Secondary Intracerebral Hemorrhage Due to Early Initiation of Oral Anticoagulation After Ischemic Stroke

An Experimental Study in Mice

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Background and Purpose—The uncertain risk of secondary intracerebral hemorrhage (sICH) frequently keeps clinicians from initiating oral anticoagulation (OAC) early after ischemic cardioembolic stroke. The goal of this experimental study was to determine the risk of sICH depending on the timing of OAC initiation relative to stroke onset and to address the role of hematogenous macrophages for repair processes preventing OAC-associated sICH.

Methods—C57BL/6 mice were subjected to transient middle cerebral artery occlusion. Subgroups were treated with either the vitamin K antagonist (VKA) phenprocoumon or the direct thrombin inhibitor dabigatran etexilate. Hematogenous macrophages were depleted using intraperitoneal injections of clodronate-filled liposomes.

Results—Time to therapeutic OAC was 48 hours with VKA and 0.5 hours with dabigatran etexilate treatment. In VKA-treated mice, the risk of sICH was high if effective OAC was already present at stroke onset or achieved within 48 hours after ischemia. With more delayed OAC, the risk of sICH rapidly decreased. Compared with VKA treatment, effective anticoagulation with dabigatran etexilate was associated with a significantly reduced extent of sICH, either if present at stroke onset or if achieved 48 hours later. Partial depletion of macrophages greatly increased the extent of OAC-associated sICH in the subacute stage of 3 to 4 days after ischemia.

Conclusions—Our findings suggest that repair mechanisms involving hematogenous macrophages rapidly decrease the risk of OAC-associated sICH in the first days after ischemic stroke. The lower risk of sICH under dabigatran etexilate compared with VKA treatment may facilitate early initiation of OAC after cardioembolic stroke. **(*Stroke*.2012;43:3352-3357.)**

Key Words: anticoagulation ■ cerebral ischemia ■ inflammation ■ intracerebral hemorrhage ■ macrophage

Oral anticoagulation (OAC) is a highly effective means for the prevention of cardioembolic stroke but complicated by an increased risk of intracerebral hemorrhage (ICH).[1–3] OAC-associated ICH may occur either as primary ICH or as secondary hemorrhagic transformation of brain infarctions. To date, little is known about the optimal timing of OAC initiation relative to stroke onset. The uncertain risk of secondary ICH (sICH) frequently prevents clinicians from initiating OAC early after cardioembolic stroke. This increases the rate of recurrent thromboembolism at least in high-risk patients.[4] Compared with vitamin K antagonists (VKA), such as warfarin or phenprocoumon, new oral anticoagulants, such as the direct thrombin inhibitor dabigatran etexilate (DE), exhibit reduced rates of intracranial hemorrhage,[5,6] indicating a potentially better benefit/risk profile for stroke prevention.

Clinical trials addressing the occurrence of sICH depending on the timing of OAC initiation relative to stroke onset are currently not available and may be difficult to conduct without additional preclinical data. Genetic studies revealed strong evolutionary conservation of coagulation factors in vertebrates,[7] enabling an experimental approach to OAC-associated ICH in laboratory animals. A pivotal study in a mouse model of warfarin anticoagulation addressed the rate of sICH in animals already effectively anticoagulated at the time point of stroke induction. Hemorrhagic infarct transformation occurred in all anticoagulated mice within 24 hours of transient middle cerebral artery occlusion (tMCAO).[8] However, more delayed stages of stroke lesion development were not addressed in this study. Regarding DE treatment, experimental findings in a model of primary ICH showed that in contrast to warfarin, pretreatment with DE did not increase hematoma volume within 24 hours after ICH induction.[9] Furthermore, warfarin but not DE pretreatment increased the rate of thrombolysis-associated sICH in the tMCAO model of ischemic stroke.[10] Thus, available evidence suggests a lower risk of hemorrhagic

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complications under DE. However, only the hyperacute injury phase has been studied experimentally to date.

The mechanisms provoking or limiting sICH in various stages after the primary ischemic insult are largely unknown. Stroke-induced neuroinflammation may cause exacerbation of ischemic brain injury but may also foster repair and tissue protection.[11–16] Recently, we showed that hematogenous recruitment of monocyte-derived macrophages is a key mechanism of brain repair in subacute stroke lesions, mainly via transforming growth factor-β–dependent stabilization of neovessels in the infarct border zone.[17] We therefore postulated that interference with hematogenous macrophage recruitment may increase the risk of sICH after initiation of OAC early after ischemia. To address this issue, we first defined the temporal risk profile of OAC-associated sICH under either VKA or DE treatment in a murine tMCAO model of moderate cerebral ischemia. To elucidate protective mechanisms counteracting sICH, we then studied the impact of partial macrophage depletion on sICH occurrence in a delayed time window after ischemia.

