Protective effects of angiopoietin-like 4 on cerebrovascular and functional damages in ischaemic stroke

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Aims
Given the impact of vascular injuries and oedema on brain damage caused during stroke, vascular protection represents a major medical need. We hypothesized that angiopoietin-like 4 (ANGPTL4), a regulator of endothelial barrier integrity, might exert a protective effect during ischaemic stroke.

Methods and results
Using a murine transient ischaemic stroke model, treatment with recombinant ANGPTL4 led to significantly decreased infarct size and improved behaviour. Quantitative characteristics of the vascular network (density and branchpoints) were preserved in ANGPTL4-treated mice. Integrity of tight and adherens junctions was also quantified and ANGPTL4-treated mice displayed increased VE-cadherin and claudin-5-positive areas. Brain oedema was therefore significantly decreased in ANGPTL4-treated mice. In accordance, vascular damage and infarct severity were increased in angptl4-deficient mice thereby providing genetic evidence that ANGPTL4 preserves brain tissue from ischaemia-induced alterations. Altogether, these data show that ANGPTL4 protects not only the global vascular network, but also interendothelial junctions and controls both deleterious inflammatory response and oedema.

Mechanistically, ANGPTL4 counteracted VEGF signalling and thereby diminished Src-signalling downstream from VEGFR2. This led to decreased VEGFR2–VE-cadherin complex disruption, increased stability of junctions and thus increased endothelial cell barrier integrity of the cerebral microcirculation. In addition, ANGPTL4 prevented neuronal loss in the ischaemic region.

Conclusion
These results, therefore, show ANGPTL4 counteracts the loss of vascular integrity in ischaemic stroke, by restricting Src kinase signalling downstream from VEGFR2. ANGPTL4 treatment thus reduces oedema, infarct size, neuronal loss, and improves mice behaviour. These results suggest that ANGPTL4 constitutes a relevant target for vasculoprotection and cerebral protection during stroke.

Keywords
Acute cerebral infarction • Endothelium • Vascular biology • Angiogenesis • Ischaemia • Hypoxia

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Introduction

The morbi-mortality of stroke is of major importance in the world: 15 million people suffer stroke each year. Of these, 5 million die and another 5 million are disabled. Despite a decline in the incidence of stroke, the overall rate of stroke may increase over the next 2 decades due to ageing of the population.1 Timely recanalization of the occluded artery is the only effective treatment for acute ischaemic stroke. Whereas i.v. thrombolysis is approved by the FDA, its effectiveness is limited in large and mid-size arteries and it is associated with the risk of severe, life-threatening haemorrhage.2 Therefore, novel approaches in the treatment of ischaemic stroke are necessary. The blood–brain barrier (BBB) plays a critical role and should be the target of new protective therapies.3,4

Expression of hypoxia-inducible factors (HIFs) and downstream targets such as VEGF, a powerful mediator of permeability, is induced during ischaemic stroke.5,6 Whereas HIFs are protective in long-term responses to hypoxic injury, VEGF also increases BBB leakage and oedema at the acute phase and thereby exacerbates tissue damage.7,8 Given the contribution of vascular leakage and oedema formation to tissue damage during stroke,6 a relevant therapeutic approach would be to specifically target VEGF-induced permeability at the acute phase of ischaemia. Anti-VEGF therapies might be promising in stroke because oedema formation and brain tissue damage were significantly reduced in an experimental model.9 Src kinases were involved in VEGF-mediated vascular permeability10 and the blockade of Src in mice provided cerebral protection following stroke due to reduced brain oedema.11 Nevertheless, it is as yet unclear whether VEGF modulation would be translatable to humans. Indeed, anti-VEGF therapies and inhibition of Src kinases, which are involved in numerous signalling pathways, have side-effects.12

It is postulated that other ischaemia-induced factors play a role in controlling vascular integrity. Angiopoietin-like protein 4 (ANGPTL4) is a secreted protein induced by hypoxia in vascular cells.13 We recently described ANGPTL4 as a therapeutic approach in vasculoprotection, counteracting VEGF-induced permeability in acute myocardial infarction (AMI).14 Here, we show that treatment with recombinant human ANGPTL4 in a mouse model of ischaemic stroke, reduces infarct size, and improves mouse behaviour. ANGPTL4 counteracts the loss of vascular integrity, and consequently diminishes vascular leakage and cerebral oedema. Altogether, we provide evidence that ANGPTL4 protects blood vessel integrity and confers cerebroprotection. ANGPTL4 might, therefore, constitute a relevant new therapeutic approach in stroke.

