlfs, O. Bajenaru, B. O. Popescu, D. Muresanu, *Neurol. Sci.* **2018**, *39*, 629; c) J. C. Vester, A. D. Buzoianu, S. I. Florian, V. Hömberg, S.-H. Kim, T. M. C. Lee, C. Matula, W. S. Poon, D. Sandesc, N. Von Steinbüchel, S. Strilciuc, P. E. Vos, K. Von Wild, D. Muresanu, *Neurol. Sci.* **2021**, *42*, 4531; d) S. Strilciuc, L. Vécsei, D. Boering, A. Pražnikar, O. Kaut, P. Riederer, L. Battistin, *Pharmaceuticals* **2021**, *14*, 1297.

[12] C. Zhang, M. Chopp, Y. Cui, L. Wang, R. Zhang, L.i Zhang, M. Lu, A. Szalad, E. Doppler, M. Hitzl, Z. G. Zhang, *J. Neurosci. Res.* **2010**, *88*, 3275.

[13] L.i Zhang, M. Chopp, D. H. Meier, S. Winter, L. Wang, A. Szalad, M. Lu, M. Wei, Y. Cui, Z. G. Zhang, *Stroke* **2013**, *44*, 1965.

[14] M. Hartbauer, B. Hutter-Paier, G. Skofitsch, M. Windisch, *J. Neural Transm.* **2001**, *108*, 459.

[15] Y. Zhang, M. Chopp, Y. Meng, Z. G. Zhang, E. Doppler, A. Mahmood, Y. Xiong, *J. Neurosurg.* **2013**, *118*, 1343.

[16] X. Anton Alvarez, C. Sampedro, R. Cacabelos, C. Linares, M. Aleixandre, M. García-Fantini, H. Moessler, *Int. J. Neuropsychopharmacol.* **2009**, *12*, 867.

[17] E. Rockenstein, K. Ubhi, E. Doppler, P. Novak, H. Moessler, B. Li, J. Blanchard, I. Grundke-Iqbal, K. Iqbal, M. Mante, A. Adame, L. Crews, E. Masliah, *J. Alzheimers Dis.* **2011**, *27*, 743.

[18] K. Ubhi, E. Rockenstein, R. Vazquez-Roque, M. Mante, C. Inglis, C. Patrick, A. Adame, M. Fahnestock, E. Doppler, P. Novak, H. Moessler, E. Masliah, *J. Neurosci. Res.* **2013**, *91*, 167.

[19] a) H. Chen, Y.-C. Tung, B. Li, K. Iqbal, I. Grundke-Iqbal, *Neurobiol. Aging* **2007**, *28*, 1148; b) E. Rockenstein, K. Ubhi, E. Pham, S. Michael, E. Doppler, P. Novak, C. Inglis, M. Mante, A. Adame, X. A. Alvarez, H. Moessler, E. Masliah, *J. Neurosci. Res.* **2011**, *89*, 1812; c) E. Masliah, E. Diez-Tejedor, *Drugs Today* **2012**, *48 Suppl A*, 3; d) X. A. Alvarez, I. Alvarez, O. Iglesias, I. Crespo, J. Figueroa, M. Aleixandre, C. Linares, E. Granizo, M. Garcia-Fantini, J. Marey, E. Masliah, S. Winter, D. Muresanu, H. Moessler, *Int. J. Neuropsychopharmacol.* **2016**, *19*, pyw024.

