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Safety of oral anticoagulants on experimental brain microbleeding and cognition

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HIGHLIGHTS

- Oral anticoagulants enhance the cerebral microhemorrhage burden.
- Only warfarin transforms cerebral microhemorrhages into deadly hematoma.
- Disseminated cerebral microhemorrhages induce cognitive impairment in healthy mice.
- Oral anticoagulant-enhanced microhemorrhages do not precipitate cognitive impairment.

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ABSTRACT

This study aims at determining the ability of clinical-based doses of four oral anticoagulants to transform the onset of a cerebral microhemorrhages (CMH) burden into a symptomatic intracerebral hemorrhage (ICH) in the healthy brain, and precipitate cognitive impairment. Wild-type mice were anticoagulated for 10 days using apixaban, rivaroxaban or dabigatran as direct oral anticoagulants (DOACs), or warfarin as vitamin K-antagonist. Meanwhile, a burden of ~20 CMHs was induced in the Sylvian territory by intra-carotid injection of cyclodextrin nanoparticles. At bleeding onset, only warfarin provoked deadly hematoma, and dramatically increased mortality (+45%). All the DOACs enhanced CMH burden through a greater number of intermediate-sized microhemorrhages (+80% to +180%). Although silent at onset, both baseline- and anticoagulant-enhanced CMH burdens increased mortality (+11% to +58%) along the following year without statistical difference among groups, and despite cessation of anticoagulation and absence of CMH progression or transformation into ICH. All survivor mice exhibited reduction in visual recognition memory from 9 months. In the healthy brain, DOACs preserve the onset of microhemorrhages from transformation into ICH, and do not precipitate cognitive impairment despite enhancement of CMH burden. High CMH burdens should however be considered for early detection and preventive memory care apart from anticoagulation decisions.

1. Introduction

Cerebral microhemorrhages (CMHs) are common in subjects likely to be exposed to anticoagulants, including patients with cerebrovascular disease or dementia, but also healthy elderly with a prevalence ranging from 5 to 23% (Hilal et al., 2017). In the latter population, the use of warfarin is now seen as an independent predictor of future intracerebral hemorrhage (ICH), which remains the most feared complication of anticoagulation (Etten et al., 2014). Indeed, dementia-free survivors of spontaneous ICH are at substantial risk of incident dementia at a term as short as 4 years after onset (Moulin et al., 2016). Likewise, as CMHs can occur after the breakdown of several cerebral microvessels, an enhanced microbleed burden (Stemer et al., 2010) has been associated to a greater risk of precipitated cognitive impairment in patients, but remains unclear in healthy elderly subjects (Cordonnier, 2010; Ding et al., 2017; Wu et al., 2014), for which anticoagulation decisions are relevant (Livingston et al., 2017). Direct oral anticoagulants (DOACs) are becoming widely adopted for their efficacy and safety profile (Barrett et al., 2017), but still lack information on their potential use in subjects at risk of presenting CMHs. We thus aimed at

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determining whether the onset of a CMH burden under clinical-based doses of DOACs can transform into a symptomatic bleeding in the healthy brain, and precipitate cognitive impairment. As CMH can be multifocally distributed within lobar and non-lobar areas of the brain parenchyma (Greenberg et al., 2009), we induced a disseminated pattern of barely visible CMHs in mice, and checked their functional consequences within the following year. During CMHs onset, mice were administered *per os* either of three DOACs (dabigatran, rivaroxaban, apixaban) or the vitamin K-antagonist warfarin.

2. Materials and methods

2.1. Ethical aspects

All experiments were approved by the national Ethical Committee in Animal Experimentation (CEEA n°75, comité d'éthique en experimentation animale), from the French Ministry for Education and Research (agreement number: n°2016022510527156-V2) and were performed in strict compliance with the European Union Directive 2010/63/EU. Experiments were reported in accordance with the ARRIVE guidelines for reporting experiments involving animals.

2.2. Animals

All mice in this study were adult male C57Bl6j (Janvier SAS, Le Genest-St-Isle, France) weighing 28–30 g (12 weeks old). As the number of experimental groups was high (6-7), only male mice were included to avoid animal overuse. Moreover, no sex differences have been previously reported in the number of CMHs induced by anticoagulants in another mouse model (Marinescu et al., 2017a). Animals were housed under controlled laboratory conditions, with a 12-h dark-cycle, a temperature of 21 ± 2 °C and a humidity of 60–70%.