Methods

For more details, see Supplementary material online, Methods.

Animals

Male C57BL/6J mice (Charles River) (8 weeks, 20–25 g) were used to assess the therapeutic potential of ANGPTL4. To provide genetic evidence of the role of ANGPTL4, angptl4-genetically modified mice were used15 and genotyped as previously described.16 Eight- to twelve-week-old male mice were used. The experiments were performed in accordance with the official regulations edicted by the French Ministry of Agriculture. This study conforms to the standards of INSERM regarding the care and use of laboratory animals and was performed in accordance with European Union Council Directives (86/609/EEC).

Ischaemic stroke model

Mice were anaesthetized with i.p. ketamine (50 mg/kg) and xylazine hydrochloride (6 mg/kg) under spontaneous respiration. Body temperature was monitored by a rectal probe and mice were maintained at 37 ± 0.5°C with a heating pad (Harvard Apparatus).

Transient focal cerebral ischaemia was induced by occlusion of the left middle cerebral artery (MCA) using an intraluminal filament technique as previously described.17 Occlusion of the MCA was controlled by laser Doppler flowmetry (Moor Instruments Ltd.). One hour after the onset of stroke, the occluding filament was pulled back to allow reperfusion.

All experiments were performed blindly, meaning that the experimenters were unaware of treatment allocation and of mouse genotype.

Assessment of the therapeutic cerebroprotective effects of angiopoietin-like 4

Angiopoietin-like 4 was produced in the laboratory as previously described.18 Mice randomly received single i.v. injection in the tail vein of either vehicle or recombinant human ANGPTL4 (40 μg/kg body weight) 5 min before the 1 h occlusion step of the MCA. A supplemental group of mice received i.v. injection at reperfusion (1 h after the onset of stroke). Twenty-four hours after the onset of stroke, an experimenter blinded to the group allocation evaluated the neurological deficit using a modified Neurological Severity Score on 34 points (Supplementary material online, Figure S1). A subgroup of Sham mice was also examined for behavioural testing.

After neurological evaluation, infarct volume and brain oedema were measured using pathology (Supplementary material online, Methods). Infarct volume was also assessed using cerebral magnetic resonance imaging (MRI) (Supplementary material online, Methods).

Statistical analysis

Data are presented as mean ± SD for continuous variables and percentages for qualitative variables except for infarct volumes and neurological score which are presented as medians (quartiles). We analysed data using either a Mann–Whitney U test or, in cases in which more than two groups are compared, a Kruskal–Wallis test followed if \( P < 0.05 \), by Dunn’s multiple comparison test. A two-tailed value of \( P < 0.05 \) was considered statistically significant. For correlation analyses, a Spearman test was used. The number of mice is provided in the figure legends.

Results

Angiopoietin-like 4 is expressed in the brain during stroke both in mice and humans

It has been reported that angptl4 mRNA is induced in a mouse model of hypoxia-induced brain damage.19 We wondered here whether ANGPTL4 might be expressed in ischaemic stroke. Western blot analyses carried out in mice subjected to stroke showed that ANGPTL4 expression was induced in the infarcted hemisphere compared with the contralateral hemisphere by a three-fold increase (Supplementary material online, Figure S2A). Moreover, using immunostaining, we demonstrated ANGPTL4 expression in endothelial...
Angiopoietin-like 4 confers cerebral protection in stroke with beneficial effects on infarct volume and neurological deficit

We compared infarct volumes at 24 h after i.v. injection of vehicle or recombinant human ANGPTL4, 5 min prior to the onset of stroke. Regional cerebral blood flow, arterial blood pressure, heart rate, vascular reactivity, and blood glucose which could influence infarct volume were monitored and were not significantly different between the two groups (Supplementary material online, Figure S2 and data not shown). Infarct volumes assessed by TTC staining and cerebral MRI were significantly decreased in ANGPTL4-treated mice compared with controls (Figure 1A–D). The Spearman test showed a strong correlation between these two quantification methods ($r = 0.79$) (Figure 1E).

We hypothesized that ANGPTL4 treatment may lead to the decrease of neurological deficit following stroke, using the previously described Neurological Severity Score, evaluated at 24 h after the onset of stroke. ANGPTL4-treated mice showed a significantly better score compared with control mice thereby revealing an improved behavioural performance (Figure 1F).