[20] a) D. F. Muresanu, W.-D. Heiss, V. Hoemberg, O. Bajenaru, C. D. Popescu, J. C. Vester, V. W. Rahlfs, E. Doppler, D. Meier, H. Moessler, A. Guekht, *Stroke* **2016**, *47*, 151; b) K. Gharagozli, A. Harandi, S. Houshmand, N. Akbari, D. Muresanu, J. Vester, S. Winter, H. Moessler, *J. Med. Life* **2017**, *10*, 153; c) E. Beghi, H. Binder, C. Birle, N. Bornstein, K. Diserens, S. Groppa, V. Homberg, V. Lisnic, M. Pugliatti, G. Randall, L. Saltuari, S. Strilciuc, J. Vester, D. Muresanu, *Eur. J. Neurol.* **2021**, *28*, 2831; d) S. R. Deboer, R. Hubbard, M. Mersha, G. Pinilla Monsalve, S. Winter, S. R. Zeiler, *Neurorehabil. Neural Repair* **2021**, *35*, 525; e) I. A. Mureșanu, D. A. Grad, D. F. Mureșanu, E. Hapca, I. Benedek, N. Jemna, ș. Strilciuc, B. O. Popescu, L. Perju-Dumbrava, R. M. Chereches, *Medicina* **2022**, *58*, 648.

[21] M. Ghosh, M. Balbi, F. Hellal, M. Dichgans, U. Lindauer, N. Plesnila, *Ann. Neurol.* **2015**, *78*, 887.

[22] A. Joutel, M. Monet-Leprêtre, C. Gosele, C. Baron-Menguy, A. Hammes, S. Schmidt, B. Lemaire-Carrette, V. Domenga, A. Schedl, P. Lacombe, N. Hubner, *J. Clin. Invest.* **2010**, *120*, 433.

[23] F. Ehret, R. Moreno Traspas, M.-T. Neumuth, B. Hamann, D. Lasse, G. Kempermann, *Front. Aging Neurosci.* **2021**, *13*, 223.

[24] R. M. Rajani, N. Dupré, V. Domenga-Denier, G. Van Niel, X. Heiligenstein, A. Joutel, *Neuropathol. Appl. Neurobiol.* **2021**, *47*, 694.

[25] T. Melnikova, A. Savonenko, Q. Wang, X. Liang, T. Hand, L. Wu, W. Kaufmann, A. Vehmas, K. Andreasson, *Neuroscience* **2006**, *141*, 1149.

[26] C. W. Baumann, D. Kwak, L. V. Thompson, *Physiology* **2020**, *35*, 405.

[27] K. Flurkey, J. M. Currer, D. Harrison, in *The Mouse in Biomedical Research*, (Eds: J. G. Fox, M. T. Davisson, F. W. Quimby, S. W. Barthold, C. E. Newcomer, A. L. Smith) Elsevier, Burlington, San Diego, London **2007**, pp. 637–672.

[28] Y. Han, M. Eipel, J. Franzen, V. Sakk, B. Dethmers-Ausema, L. Yndriago, A. Izeta, G. De Haan, H. Geiger, W. Wagner, *Elife* **2018**, *7*, 37462.

[29] a) A. Matrone, C. Gambale, A. Prete, P. Piaggi, V. Cappagli, V. Bottici, C. Romei, R. Ciampi, L. Torregrossa, L. De Napoli, E. Molinaro, G. Materazzi, F. Basolo, R. Elisei, *Cancers (Basel)* **2020**, *13*, 94; b) M. B. Schultz, A. E. Kane, S. J. Mitchell, M. R. MacArthur, E. Warner, D. S. Vogel, J. R. Mitchell, S. E. Howlett, M. S. Bonkowski, D. A. Sinclair, *Nat. Commun.* **2020**, *11*, 4618; c) N. R. Smoll, O. P. Gautschi, B. Schatlo, K. Schaller, D. C. Weber, *Neuro-Oncology* **2012**, *14*, 1062.

[30] a) J. W. Rutten, R. R. Klever, I. M. Hegeman, D. S. Poole, H. G. Dauwerse, L. A. M. Broos, C. Breukel, A. M. Aartsma-Rus, J. S. Verbeek, L. Van Der Weerd, S. G. Van Duinen, A. M. J. M. Van Den Maagdenberg, S. A. J. Lesnik Oberstein, L. van der Weerd, *Acta Neuropathol. Commun.* **2015**, *3*, 89; b) C. Ayata, *Stroke* **2010**, *41*, 129.