Methods

Experimental procedures are detailed in the Supplementary Methods section. All animal experiments were approved by local authorities and were performed in accordance with international guidelines on handling laboratory animals. Focal cerebral ischemia was induced in male C57BL/6 mice by 55 minutes of tMCAO.[18] Phenprocoumon or DE was administered via gastric tubing. In phenprocoumon-treated mice, international normalized ratio was determined at day 2 and day 4 after initiation of OAC using a point-of-care device (CoaguChek; Roche, Basel, Switzerland). In DE-treated mice, activated partial thromboplastin time was monitored using standard laboratory testing. Hematogenous macrophages were depleted by means of clodronate-filled liposomes according to a previously described protocol.[17] Infarct volumes and bleeding rates were assessed on 2-mm-thick slices stained with 2% 2,3,5-triphenyltetrazolium chloride. Bleeding volumes were determined photometrically in supernatants of brain tissue homogenates. All analyses were performed by an investigator blinded for treatment allocation, and results are presented as mean±SD. Statistical analyses were conducted using GraphPad Prism software (GraphPad Software Inc, La Jolla, CA).

Results

Blood Coagulation Parameters

In untreated mice, INR was 0.97±0.07 (n=9). Once-daily administration of 0.8 mg/kg phenprocoumon led to effective anticoagulation 48 hours later (INR 2.51±0.38, n=9), which could be maintained in a therapeutic range throughout the experiment (Figure 1A).

In mice treated with DE (Figure 1B), we used standard laboratory assessment of aPTT for establishment of a dose–response curve over time. Thirty minutes after administration of DE at 75 mg/kg body weight, aPTT increased 2.5-fold above untreated controls (62.20±24.26 versus 25.14±3.9 seconds, n=5), decreasing to 1.59-fold at 8 hours (38.43±12.33 seconds, n=4), which is close to the mean aPTT elevation in humans in the steady state before administration of the next dose.[19]

Time-Dependent Risk of OAC-Associated sICH

We first performed macroscopic assessment of infarct development and bleeding rate in tMCAO mice treated with phenprocoumon at various stages before or after ischemia induction (Figure 2A and 2B). When effective OAC was present at the time point of stroke induction (ie, initiation of VKA treatment 2 days before tMCAO), 6 of 9 phenprocoumon-treated mice developed severe parenchymal hemorrhage and 3 of 9 developed hemorrhagic transformation. Hemorrhage was restricted to ischemic tissue areas. When effective OAC was achieved 48 hours after ischemia, 4 of 9 mice developed parenchymal hemorrhage and 3 of 9 developed hemorrhagic transformation. At both 72 and 96 hours, none of the mice developed parenchymal hemorrhage. Hemorrhagic transformation occurred in 5 of 10 mice in the 72-hour group and was absent in the 96-hour group.

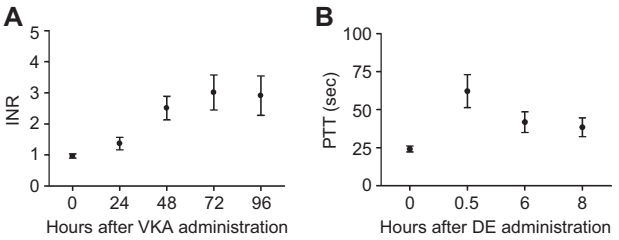

Figure 1. A, INR measurements at indicated time points after daily administration of phenprocoumon at 0.8 mg/kg body weight. Effective oral anticoagulation is achieved after 48 hours and maintained into later stages (n=9). **B**, After administration of dabigatran etexilate (DE) at a dose of 75 mg/kg body weight, aPTT increases within 30 minutes and reaches steady-state levels after 6 to 8 hours (n≥4, mean±SD). VKA denotes vitamin K antagonist.