2.3. Induction of microhemorrhages and drug administration

Cerebral microhemorrhages were induced by injecting Randomly methylated-β-Cyclodextrins (RAME-CD) diluted in saline solution (NaCl 0.9%) at the concentration of 200 mg/mL. Cyclodextrins are a group of structurally related nanoparticles, which are natural products of the bacterial digestion of cellulose, used as pharmaceutical excipients. Among them, β-CD and their methylated derivatives are regarded as safe and well eliminated since water-soluble (Jambhekar and Breen, 2016). These methylated nanoparticles have been reported not to cross the blood-brain barrier (BBB) *in vitro* but to release cholesterol and phospholipids from the cell membrane, thus damaging the BBB endothelium *in vitro* (Monnaert et al., 2004). This ability was exploited here through the injection of RAME-CD (courtesy of Dr Hervé Bricout, UCCS Laboratory, Lens, France) in the carotid artery in order to erode the membrane of the endothelium of the downstream vasculature of the Sylvian territory. For this induction, mice were anesthetized with isoflurane (induction: 3%; maintenance: 2%) and body temperature was kept constant around 37 °C. The right common carotid arteries were exposed through a midline cervical incision. The RAME-CD solution (200 mg/ml) was injected in the common carotid via a 29G insulin syringe (0.3 g/kg) to allow a circulation into the circle of Willis and distribution to both hemispheres. Then, the suture was carefully removed and the mouse was placed under a red warm light to recover. Several tests were performed to optimize the dose, injection route and delay before visualizing the first CMHs, which appeared from 3 days after injection (data not shown). A maximum pattern of around 20 CMHs per brain was observed at 7 days after injection, and used for the present study.

Two studies were carried out in parallel (Fig. 1):

- a short-term protocol was designed for the induction of a cerebral CMH burden in the brain of mice, and parallel treatment with oral anticoagulants at onset (Fig. 1A). Mice were randomly divided into 6 groups (n = 15–20 per group) treated with: vehicle (Veh, NaCl 0.9%, once a day), warfarin (War, 0.2 mg/kg, once a day), apixaban (Api, 5 mg/kg per day, twice a day), rivaroxaban once (Riv1, 2 mg/kg per day, once a day), rivaroxaban twice (Riv2, 4 mg/kg per day, twice a day) and dabigatran (Dab, 20 mg/kg per day, twice a day). Doses were chosen according to community-medicine practice, further adapted to the mouse as found in experimental studies (Kono et al., 2014; Sun et al., 2013). Each drug was administered orally (gavage) for 10 days (3 days before the injection of RAME-CD, and 7 days after). Mice were kept for behavioral studies in order to check for the functional impact of CMHs, and then sacrificed for the determination of the CMH burden or for histological examination. A group of sham-operated mice was inserted as a non-lesioned control group (nl: n = 10), since injected saline.

- a long-term protocol (Fig. 1B) ensured the follow-up study of the effects of baseline or anticoagulant-modified CMH burdens apart from anticoagulation. The same design and protocol as for the short-term study were applied to 8-12 mice per group (Veh, War, Api, Riv1, Riv2 and Dab), then anticoagulation treatment was stopped, and behavioral tests were performed at 3-6-9- and 12-month time points. At the end, mice were sacrificed for the determination of the CMH burden or for histological examination. A group of sham-operated mice was inserted as a non-lesioned control group (nl: n = 10), since injected saline.

