

Galectin-3 ablation does not affect infarct size or inflammatory cytokines after experimental stroke in 24-month-old female mice

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Background The tissue damage following a focal stroke causes an inflammatory response that is thought to aggravate the disease state. Galectin-3 is a proinflammatory molecule that has been shown to play a significant role in the inflammatory responses in brain diseases and following experimental stroke. In most animal experiments, young animals are used, although attempts are often made to model diseases that affect the elderly. Therefore, in this project, we intended to investigate the role of Galectin-3 in experimental stroke in older mice.

Methods In this project, 24-month-old (aged) female mice were subjected to an experimental stroke (permanent middle cerebral artery occlusion) 7 days before sacrifice. We wanted to investigate whether the absence of the inflammatory protein Galectin-3 could affect motor phenotype, neuroinflammation and infarct size. Number of mice without Galectin-3 (Galectin-3 KO)=9, number of wildtype controls of the same age=6.

Results In our aged female mice, we could not observe any significant differences between Galectin-3 KO and wildtype regarding the inclined plane test or cylinder test.

We could not observe different infarct sizes between the two genotypes. In brain homogenates, we measured levels of 10 inflammatory cytokines, but we could not see any significant differences in any of them.

Conclusion In summary, it can be said that the absence of the inflammatory mediator Galectin-3 does not seem to have a strong poststroke effect in aged females. Unfortunately, we could not analyze these mice with immunohistochemistry, which limited our study. *NeuroReport* 33: 266–271 Copyright © 2022 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

Age is the most substantial nonmodifiable risk factor for ischemic stroke [1], and in the elderly, women are more affected than men [1]. Neuroinflammation mediated by microglia, the main innate immune cells of the central nervous system, has emerged as a key event in the pathophysiology of stroke disease. In response to the neuronal damage initially caused by deprivation of oxygen and essential nutrients, these cells are rapidly activated and recruited to the injury site [2]. The activated microglial cells express Galectin-3 [3], a β -galactosidase binding protein that has been suggested to play a significant role in regulating the inflammatory response [4] and the outcome after brain ischemia in experimental models [5,6]. For example, Lalancette-Hébert showed that the infarct lesion was increased in Galectin-3 null mice after

transient middle cerebral artery occlusion (tMCAO) [5], while our group has shown that neuronal survival was higher after global ischemia [6]. However, these studies have been performed in young male mice, and it is not known how age or age combined with sex affects similar insults. Therefore, we aimed to measure the outcome after experimental stroke in aged female mice, with and without the expression of Galectin-3.

Methods

Animals and surgery

All proceedings and animal treatment were by the guidelines and requirements of the government committee on animal experimentation at Lund University (2012, Dnr: M427-12). Animals were housed in groups of 3–6 mice per cage under a 12:12 light-dark cycle with access to water and food ad libitum. In this study, we used female wildtype ($n=6$) and Galectin-3 knockout ($n=9$) C57BL/6 mice, aged 24 months. Galectin-3 KO mice were originally obtained for Dr. K. Sävman from Gothenburg University, where they were generated as

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described previously [7]. The genotype of each mouse was confirmed with PCR as described before [8]. Mice were subjected to a left-sided permanent middle cerebral artery occlusion (pMCAO) as previously described [9]. Mice were anesthetized with 5% isoflurane in oxygen, followed by 2% isoflurane in oxygen during the whole surgical procedure. A small incision was made between the left eye and ear, and the temporal muscle was separated from the skull. The middle cerebral artery was made visible by drilling a small hole in the skull. A distal permanent occlusion was made using a vessel cauterizer. The incision was sutured (nylon), and animals were returned to their cages.

Behavioral tests

Grip strength

The grip strength test was performed prior to and 6 days after pMCAO. The mouse was lowered over a metallic grid connected to a force sensor whereby it was allowed to attach to the grid with only its forelimbs. The mouse was then gently pulled back by its tail until the grip was released, and the displayed grip strength value was recorded. The grip strength of the left forelimb, right forelimb and both forelimbs was determined. For each condition, the mean value of three trials was calculated and used for analysis.