In line with the macroscopic findings, photometrically determined blood volumes decreased from 11.0±4.5 µL (n=6) in mice effectively anticoagulated at the time of tMCAO to 5.1±4.2 µL (n=6) with OAC achieved at 48 hours and to 3.5±1.5 µL (n=6) at 72 hours (Figure 2C). Effective OAC achieved at 96 hours was associated with very discrete hemorrhagic transformation (1 µL blood volume) in only 1 animal. Infarct volumes were not different between groups (effective OAC VKA d0, 88.5±31.93 mm^3; VKA d2, 92.75±36.17 mm^3; VKA d3, 85.86±30.59 mm^3; VKA d4, 91.5±19.07 mm^3).

Reduced Extent of sICH in DE-Treated Mice

For a comparative analysis of sICH under DE treatment, mice were started on DE at various time points before and after tMCAO. Compared with phenprocoumon treatment, mice pretreated with DE at a dose of 75 mg/kg body weight for 2 days before tMCAO (corresponding to effective anticoagulation with an aPTT of 38.43±12.33 seconds at the time point of stroke induction) showed a trend of decreased parenchymal hemorrhage with reciprocally increased hemorrhagic transformation (Figure 3A). This qualitative finding was corroborated by a significant decrease in bleeding volume assessed photometrically in a separate experiment (11.0±4.5 µL in VKA-pretreated mice versus 3.8±3.5 µL in DE-pretreated mice, n=6; Figure 3B). Infarct volumes were not significantly different between groups (VKA, 88.5±31.93 mm^3; DE, 82.67±22.25 mm^3).

If effective anticoagulation was achieved 48 hours after ischemia (ie, DE treatment initiated 47 hours after tMCAO), none of the DE-treated mice developed parenchymal hemorrhage and

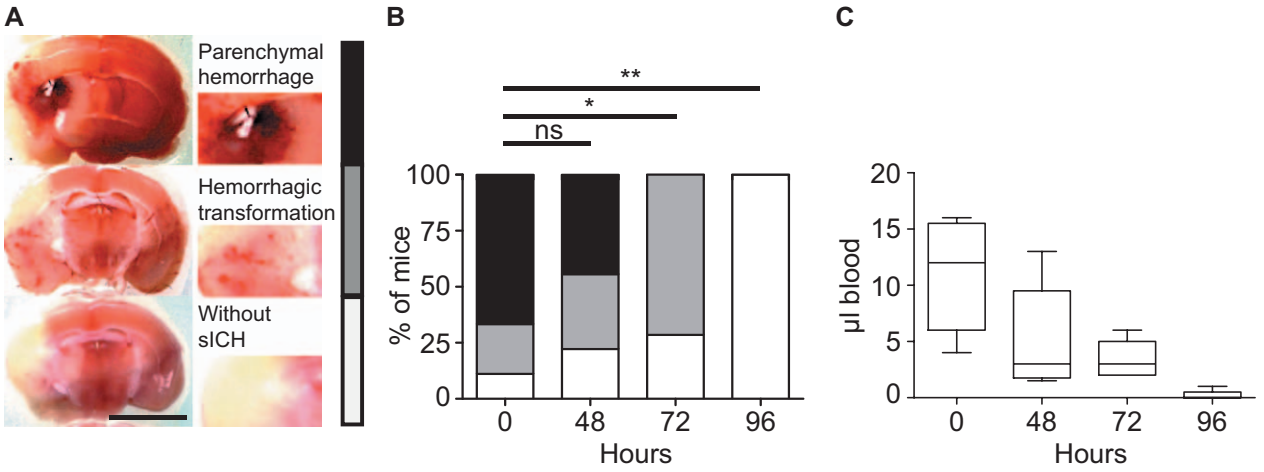

Figure 2. A, Brain slices illustrating representative findings of parenchymal hemorrhage, hemorrhagic transformation, or absence of secondary intracerebral hemorrhage (sICH) after transient middle cerebral artery occlusion. Bar=5 mm, with ×2 magnifications shown on the right. **B**, Bleeding risk rapidly decreases with increasing latency between stroke onset and the day when effective oral anticoagulation (OAC) is achieved (n≥9). Black bars indicate parenchymal hemorrhage; gray bars, hemorrhagic transformation; and white bars absence of sICH. The time point of effective OAC relative to stroke onset is indicated on the *x*-axis. **P*<0.05, ***P*<0.01 (χ^2 test). **C**, Bleeding volumes also decrease with increasing latency from stroke onset (n=6). The whiskers of the box-plot indicate the extreme values.

only 6 of 11 showed hemorrhagic transformation (Figure 3C). In photometric analysis, bleeding volume in the 48 hours group was also reduced in DE-treated versus phenprocoumon-treated mice (0.8±1.0 µL versus 5.1±4.2 µL, n=6; Figure 3D). Infarct volumes were not different between groups (VKA, 92.75±36.17 mm^3; DE, 93.63±35.68 mm^3). Rotarod testing performed at day 5 in mice effectively anticoagulated with either VKA or DE from 48 hours after stroke did not reveal significant differences in clinical outcome.