2.4. Drug activity in plasma

Since prothrombin time assessment is not specific when DOACs are used (Adcock and Gosselin, 2015), Anti-Xa (for Riv and Api groups) and anti-IIa (for Dab group) activities were determined with specific kits (Hyphen Heparin and Hemoclot Thrombin Inhibitor, Hyphen Biomed). Despite DOACs are well known for their rapid onset/offset action, blood was collected at the end of the protocol (at day 9) in order to check for possible cumulative effect of daily administrations which might lead to overdose and thus over-anticoagulation in mice. Briefly, blood was collected from the retroorbital sinus at 1 h after the first gavage, plus just before the second gavage, and before sacrifice (day 10). Different doses were tested (Api: 1.25–2.5 and 5 mg/kg/gavage; Riv: 1-2 and 4 mg/kg/gavage; Dab: 10-20 and 50 mg/kg/gavage). For warfarin and vehicle groups, hemostatic state was checked through prothrombin time assessment along the protocol. Blood samples were thus collected at day 0, day 3 and before sacrifice at day 10. Three mice per group were used for all tests (Fig. 2).

2.5. Determination of the cerebral microhemorrhage burden

At the end of the protocols, mice were killed with an overdose of pentobarbital (200 mg/kg, intraperitoneal). Brains were rapidly removed and placed in ice-cold isopentane solution, frozen and coronally dissected into 20 µm-thick slices on cryostat. Then, CMH were counted blinded to treatment on each brain slice, and characterized according to size as described previously by Haddad et al. (2008). Briefly, CMHs that were evident to the eye aided by a magnifying glass x2, and found in only 1 slice, were defined as type 1 CMHs. When evident to the naked eye and found again in the next slice, CMHs were defined as type 2. When larger and found again in more than 2 slices, CMHs were defined as type 3. A total hemorrhagic score was calculated as the sum of all CMHs found throughout the brain, and considered as the CMH burden. Mice with less than 10 CMHs were excluded from the study.

2.6. Behavioral assessment

Mice of each group underwent a battery of tests evaluating spontaneous motor activity, visual recognition memory and working memory. These tests were chosen according to the brain areas affected by CMHs, which are those of the Sylvian territory. During experiments, the equipment was cleaned with 70% ethanol after each trial so that

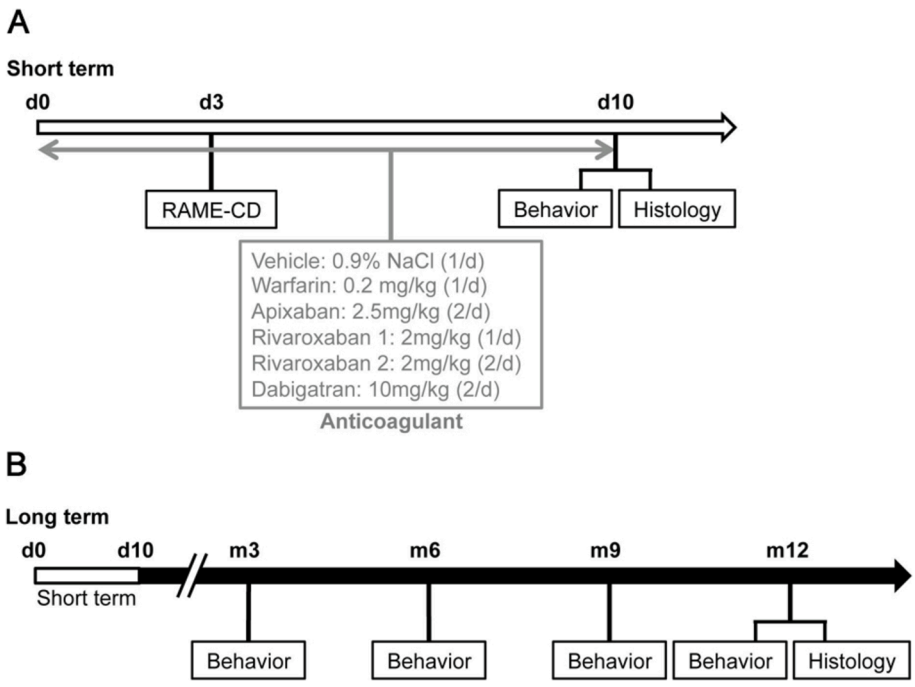


Fig. 1. ***Overview of study design.*** **A.** Short-term protocol for the induction of cerebral microhemorrhages (CMH) burden and treatment of mice with oral anticoagulants at onset. RAME-CD: Randomly methylated-β-Cyclodextrins. **B.** Long-term protocol for the follow-up study of the effects of baseline- or anticoagulant-modified CMH burdens.

olfactory cues did not bias the results.