Inclined plane test

The inclined plane test was performed prior to and 6 days after pMCAO. Each mouse was placed head down on an inclined plane platform with a grooved plastic surface. The angle was gradually increased until the mouse was unable to hold its position. The last angle that the mouse was able to hold was recorded. Three rounds per mouse were performed and the mean angle was calculated and used for analysis.

Cylinder test

To assess for asymmetric use of forelimbs, the cylinder test [10] was performed 2 days after the pMCAO. Mice were put in a glass cylinder with a 90° angled mirror behind it for 3 min, and movements were recorded. Right and left forelimb use was analyzed as one weight-bearing contact with the glass wall accounting for one 'touch unit', using a VLC media player.

Cryosectioning

Animals were sacrificed on day 7 following surgery by isoflurane anesthesia and transcardial perfusion with PBS. Brains were then carefully removed and stored at -80 °C. Using a cryostat (Leica CM3050 S Cryostat, Wetzlar, Germany), a series of six 30 µm thick coronal brain sections was obtained, mounting sections in series 1-4 directly on glass. The remaining two sections (series 5 and 6) were put in an Eppendorf tube each. Sections were collected from approximately Bregma -2 to Bregma 2 (www.mbl.org). Glasses were dried and stored at -80°C. Tubes were stored at -80°C.

Morphology staining

Sections were fixed in paraformaldehyde (4%, HistoLab Products AB, Gothenburg, Sweden) for 10 min and rinsed in PBS for 2×3 min. Sections were incubated in Mayers-HTX (HistoLab) solution for 3 min and rinsed in a continuous flow of tap water for at least 5 min. Sections were incubated in eosin (Sigma-Aldrich, St Louis, Missouri, USA) for 1 min, and then rinsed in 70% ethanol (SOLVECO, Rosersberg, Sweden) for 1 min, followed by a rinse in 95% ethanol (SOLVECO) for 1 min and then rinsed in absolute ethanol (SOLVECO) for 2×1 min. Sections were incubated in HistoLab-Clear (HistoLab) for 3×1 min plus 1 min. Sections were mounted with Pertex (HistoLab) and air-dried on plastic, then stored at room temperature.

Infarct volume

The hematoxylin/eosin stainings were scanned, and image analysis was performed. Using ImageJ (<https://imagej.nih.gov>), whole coronal face areas of the contralateral hemisphere, the intact part of the ipsilateral hemisphere, and the entire ipsilateral hemisphere measured in all slides containing a distinguishable infarct (approximately responding to Bregma: 2 to -2 mm). Two infarct volumes, direct and indirect, were calculated. Direct volume was obtained by subtracting the intact ipsilateral area from the entire ipsilateral and indirect infarct area by subtracting the intact ipsilateral from the contralateral area.

Brain tissue homogenization

Every 5th and 6th slice at cryosectioning from whole brain tissue were frozen in -80 °C. Tissue mass weight was calculated for each tube. radioimmunoprecipitation assay (RIPA) buffer was prepared by adding one complete tablet (Roche, Basel, Switzerland) and one PhosStop (Roche) tablet to 10 mL of RIPA solution (Sigma-Aldrich). RIPA buffer was added in ratio 1 mL RIPA buffer/100 mg of brain tissue and stored on ice. Samples were manually homogenized using a plastic grinder. A sonicator (Branson, Ferguson, USA) was set on 10 s pulses with 60% amplitude and three pulses with 10-s intervals, and then stored on ice for a minimum of 30 min. Homogenized samples were centrifuged (Eppendorf Centrifuge 5430 R, Hamburg, Germany) on 10.000 rcf for 20 min. Supernatants were collected in Eppendorf tubes. Later, protein concentration was measured using a bicinchoninic acid (BCA) kit according to the manufacturer's protocol (BCA Protein Assay-Kit, ThermoScientific, Sweden).

Cytokine assay

MesoScale plates were used to evaluate the cytokine levels (proinflammatory panels for IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 and tumor necrosis factor-α) from every whole brain homogenate (see in Brain tissue homogenization). Analyses were carried out according to the protocol provided by the manufacturer, as described before [11,12]. Samples were prepared by a 2-fold dilution of sample protein solution (containing 120 mg protein) in diluent 41.