To elucidate the impact of intensity of OAC, we introduced an additional group of mice treated with a lower dose of phenprocoumon (0.64 mg/kg) from day 0 after tMCAO, reaching effective OAC 48 hours after stroke (INR 2.24±0.28, n=9). In both dose groups, bleeding complications occurred to a similar extent (Figure 3C). Similarly, when all mice from both dose groups were pooled and stratified by INR range (INR 1.8–2.5 versus INR 2.6–3.5), no differences between groups were evident. Thus, bleeding rates were independent from the intensity of OAC. We, furthermore, studied the effect of a higher dose of DE (112.5 mg/kg body weight). DE at 112.5 mg/kg caused a 2.76-fold elevation of the aPTT 6 hours later (66.68±25.21 seconds, n=4), thereby resembling an overdose of DE in a clinical setting.[19] In this high-dose DE-treated group, a high rate of severe sICH occurred, which was similar to the VKA-treated groups (Figure 3C and 3D). Thus, supratherapeutic dosage of DE caused an increase in sICH comparable with VKA treatment, which is in line with previous findings in thrombolysis-associated infarct hemorrhage[8] and collagenase-induced hemorrhage.[20]

Partial Depletion of Hematogenous Macrophages Increases OAC-Associated sICH

Taken together, our results suggested the existence of a repair process that leads to a rapid decrease in the rate of OAC-associated sICH within 72 hours after tMCAO. In a recent study, we showed that depletion of monocyte-derived macrophages by means of clodronate-filled liposomes injected intraperitoneally at days 0 and 1 after tMCAO causes severe sICH of tMCAO-induced brain infarctions in nonanticoagulated mice.[17] To address a possible role of macrophages for the prevention of OAC-associated sICH, we chose a submaximal dose of clodronate liposomes (75% of maximum dose), which caused a less pronounced reduction of monocyte-derived macrophages in multicolor flow cytometric analysis of leukocytes isolated from the infarctions (Figure 4A). In these analyses, hematogenous monocyte-derived macrophages were identified as CD11b-positive cells expressing high levels of CD45 ($CD45^{hi}$, as opposed to brain-resident microglia expressing intermediate levels of CD45) but not expressing the granulocyte marker Ly-6G. In the partially depleted mice, we observed only discrete hemorrhagic transformation but not severe parenchymal hemorrhage (Figure 4B). However, partially depleted mice additionally started on phenprocoumon from 24 hours after tMCAO (ie, reaching effective anticoagulation at day 3 after ischemia) exhibited a striking increase of parenchymal hemorrhage that exceeded the rate under either VKA or clodronate treatment alone (Figure 4B). Similar results were obtained in partially depleted mice effectively anticoagulated with DE at 48 hours after tMCAO (Figure 4B). The increase in bleeding severity was paralleled by an increase in bleeding volume (Figure 4C). Thus, partial depletion of monocyte-derived macrophages and OAC with either VKA or DE synergistically provoked sICH in subacute brain infarction.

Discussion

As a main finding, we show that the risk of sICH upon initiation of OAC early after tMCAO in mice rapidly decreases within 3 days of stroke onset, suggesting effective repair mechanisms that reestablish at least partial integrity of the neurovascular unit in early subacute stroke stages. Relative to VKA treatment, OAC with the direct thrombin inhibitor