2.6.1. - Spontaneous locomotor function

Spontaneous locomotor activity was assessed in an open field test, using an infrared actimeter (Bioseb, USA). The apparatus consisted of a square arena (45 × 45 cm) with a black polymethyl methacrylate floor and transparent 34-cm-high polymethyl methacrylate walls. Mice were placed in the center of the arena and allowed to explore freely for 10 min. Activity was recorded by two rows of infrared photocell sensors and processed with Actitrack software (Bioseb). The total distance covered (in cm), the duration of inactivity (resting time, in seconds) and the number of rearings were measured.

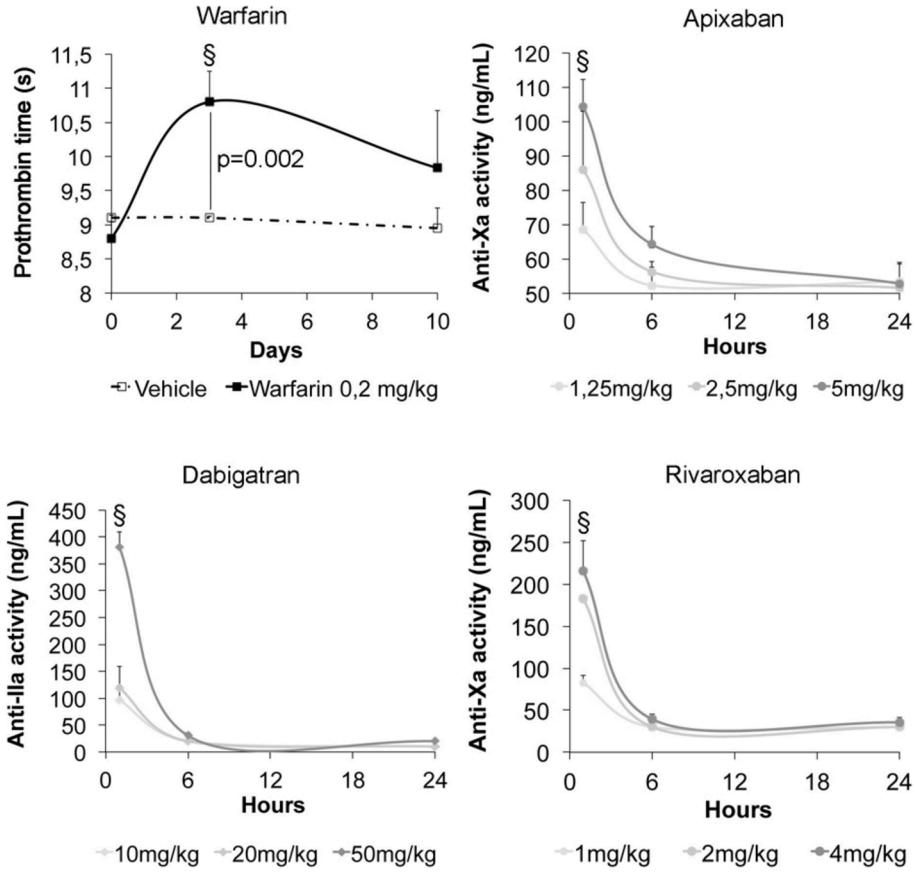


Fig. 2. ***Pharmacological activity of oral anticoagulants.*** For vehicle(Veh)-treated and warfarin (War)-treated animals, prothrombin time was measured at the beginning of the experiment, then 3 days and 10 days after. Prothrombin time was significantly higher at 3 days in the War group compared to Veh group (*t*-test, $p = 0.002$). For direct oral anticoagulants (DOACs), anti-Xa activity (for apixaban and rivaroxaban) and anti-IIa activity (for dabigatran) were measured at 1 h, 6 h and 24 h after gavage. For each DOAC and dose, drug activity significantly increased at 1 h according to a two-way ANOVA followed by a Tukey post-hoc test ($p < 0.001$). Cerebral microbleeds were induced after the injection of randomly methylated cyclodextrins (RAME-CD) at the time point of high anticoagulant activity (§). n = 3 mice per group.