Statistical analysis

All statistical analyses were performed in GraphPad Prism 9 (GraphPad, San Diego, California, USA). *P* values ≤ 0.05 were considered significant. Since the wild-type group *N* was too low for normality testing, we used Mann-Whitney rank tests for pairwise comparisons. For some behavioral tests with more than one variable, we used 2-way analysis of variance (ANOVAs).

Results

Behavioral tests

We first set out to study sensorimotor function in established experimental stroke tests previously used by us [13-16].

Grip strength with both front paws was decreased after pMCAO (Fig. 1a; 2-way ANOVA; $F(1, 22)=16.72$; $P=0.0005$), and interestingly, was overall lower in Galectin-3 KO females (Fig. 1a; 2-way ANOVA; $F(1, 22)=8.889$; $P=0.0069$). Furthermore, it was only in mice without Galectin-3 where grip strength was significantly lower after pMCAO (Fig. 1a; Sidak multiple comparison; $P=0.023$). Grip strength with the injured (right) forepaw was not significantly weaker 6 days after pMCAO (Fig. 1b). In addition, there was no genotype difference with regards to right vs. left paw grip strength, 6 days after stroke (Fig. 1b).

Use of impaired forelimb after stroke was determined with the cylinder test. There was a nonsignificant trend

of higher use of the impaired forelimb in the Galectin-3 KO mice (Fig. 2a; Mann-Whitney; $P=0.1447$).

To further analyse limb motor function, we subjected the mice to an inclined plane test before and after pMCAO. We did not observe any differences regarding genotype or stroke (Fig. 2b; 2-way ANOVA). Taken together, we are not able to find a clear effect of Galectin-3 ablation on sensorimotor behavior following stroke.

Infarct volume

Infarct size was measured with a series of HTX slices. The absence of Galectin-3 did not significantly affect direct or indirect infarct volume 7 days after pMCAO in 24-month-old females (Fig. 3a,b; Mann-Whitney; $P=0.8639$ and 0.3884 , respectively).

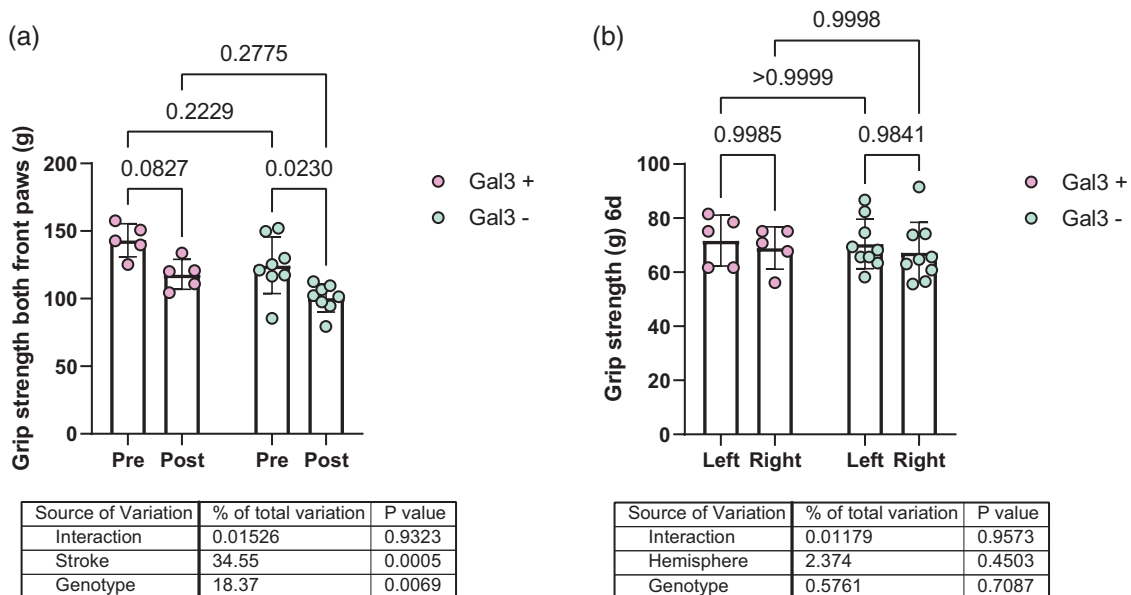
Immunohistochemistry

We tried to label these brains with microglial markers, using multiple protocols, but we were not successful.