[31] F. J. Heredia-López, F. J. Álvarez-Cervera, J. G. Collí-Alfaro, J. L. Bata-García, G. Arankowsky-Sandoval, J. L. Góngora-Alfaro, *Behav. Res. Methods* **2016**, *48*, 1631.

[32] L. P. Breitling, K.-U. Saum, L. Perna, B. Schöttker, B. Holleczek, H. Brenner, *Clin. Epigenet.* **2016**, *8*, 21.

[33] Y. Zheng, M. Habes, M. Gonzales, R. Pomponio, I. Nasrallah, S. Khan, D. E. Vaughan, C. Davatzikos, S. Seshadri, L. Launer, F. Sorond, S. Sedaghat, D. Wainwright, A. Baccarelli, S. Sidney, N. Bryan, P. Greenland, D. Lloyd-Jones, K. Yaffe, L. Hou, L. Launer, *Aging (Albany N. Y.)* **2022**, *14*, 1691.

[34] R. F. Hillary, A. J. Stevenson, S. R. Cox, D. L. Mccartney, S. E. Harris, A. Seeboth, J. Higham, D. Sproul, A. M. Taylor, P. Redmond, J. Corley, A. Pattie, M. D. C. V. Hernández, S. Muñoz-Maniega, M. E. Bastin, J. M. Wardlaw, S. Horvath, C. W. Ritchie, T. L. Spires-Jones, A. M. Mcintosh, K. L. Evans, I. J. Deary, R. E. Marioni, P. Redmond, *Mol. Psychiatry* **2021**, *26*, 3806.

[35] a) P. Bouchard, T. Maurice, S. St-Pierre, A. Privat, R. Quirion, *Eur. J. Neurosci.* **1997**, *9*, 2142; b) H. Li, J. Qu, H. Zhu, J. Wang, H. He, X. Xie, R. Wu, Q. Lu, *Front. Cell Dev. Biol.* **2021**, *9*, 675503; c) G. Vitale, G. Pellegrino, M. Vollery, L. J. Hofland, *Front. Endocrinol. (Lausanne)* **2019**, *10*, 27; d) S. A. Galle, I. K. Geraedts, J. B. Deijen, M. V. Milders, M. L. Drent, *J. Prev. Alzheimer's Dis.* **2020**, *7*, 265; e) A. Roichman, S. Elhanati, M. Aon, I. Abramovich, A. Di Francesco, Y. Shahar, M. Avivi, M. Shurgi, A. Rubinstein, Y. Wiesner, *Nat. Commun.* **2021**, *12*, 3208; f) A. Garcia-Venzor, D. Toiber, *Front. Aging Neurosci.* **2021**, *13*, 747989; g) A. Roichman, Y. Kanfi, R. Glazz, S. Naiman, U. Amit, N. Landa, S. Tinman, I. Stein, E. Pikarsky, J. Leor, *J. Gerontol., Ser. A* **2017**, *72*, 603; h) J. Rahmani, A. Montesanto, E. Giovannucci, H. Zand, M. Barati, J. J. Kopchick, M. G. Mirisola, V. Lagani, H. Bawadi, R. Vardavas, *Aging Cell* **2022**, *21*, 13540.

[36] C. Capone, E. Cognat, L. Ghezali, C. Baron-Menguy, D. Aubin, L. Mesnard, H. Stöhr, V. Domenga-Denier, M. T. Nelson, A. Joutel, *Ann. Neurol.* **2016**, *79*, 387.

[37] A. Viswanathan, A. Gschwendtner, J.-P. Guichard, F. Buffon, R. Cumurciuc, M. O'sullivan, M. Holtmannspotter, C. Pachai, M.-G. Bousser, M. Dichgans, H. Chabriat, M. Dichgans, *Neurology* **2007**, *69*, 172.