2.6.2. - Visual recognition memory

The novel object recognition test is based on the tendency of mice to explore a novel object rather than a familiar one. Mice were habituated to a square arena (50 × 50 × 25 cm) for 10 min on the first day. On day 2, mice were exposed to the arena containing two identical objects. On day 3, mice were exposed, in a first phase ("sample phase"), to two novel and identical objects for 15 min. Animals returned in their cages. After 1 h, they were placed back in the testing arena for 5 min ("test phase"), but one of the familiar object had been previously replaced by a novel object. Performance of the mice was video recorded (Ethovision XT, Noldus, Wageningen, The Netherlands). Exploratory behaviour was defined as the animal directing its nose toward the object at a distance below 2 cm. Any subject that failed to complete a minimum of 20 s of exploration during the sample phase was excluded from the analysis. A discrimination ratio (index) was calculated as the difference between the time spent exploring the novel object minus the time spent exploring the familiar object divided by the sum of both (Antunes and Biala, 2012).

2.6.3. - Working memory

The spontaneous alternation test was performed using a Y-maze (made of white polyvinyl chloride) and a closed-circuit video camera (Ethovision XT, Noldus, Wageningen, The Netherlands). All three arms were of the same size (30 × 8 × 15 cm) and were oriented at an angle of 120° to each other. The Y-maze was placed in a room with no environmental cues. Each mouse was placed at the end of one arm and was allowed to move freely between the maze's three arms for 8 min. A visit to an arm was scored when all four of the mouse's paws were within the arm area. The sequence of the arm visits was recorded, and an alternation response was scored when the animal entered the least recently visited arm. The alternation score was calculated as the ratio between actual and possible alternations (defined as the total number of arm visits minus 2), multiplied by 100 (Hidaka et al., 2008). This calculation was performed only for mice making more than 20 arm visits.

2.7. Histological analysis

At the end of protocols, mice that were not used for the determination of the CMH burden (n = 2–3 per group), were euthanized with an overdose of pentobarbital (200 mg/kg, intraperitoneal). For tissue staining procedure, mice were transcardially perfused with heparinized physiologic saline for 5 min and decapitated. Subsequently, brains were isolated and fixed in methacarn solution (60% methanol-30% chloroform-10% acetic acid) at 4 °C for 1 day, then in 70% ethanol at 4 °C for 1 day, followed by paraffin embedding. Brains were serially sliced at 8 μm thickness. Every twentieth slice was collected and stained with diaminobenzidine (DAB) to highlight the presence of fresh CMHs or with Prussian blue staining to detect hemosiderin (a marker of subacute hemorrhage). Approximately 20 brain sections were analyzed per mouse. For DAB staining, sections were stained with hematoxylin solution for 5 min to reveal the brain structure, and immersed in fresh DAB solution for 15 min which turned into dark brown after reacting with red blood cell (RBC) peroxidases, thus enabling the detection of extravasated RBCs (intact of lyzed) in brain parenchyma (Toth et al., 2015). For Prussian blue staining, sections were stained with freshly prepared 20% potassium hexacyanoferrate trihydrate and 20% hydrochloric acid for 4 h, rinsed in water and counterstained with hematoxylin-eosin solution for 5 min. Digitalized images were obtained with an automated microscope (AxioScan digital slide scanner Zeiss microscopy, Marly le Roi, France) and analyzed by an observer blinded to experiment.

2.8. Statistical analysis

All values were expressed as mean ± standard error of the mean (SEM). For the short-term study, behavioral continuous variables were compared with a one-way ANOVA followed by a post-hoc Tukey's Multiple Comparison Test if variance analysis was significant. For the long-term study, behavioral continuous variables were compared with a two-way ANOVA followed by a post-hoc Tukey's Multiple Comparison Test if variance analysis was significant. For survival curves, a Mantel-Cox test was performed. For the CMB score, a Pearson's Chi-squared test was realized. In each case, a value of $p < 0.05$ indicated statistical significance.