Cytokine expression

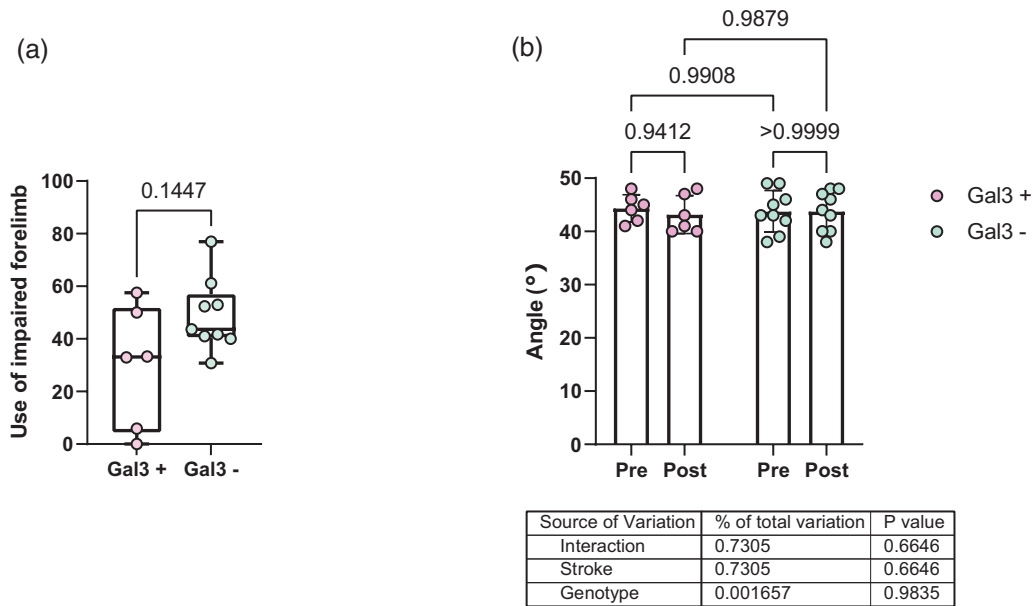
With a mesoscale U-plex Proinflammatory panel, we measured inflammatory cytokines from brain homogenates (from slices). Except for a few analytes (IFN- γ and IL12p70), we could read values within the detection range for all samples. Trends with lower expression in Galectin-3 KO could be seen for IL-1b and IL-6, but these differences were not statistically significant (Fig. 4b,f; Mann-Whitney; $P=0.18$ and 0.0879 , respectively).

Fig. 1



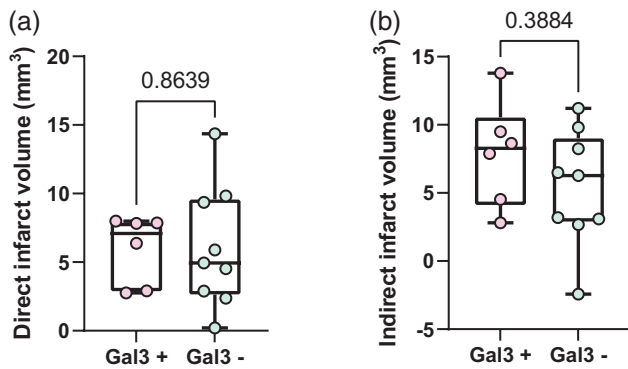
Grip strength. Wild-type $n=5$ (one mouse did not grip with right forelimb), Galectin-3 KO $n=9$. (a) Grip strength before and after pMCAO. (b) Grip strength with left and right (injured) front limb 6 days after pMCAO. 2-way ANOVA with Sidak multiple comparisons. Error bars SD, and exact P-values on top of comparison brackets. ANOVA, analysis of variance; pMCAO, permanent middle cerebral artery occlusion.