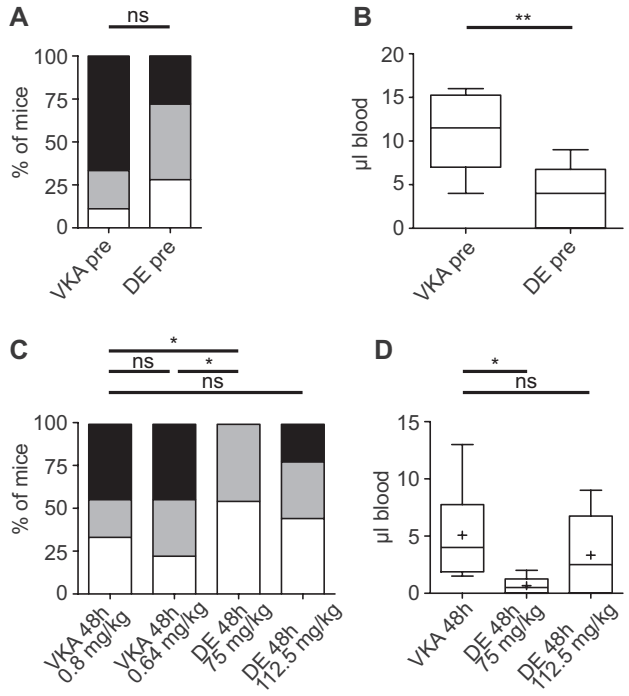

Figure 3. A, Comparison of mice treated with vitamin K antagonist (VKA; n=9) or dabigatran etexilate (DE; n=7) before transient middle cerebral artery occlusion (tMCAO) reveals a nonsignificant shift (χ^2 test) from parenchymal hemorrhage toward hemorrhagic transformation in DE-pretreated animals. Black bars indicate parenchymal hemorrhage; gray bars, hemorrhagic transformation; and white bars absence of sICH. **B**, Significant reduction of bleeding volume after tMCAO in DE-pretreated versus VKA-pretreated mice (n=6). $^{**}P<0.01$ (Student *t* test). **C**, If effective OAC is reached 48 hours after stroke onset, significantly lower bleeding rates occur in DE-treated compared with VKA-treated mice. However, the difference is only evident at a DE dose of 75 mg/kg body weight but not at 112.5 mg/kg (n=9; $^{*}P<0.05$ in χ^2 test). In contrast to DE, bleeding pattern in VKA-treated mice is independent from the dosage administered. **D**, Relative to VKA treatment, bleeding volume is likewise reduced with DE at 75 mg/kg but not at 112.5 mg/kg (n=6, $^{*'}P<0.05$, Kruskal-Wallis test with the Dunn post hoc test). The whiskers of the box-plots throughout indicate the extreme values, and + indicates the mean.

DE is associated with a reduced risk of sICH both at the time of stroke onset and in the subacute stage of 48 hours after tMCAO. This finding is basically in line with the reduced rate of intracranial bleeding with DE treatment in clinical trials of stroke prevention, as well as with previous studies addressing DE effects in experimental ICH.[5,6,9] Compared with these previous studies, our present study for the first time addressed the clinically relevant situation of OAC initiation early after ischemic stroke. In this subacute time window, clinicians are frequently faced with the therapeutic dilemma of reducing embolic stroke recurrence at the expense of increased intracranial bleeding complications, particularly in patients at high risk of thrombembolism. Our results indicate that the risk of sICH associated with classical VKA treatment may be relatively low beyond day 3 after the onset of ischemia and may be even lower with the newer anticoagulants, such as DE.

Regarding possible repair mechanisms, we have previously shown that extensive depletion of hematogenous macrophages using high-dose clodronate liposome treatment of nonanticoagulated mice provokes a high rate of ≈50% parenchymal hemorrhage after tMCAO.[17] The clodronate liposome technique is a well-characterized approach for specific depletion of phagocytic cells.[21] Of note, clodronate liposomes do not deplete platelets but rather increase thrombopoietic activities, excluding that the observed bleedings are caused by a loss of platelets.[22] In our previous study in nonanticoagulated mice, infarct hemorrhage after extensive macrophage depletion occurred between days 2 and 3 after stroke onset,[17] corresponding to the temporal risk profile delineated in our present study. This suggested that macrophage-dependent brain repair may be involved in the prevention of OAC-associated sICH. To test this hypothesis, we reduced the liposome dose to a point where no overt bleeding was induced in nonanticoagulated mice and combined this low-dose liposome regimen with phenprocoumon treatment from day 1 onward, normally also leading to only slight hemorrhagic transformation. Together, these 2 subthreshold conditions of macrophage depletion and anticoagulation synergistically increased the bleeding severity. Similar results were obtained with delayed DE treatment in macrophage-depleted mice, indicating that macrophage-dependent repair processes are also essential for preventing sICH under the new anticoagulants.