3. Results

3.1. Drugs activity in the blood

Pharmacological activity of anticoagulants is represented in Fig. 2. For warfarin-treated mice, a significant increase in the Prothrombin time was observed at day 3 compared with vehicle-treated controls ($p = 0.002$), and tended to fall over time. In the DOAC-treated mice, whatever the dose tested, anti-Xa (Riv and Api groups) and anti-IIa (Dab group) activities significantly increased 1 h after the first gavage (Riv: $F_{2,12} = 86.39$, $p < 0.001$; Api: $F_{2,10} = 35.80$, $p < 0.001$; Dab: $F_{2,12} = 77.659$, $p < 0.001$), followed by a gradual decrease after 6 h, down to zero at 24 h.

3.2. Effects of oral anticoagulants at onset of cerebral microhemorrhages

All the mice injected with RAME-CD nanoparticles and treated with vehicle (Veh) have survived after 7 days. Only 1 mouse out of the whole cohort has displayed 9 CMHs (Veh group) and has thus been excluded from the study. Forty-five percent of mice treated with warfarin underwent premature death (9/20), whereas the 3 DOACs led to a low mortality rate (Api: 2/10, 20%; Riv1: 1/20, 5%; Dab: 1/15, 6.6%). No death was found in Riv2 group (Fig. 3A). Furthermore, only warfarin was able to provoke a hematoma in 3 mice out of the 9 mice that died prematurely. Only the warfarin-induced mortality was significantly different from the other groups (Mantel-Cox Test, $p = 0.0003$). In all the survivors, visual CMHs were found in all areas of the Sylvian territory of the brain. In the vehicle-treated mice, a maximum pattern of around 20 CMHs per brain was observed at 7 days after injection. Microhemorrhages were mostly of small (type1) and medium (type 2) sizes. The type 3 CMH was observed only in the brains of anticoagulant-treated mice (Fig. 3B and C). A significant aggravation of CMH burden was noticed in warfarin-treated mice compared to control (Fig. 3D), through a higher contribution of medium-sized (type 2) CMHs ($\chi^2_{(2.509)} = 9.83$, $p = 0.007$, chi-squared of type 2 CMHs contribution = 3.58). Among mice treated with DOACs, the CMH burden was significantly higher in the Api group ($\chi^2_{(2.597)} = 6.62$, $p = 0.03$, chi-squared of type 2 CMHs contribution = 2.67) and the Riv2 group ($\chi^2_{(2.564)} = 9.11$, $p = 0.01$, chi-squared of type 2 CMHs contribution = 3.57). No significant difference was noticed in Riv1 ($p = 0.1$) and Dab ($p = 0.2$) groups compared to Veh.

3.3. Long-term mortality and evolution of the microhemorrhage lesions

Vehicle-treated mice underwent a 20% death rate (2/10) after 12 months. Fifty percent of mice treated with warfarin died within the first 3 months (6/12) and another died at 6 months, amounting to 58% of mortality in the War group (Fig. 4A). Mortality was observed in mice treated with either of the 3 DOACs, reaching 50% for the Api group (5/10), 11.1% for the Dab group (1/9), 40% for the Riv1 group (4/10) and 50% for the Riv2 group (5/10). No hematoma was found in the brain of prematurely died animals (data not shown). Non-lesioned control mice (nl) did not die along the protocol. Mortality did not differ significantly among all groups (Mantel-Cox test, $p = 0.21$). The visual CMH burden of the survivors was determined for each mouse (Fig. 4B). At the final time, no difference was noticed between all groups compared to Veh, even for the War (Pearson's Chi-squared test = 1.73, $p = 0.18$) and