We next asked whether ANGPTL4 administration could also be effective at reperfusion, i.e. 1 h after vascular occlusion. Relative to control mice, the administration of ANGPTL4 at reperfusion also significantly reduced infarct size and improved neurological performance at 24 h after the onset of stroke (Supplementary material online, Figure S4).

Angiopoietin-like 4 treatment preserves cerebral vasculature in stroke

Given the impact of vascular leakage in cerebral tissue damage, we here asked whether the beneficial effects of ANGPTL4 could be related to the preservation of vascular integrity. Using PECAM staining, we analysed endothelial networks in control and ANGPTL4-treated mice, both in the infarcted and contralateral healthy hemispheres (Figure 2A). In the contralateral hemisphere, there was no significant difference in the EC surface area between the two groups. In the infarcted area, in contrast, the EC surface area was significantly increased in ANGPTL4-treated mice (Figure 2B). Branching of vascular networks was also quantified. Whereas there was no difference in the contralateral hemisphere in both groups, significantly increased branching was observed in the infarcted zone of ANGPTL4-treated mice (Figure 2C).

Western blot analyses further confirmed the increase in PECAM in infarcted hemispheres of ANGPTL4-treated mice compared with controls (Supplementary material online, Figure S5).

Angiopoietin-like 4 protects interendothelial junctions from disruption following stroke

To provide genetic evidence that ANGPTL4 protects vascular integrity during stroke, $\text{angptl4}^{+/+}$, $\text{angptl4}^{-/-}$, and $\text{angptl4}^{+/+}$ mice were subjected to transient cerebral ischaemia. $\text{angptl4}^{-/-}$ mice displayed a significantly increased infarct volume compared with both $\text{angptl4}^{+/+}$ and $\text{angptl4}^{-/-}$ mice (Supplementary material online, Figure S6).

Altogether, these data imply that ANGPTL4 not only protects from global vascular damage, but also specifically protects from ischaemia-induced EC-vadherin and claudin-5 disruption following stroke.

Infarct size is increased in $\text{angptl4}^{-/-}$ mice

Acute exposure to VEGF mimicks in vivo ischaemia-induced adherens junction disruption via VE-cadherin phosphorylation, leading to permeability. To assess ANGPTL4 protective effects on endothelial junctions, ECs grown to confluence, treated with VEGF, with or without ANGPTL4 were stained with VE-cadherin antibody (Figure 4A). As reported, VEGF induces VE-cadherin redistribution in a zig-zag pattern that evidences cell–cell junction disassembly. Co-treatment of VEGF and ANGPTL4 inhibited VE-cadherin disorganization. VE-cadherin-positive areas were semi-automatically quantified (Figure 4B). VEGF treatment alone induced a significant increase in the destabilization of VE-cadherin junctions, illustrated by a larger positive area compared with the control, which was inhibited by adding ANGPTL4. ANGPTL4 thus counteracts VEGF effects on VE-cadherin junction breakdown.

The role of Src kinase in dissociating the VEGFR2–VE-cadherin complex is well established in stroke. To decipher mechanistic insights of the ANGPTL4-mediated protection of vascular junction
disruption, Src-signalling downstream of VEGFR2 was analysed in vivo in the brains of ANGPTL4-treated mice after stroke (Figure 4C). Immunoprecipitation of VEGFR2 showed a diminished recruitment of phospho-Src to VEGFR2 (expressed as the ratio between phospho-Src and Src) in infarcted hemispheres of ANGPTL4-treated mice compared with controls (Figure 4D). As a consequence, the amount of VE-cadherin co-immunoprecipitated with VEGFR2 was significantly increased in the infarcted hemisphere of ANGPTL4-treated mice (Figure 4E). The results, therefore, show that the VEGFR2–VE-cadherin complexes, and thereby the endothelial-barrier function, are preserved by ANGPTL4.