Fig. 2



Cylinder test and inclined plane test. Wildtype $n=6$, Galectin-3 KO $n=9$. (a) Cylinder test with Mann-Whitney. Error bars range and exact P -values on top of comparison bracket. (b) Inclined plane test. 2-way ANOVA with Sidak multiple comparisons. Error bars SD, and exact P -values on top of comparisons brackets. ANOVA, analysis of variance.

Fig. 3



Infarct measurements. (a) Direct ($V_{\text{ipsilateral}} - V_{\text{unaffected ipsilateral}}$) and (b) indirect ($V_{\text{ipsilateral}} - V_{\text{contralateral}}$) infarct volumes in cubic millimeters. Wildtype $n=6$, Galectin-3 KO $n=9$. Mann-Whitney test is used for pairwise comparison. Error bars=min/max, and exact P -value on top of comparison bracket.

Likewise, levels of IFN- γ and IL-4 appeared higher in KO females, but differences were not statistically significant (Fig. 4a,d; Mann-Whitney; $P=0.2284$ and 0.1135 , respectively).

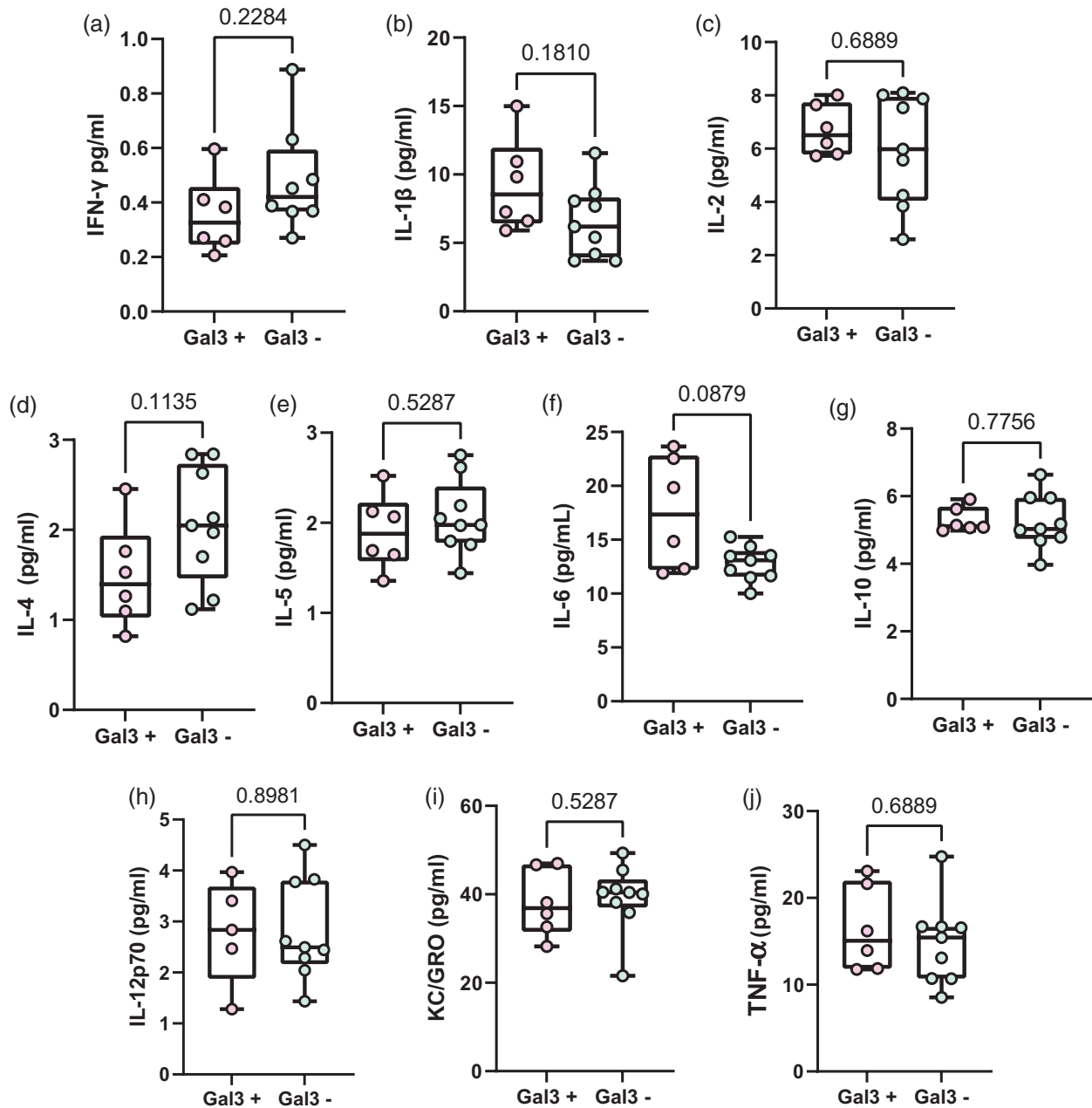
Discussion

Many experimental studies show that neuroinflammation after cerebral focal ischemia can exacerbate the insult [17]. However, most studies have been performed

in adolescent mice when modeling these stroke-related diseases that usually affect the elderly [18]. Galectin-3 is an inflammatory mediator that has been implicated in many diseases, including brain ischemia. In some conditions, it exerts protective and antiapoptotic effects [5], and in others, it exacerbates inflammation and injury [6,19]. Our group has previously shown that Galectin-3 ablation improves outcomes in experimental models of neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease [6,8,20]. In this study, we subjected 24-month-old female mice to pMCAO. In contrast to a previous study by Lalancette-Hébert *et al.*, (2012), we do not report differences in lesion size between the genotypes [5]. Of course, discrepancies between our observations and Lalancette-Hébert were not only limited to the age and sex of the mice but could also be related to differences in the stroke model (permanent versus transient) and duration (7 days and 72 h) [5]. Our study design enabled us to perform several behavioral tests, and we did detect a significant genotype effect in grip strength with both front paws, where Galectin-3 ablated females were weaker. Both genotypes were weaker after stroke, but Galectin-3 KO mice were weaker before and decreased more after the pMCAO surgery. In the grip strength test with only one paw and the inclined plane test, we could not detect any effect regarding either genotype or injury/stroke.