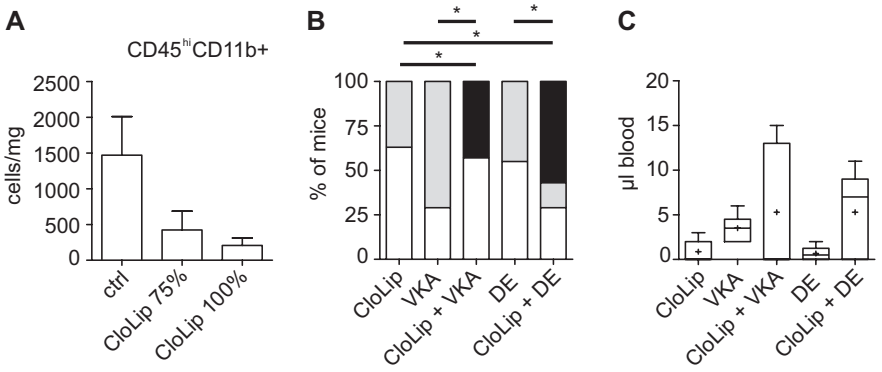

Figure 4. A, Flow cytometric analysis of hematogenous macrophages 72 hours after transient middle cerebral artery occlusion. The full dose (100%) of clodronate liposomes (CloLip) strongly reduces macrophage numbers in the infarctions. Less efficient reduction is observed with the 75% liposome dose. **Bars** denote mean±SD. **B**, Partial macrophage depletion alone, as well as effective oral anticoagulation (OAC) alone (either vitamin K antagonist [VKA] or dabigatran etexilate [DE]), causes only discrete hemorrhagic transformation of the infarctions. When delayed OAC and macrophage depletion are combined, high rates of severe parenchymal hemorrhage are found in both the VKA-treated and DE-treated mice. Black bars indicate parenchymal hemorrhage; gray bars, hemorrhagic transformation; and white bars absence of sICH. $^{*}P<0.05$, χ^2 test. **C**, Bleeding volumes are higher in macrophage-depleted and anticoagulated mice compared with each treatment alone (n=6). The **whiskers** of the box-plot indicate the extreme values, and + indicates the mean.

Our findings are in line with accumulating evidence from other models of tissue injury describing profound impairment of tissue repair and hemorrhagic complications after depletion of macrophages.[23–25] Interestingly, bleeding into skin wounds of macrophage-depleted mice specifically occurred in a delayed time window after injury,[24] comparable with our results. In contrast, most previous studies in stroke models focused on the acute stage of neuroinflammation, but did not specifically address the role of hematogenous macrophages for delayed repair mechanisms.[14,15] The critical relevance of the time window was underscored by experiments addressing the role of matrix metalloproteinase-9 in neurovascular remodeling after stroke.[26] Although inhibition of matrix metalloproteinase-9 was beneficial in the early stage, delayed inhibition increased brain injury and—of particular importance for our study—caused hemorrhage into the ischemic area.

Under a translational perspective, several endogenous as well as exogenous factors can be envisaged to interfere with macrophage-dependent repair and thereby increase the risk of OAC-associated sICH in stroke patients. Diabetes mellitus and hyperglycemia are associated with a worse prognosis of ICH[27,28] and an increased rate of hemorrhagic transformation after systemic thrombolysis in patients with ischemic stroke.[29,30] Although direct influences on the coagulation system may at least partially explain the increased bleeding risk,[31] several studies showed that macrophage responses critical for wound healing are also impaired in diabetes mellitus.[32,33] Regarding potential exogenous factors, cardiovascular drugs such as statins or angiotensin-II type 1 receptor blockers also interfere with macrophage recruitment into inflammatory tissue lesions.[34,35] Although the pharmacological suppression of macrophage recruitment may be beneficial in atherosclerosis or autoimmunity, harmful effects causing disturbances in infarct demarcation and an increased bleeding risk after stroke also need to be considered and should be the subject of further study.

In conclusion, our study provides evidence that endogenous repair mechanisms involving hematogenous macrophages lead to a rapid decrease in the risk of OAC-associated sICH after ischemic stroke. Limitations of our study arise from the fact that it was conducted in young mice and that the impact of additional risk factors for OAC-associated ICH, in particular age and leukoaraiosis,[36,37] was not specifically addressed. Therefore, studies incorporating additional risk factors are needed for a more complete understanding of OAC-related sICH in ischemic stroke.

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Disclosures

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