VEGF activates two main signalling pathways downstream from VEGFR2: Src kinase and PI3K–Akt. To determine whether these pathways were also regulated by ANGPTL4, HUVECs were incubated with ANGPTL4. ANGPTL4 increased Src and Akt phosphorylation (Figure 4F). We first showed that ANGPTL4 could regulate VEGF-induced Src signalling. Co-immunoprecipitation experiments showed that VEGF + ANGPTL4 significantly decreased VEGF-
mediated Src and phospho-Src association to VEGFR2 when compared with VEGF alone (Figure 4G). We then studied whether ANGPTL4-induced Akt activation might regulate Src activation. On HUVECs treated with both VEGF and ANGPTL4, wortmannin, a specific inhibitor of the PI3K–Akt pathway, partially restored phospho-Src recruitment to VEGFR2 after VEGF stimulation (Figure 4G). As a control, wortmannin + ANGPTL4 inhibits Akt phosphorylation without altering Src activation downstream of ANGPTL4 (Supplementary material online, Figure S10). Mechanistically, these results strongly support the idea/theory that Akt activation mediated by ANGPTL4 exerts an inhibitory control on phospho-Src recruitment to VEGFR2 after VEGF stimulation thus protecting VEGFR2–VE-cadherin complexes from disruption and preserving vascular integrity.

**Angiopoietin-like 4 prevents VEGF-induced permeability in vitro and in vivo**

To assess the functional relevance of ANGPTL4-mediated counteraction of VEGF signalling, we developed an *in vitro* permeability assay: HDMECs were cultured on transwell plates and diffusion of Evans blue from the top to the bottom chamber was induced by VEGF. Angiopoietin-like 4 treatment prevented VEGF-driven Evans blue diffusion, confirming the anti-permeability effect of ANGPTL4. Wortmannin significantly inhibited the effect of ANGPTL4 in the presence of VEGF, restoring diffusion of Evans blue (Figure 5A). Altogether, these data confirm that the activation of the PI3K–Akt pathway by ANGPTL4 plays an important role in counteracting VEGF-induced permeability.

The protective effect of ANGPTL4 on VEGF-mediated vascular permeability was further assessed *in vivo*, in mice. Evans blue was injected into the tail vein, followed by intradermal injection of VEGF, ANGPTL4, or saline. The dye that extravasated into the skin was quantified as previously described. Treatment with VEGF + ANGPTL4 decreased dye leakage when compared with VEGF alone (Figure 5B and C). As *in vitro*, wortmannin inhibited the ANGPTL4 effect on counteracting VEGF-induced vascular permeability.

**Angiopoietin-like 4 treatment decreases hypoxia-VEGF-induced permeability through prevention of blood–brain barrier breakdown during stroke**

We then studied whether these mechanisms operated in stroke *in vivo*, in which the vascular leakage leading to brain oedema plays a critical deleterious role. Twenty-four hours after the onset of stroke, BBB breakdown was assessed by measuring brain oedema in control and ANGPTL4-treated mice. Brain oedema was significantly decreased in the ANGPTL4-treated group relative to the control (Figure 6A). To assess BBB permeability *in vivo* and label sites of vascular leakage, FITC-labelled beads were injected in both groups at 24 h after the onset of stroke. Angiopoietin-like 4 treatment prevented extravasation of the beads when compared control mice (Figure 6B). Three-dimensional rebuilt from confocal pictures
confirmed that FITC beads extravasated from blood vessels (Figure 6C). Quantification showed that extravasation was significantly decreased in the infarcted hemisphere of ANGPTL4-treated mice compared control mice (Figure 6D). This indicates that BBB integrity is less altered in ANGPTL4-treated mice.