[38] V. Navrotskaya, G. Oxenkrug, L. Vorobyova, P. Summergrad, *Am. J. Plant Sci.* **2014**, *5*, 275.

[39] J.-F. Perez-Correa, V. Tharmapalan, H. Geiger, W. Wagner, *Front. Cell Dev. Biol.* **2022**, *10*, 902857.

[40] W. Wagner, *Genome Biol.* **2017**, *18*, 107.
[41] R. Lardenoije, A. Iatrou, G. Kenis, K. Kompotis, H. W. M. Steinbusch, D. Mastroeni, P. Coleman, C. A. Lemere, P. R. Hof, D. L. A. Van Den Hove, B. P. F. Rutten, *Prog. Neurobiol.* **2015**, *131*, 21.
[42] S. Guo, A. F. Christensen, M. L. Liu, B. N. Janjooa, J. Olesen, M. Ashina, *Cephalalgia* **2017**, *37*, 114.
[43] E. D. Goldstein, N. Gopal, M. K. Badi, D. O. Hodge, A. De Havenon, P. Glover, P. L. Durham, J. F. Huang, M. P. Lin, H. Baradaran, J. J. Majersik, J. F. Meschia, H. Baradaran, *The Neurologist* **2023**, *28*, 231.
[44] a) I. De Boer, A. Maassenvandenbrink, G. M. Terwindt, *Cephalalgia* **2020**, *40*, 1676; b) S. Aradi, E. Kaiser, B. Cucchiara, B. Cucchiara, *J. Stroke Cerebrovasc. Dis.* **2019**, *28*, 104286.
[45] a) J. M. Borkum, *Headache* **2019**, *59*, 1339; b) A. F. Russo, D. L. Hay, *Physiol. Rev.* **2023**, *103*, 1565.
[46] G. H. Chan, R. R. Fiscus, presented at Proc. of the Second Int. and the Seventeenth American Peptide Symp, San Diego, California, USA, June **2001**.
[47] a) Y. Kanfi, S. Naiman, G. Amir, V. Peshti, G. Zinman, L. Nahum, Z. Bar-Joseph, H. Y. Cohen, *Nature* **2012**, *483*, 218; b) A. Bartke, *Clin. Interv. Aging* **2008**, *3*, 659.
[48] H. Kim, H.-S. Kim, B.-K. Kaang, B.-K. Kaang, *Mol. Brain* **2018**, *11*, 49.
[49] a) D. Gems, L. Partridge, *Cell Metab.* **2008**, *7*, 200; b) Z. Mao, C. Hine, X. Tian, M. Van Meter, M. Au, A. Vaidya, A. Seluanov, V. Gorbunova, *Science* **2011**, *332*, 1443; c) S. Milman, G. Atzmon, D. M. Huffman, J. Wan, J. P. Crandall, P. Cohen, N. Barzilai, *Aging Cell* **2014**, *13*, 769.
[50] a) K. Z. Young, S. J. Lee, X. Zhang, N. M. P. Cartee, M. Torres, S. G. Keep, S. R. Gabbireddy, J. L. Fontana, L. Qi, M. M. Wang, *J. Biol. Chem.* **2020**, *295*, 1960; b) K. Z. Young, G. Xu, S. G. Keep, J. Borjigin, M. M. Wang, *Am. J. Pathol.* **2021**, *191*, 1871.
[51] K. Coupland, U. Lendahl, H. Karlström, *Stroke* **2018**, *49*, 2793.
[52] M. M. Ruchoux, D. Guerouaou, B. Vandenhaute, J.-P. Pruvo, P. Vermersch, D. Leys, *Acta Neuropathol.* **1995**, *89*, 500.
[53] G. Rodriguez, S. J. Moore, R. C. Neff, E. D. Glass, T. K. Stevenson, G. S. Stinnett, A. F. Seasholtz, G. G. Murphy, V. A. Cazares, *Neurobiol. Stress* **2020**, *13*, 100262.