Our study is narrow in its scope and the lowered power due to the relatively small control group may have

Fig. 4



Inflammatory cytokines in whole brain homogenates. Wildtype $n=6$, Galectin-3 KO $n=9$. (a) IFN- γ . Here one Galectin-3 KO sample was below the detection range. (b) IL-1 β , (c) IL-2, (d) IL-4, (e) IL-5, (f) IL-6, (g) IL-10 (h) IL-12p70. One wildtype sample was below detection range (i) KC/GRO, (j) TNF- α . Mann-Whitney test is used for pairwise comparison. Error bars=min/max, and exact P value on top of comparison bracket. TNF, tumor necrosis factor.

resulted in nondetection of significant differences. In addition, it is possible that our cytokine results were diluted when we homogenized both the ipsilateral and the 'healthy' contralateral hemispheres into one sample. Moreover, we do not have a young group of mice, or any males, for comparisons to our aged female results. Initially, we aimed at having both female and male mice, but due to males killing one another in the home cages,

we were only able to focus this study on female mice. Because publications including female mice are under-represented in the primary literature, we believe that this study is important to be made available. In fact, females are especially vulnerable to stroke disease, likely related to decreasing levels of estrogen [21,22]. It is difficult to say whether the results in aged male Galectin-3 null mice would have differed from male wild-type controls, but

because the inflammatory response differs between the sexes [21], the outcome is not guaranteed to be similar to our findings.

In conclusion, the ablation of Galectin-3 did not affect infarct size and levels of brain cytokines in aged female mice. However, Galectin-3 null mice appeared to be a bit weaker in grip strength, both before and after stroke. How this relates to outcomes in human patients is not easy to decipher. But we would suggest that researchers in future studies concerning diseases of primarily aged women take age and sex into consideration when designing their experiments.

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Conflicts of interest

There are no conflicts of interest.

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