Angiopoietin-like 4 reduces inflammation in the infarcted area

Neutrophil infiltration into the cerebral parenchyma and its deleterious effects during stroke are related to BBB disruption and to the overexpression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) by ECs. 25 – 27 We first showed that ANGPTL4 limited polymorphonuclear neutrophil (PMN) recruitment and thus inflammation in stroke. The number of positive cells for myeloperoxidase (MPO) was significantly decreased in the infarcted area of ANGPTL4-treated mice compared with controls (Figure 7A and C). Western blot confirmed a significant decrease in the amount of MPO in infarcted hemispheres of ANGPTL4-treated mice compared with controls (Supplementary material online, Figure S11). As decreased PMN recruitment may also be due to reduced expression of ICAM-1, 27 ICAM-1 was immunostained. Quantification
Figure 4 Angiopoietin-like 4 counteracts VEGF-induced VEGFR2–VE-cadherin complex disruption through decreased recruitment of Src kinase to VEGFR2, under control of the PI3K–Akt pathway. (A) HDMEC stained for VE-cadherin, treated with angiopoietin-like 4, VEGF, or both. (B) Quantification of VE-cadherin-positive areas in HDMECs according to treatment. Experiments were done 3 times in duplicate. (C) VEGFR2 immunoprecipitation analyses on extracts from contralateral and infarcted hemispheres of control and angiopoietin-like 4-treated mice. (D) Ratio between Src kinase and phospho-Src normalized to VEGFR2 in contralateral and infarcted hemispheres of control and angiopoietin-like 4-treated mice. (E) Amounts of VE-cadherin normalized to VEGFR2 in contralateral and infarcted hemispheres of control and angiopoietin-like 4-treated mice (n = 6 mice per group). (F) Angiopoietin-like 4 stimulates Akt and Src phosphorylation in HUVECs. (G) Western blot analyses of HUVEC extracts stimulated with angiopoietin-like 4, VEGF, or both; combined or not with wortmannin. Error bars represent SD. Scale bar: 10 μm.
of the ICAM-1 area normalized to the EC surface area (assessed by Isolectin B4 staining) was expressed as a percentage and showed a significant decrease in ICAM-1 expression in the infarcted hemisphere of ANGPTL4-treated mice compared with controls (Figure 7B and D). These results being expressed as a ratio on the EC surface area, they cannot be explained solely by the protection of vascular network integrity. Therefore, in addition to protecting the integrity of the vascular network, ANGPTL4 also specifically reduces ICAM-1 expression, both these effects decreasing PMN recruitment in stroke, thus limiting inflammation.

As recently described, transcriptional activation of the matrix metalloproteinase 9 (MMP9) by nuclear-factor-κB (NF-κB) in the cerebral vasculature could be involved in BBB disruption. Using western blot analyses, we showed that NF-κB activation and MMP9 amount are significantly decreased in the infarcted hemisphere of ANGPTL4-treated mice compared with controls (Supplementary material online, Figure S12A and B). Decreased expression of MMP9 in the infarcted hemisphere of ANGPTL4-treated mice was confirmed by immunostaining, while gelatin zymography demonstrated a significant decrease in MMP9 activity (Supplementary material online, Figure S12C and D). To analyse whether ANGPTL4 might directly regulate NF-κB–MMP9 pathways, HUVEC cells were treated with histamine, a known activator of NF-κB, with or without ANGPTL4. Histamine-induced phosphorylation of NF-κB and MMP9 expression was not affected by ANGPTL4 (Supplementary material online, Figure S13). These results, therefore, confirm that ANGPTL4 reduces inflammation during stroke, with a decreased activation of NF-κB, but suggest that ANGPTL4 does not directly regulate the NF-κB–MMP9 pathway.

**Angiopoietin-like 4 protects from neuronal loss in stroke**

Brain oedema following stroke can contribute to subsequent neuronal injury. Given the role of ANGPTL4 in reducing brain oedema and infarct volume, we analysed its effects on neuronal loss. Mature neurons were quantified using NeuN antibody immunostaining in mice receiving ANGPTL4 or vehicle. Whereas no significant difference was found in the contralateral hemispheres of both groups, a significant increase in the number of NeuN positive cells was quantified in the infarcted hemisphere of ANGPTL4-treated mice compared with the control (Supplementary material online, Figure S14).

**Discussion**

The beneficial effects of prompt and adequate restoration of the blood flow after acute ischaemic stroke may be undermined by reperfusion injury to the cerebral microvasculature. VEGF-induced vascular permeability during stroke has been linked to the progression of ischaemic injury in the brain and to subsequent neuronal injury by inducing endothelial junction disruption and oedema. It has previously been shown that angptl4 mRNA is induced by hypoxia in ECs, cardiomyocytes, and ischaemic brain injury. We show here that ANGPTL4 expression is also induced during ischaemic stroke in humans and mice and we, therefore, propose ANGPTL4 as a new therapeutic approach specifically aimed at modulating cerebrovascular permeability during stroke.
VE-cadherin constitutes the major component of the adherens junctions in ECs, and is required to maintain vascular integrity and barrier function. We, here, provide evidence that ANGPTL4 protects interendothelial junctions from disruption, VEGF-induced in vitro, and ischaemia-induced during stroke in vivo. These findings show that ANGPTL4 represents a new strategy that targets the blood vessels. This could be particularly interesting for prevention of haemorrhagic transformation in stroke, a medical need which remains unmet. Given the importance of rtPA-induced haemorrhagic transformation in stroke, a medical need which remains unmet. Given the importance of rtPA-induced haemorrhagic transformation, which is correlated to BBB injury, the maintenance of vascular integrity by ANGPTL4 indeed represents a promising therapeutic approach for future studies.

Figure 6 Angiopoietin-like 4 treatment prevents blood–brain barrier breakdown during stroke. (A) Angiopoietin-like 4 significantly decreases brain oedema compared with control mice. (B) Isolectin B4 staining of blood vessels in the infarcted area of both groups. Angiopoietin-like 4 prevents the extravasation of fluorescent beads (arrows). Scale bar: 30 μm. (C) Three-dimensional rebuild showing extravasation of fluorescent microspheres from brain blood vessels immunostained with isolectin B4 after stroke. Scale bar: 3 μm. (D) Quantification of FITC beads extravasation in contralateral and infarcted hemispheres of control and angiopoietin-like 4-treated mice (n = 8 mice per group). Error bars represent SD.

In the present study, the mechanisms responsible for ANGPTL4-mediated vasculoprotection were studied in stroke. It has been shown that ischaemia-induced VEGF binds to VEGFR2 and dissociates the VEGFR2–VE-cadherin complex through the Src-signalling pathway leading to interendothelial junction disruption thus promoting vascular leakage. In vivo, we show that ANGPTL4 maintains vascular integrity through protection of VEGF-induced dissociation of VEGFR2–VE-cadherin complexes. Signalling pathways activated by ANGPTL4 were further analysed in vitro. Whereas ANGPTL4 alone activates Src, it leads to diminished Src-signalling downstream of VEGFR2 in the presence of VEGF, by impeding Src recruitment downstream of VEGFR2. In addition, we demonstrate that
Angiopoietin-like 4 decreases intercellular adhesion molecule-1-positive vessels and reduces polymorphonuclear neutrophil recruitment in stroke. (A) Immunostainings of myeloperoxidase-positive cells and Isolectin B4 in contralateral and infarcted hemispheres of control and ANGPT4-treated mice. (B) Immunostainings of intercellular adhesion molecule-1-positive vessels and Isolectin B4 in contralateral and infarcted hemispheres of control and ANGPT4-treated mice. (C) Quantification of myeloperoxidase-positive cells in contralateral and infarcted hemispheres of control and angiopoietin-like 4-treated mice. (D) Quantification of intercellular adhesion molecule-1 vascular coverage normalized to the EC surface area, in contralateral and infarcted hemispheres of control and angiopoietin-like 4-treated mice (n = 4 mice per group). Error bars represent SD. Scale bar: 160 μm.
ANGPTL4 activates the PI3K–Akt pathway, which participates in the control of the Src-signalling pathway. Inhibition of ANGPTL4-activated Akt by wortmannin indeed restored the recruitment of Src downstream of VEGFR2. To the best of our knowledge, we here provide the first evidence that ANGPTL4 is a regulator of cross-talk between PI3K–Akt and Src kinase signalling pathways.

In addition, cerebral infarcted areas are associated with cellular infiltration and acute inflammatory response. Intercellular adhesion molecule-1, which is up-regulated in the infarcted area of the human brain, plays a pivotal role in neutrophil recruitment and ICAM-1 deficient mice display reduced inflammatory responses and decreased brain oedema. Interestingly, we, here, show that ANGPTL4 prevents cerebral neutrophil infiltration through (i) protection of ischaemia-related BBB disruption but also (ii) inhibition of the overexpression of ICAM-1 by Ecs upon stroke injury. Subsequent deleterious post-infarction inflammatory response is, therefore, limited in ANGPTL4-treated mice and thus also expansion of the infarcted area. Significantly reduced infarct size is indeed demonstrated in mice treated with ANGPTL4 using two different quantification methods (TTC staining and cerebral MRI).

Stroke being the leading cause of acquired disability worldwide, it is of importance to note that besides the reduction of infarct volume, ANGPTL4 treatment also significantly improved behaviour. Further studies are needed to confirm the relevance of targeting interendothelial EC junctions to reduce permeability by ANGPTL4, and to investigate whether this original therapeutic approach targeting protection of the cerebral microvasculature could be applied to ischaemic stroke in humans.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

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**Conflicts of interest:** S.G. and C.M. own the patent. (Methods and pharmaceutical composition for the preservation of vascular EC barrier integrity. Patent No 11700371.5—2107).

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