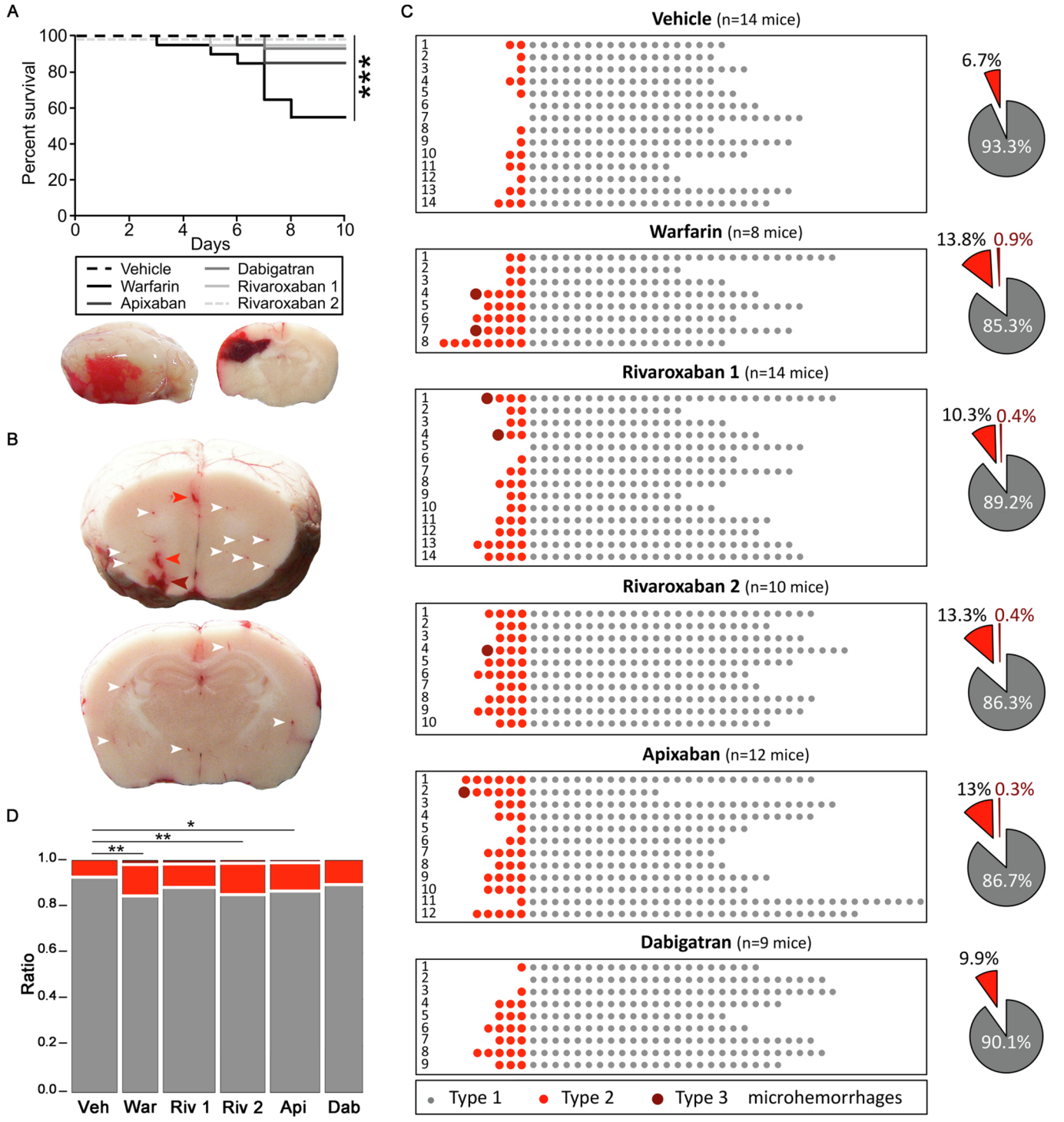


Fig. 3. ***Microbleeding profile of mice at onset.*** **A.** Kaplan-Meyer survival curves (n = 15–20). Mortality was significantly higher in War group compared to others (Mantel-Cox test, p = 0.0002, ***p < 0.001), with a representative view of hematoma (lower side of the panel). **B.** Representative view of cerebral microhemorrhages (CMHs) in the brain of an apixaban-treated mouse, defined as of type 1 (white arrowheads), type 2 (red arrowheads), or type 3 (brown arrowheads) according to size. **C.** Dot plot of CMHs obtained for each mouse with corresponding proportions (right hand side of the panel). **D.** Mosaic plot of CMHs obtained in each group (n = 8–15). The ratio refers to the percentage of each type of microhemorrhage among all observed CMHs. Repartition of CMHs was significantly different according to Pearson's Chi-squared test. **p < 0.01, *p < 0.05 *vs* Veh.

Riv2 (Pearson's Chi-squared test = 3.77, p = 0.15) groups, which displayed the most enhanced CMH burdens of this long-term study (Fig. 4C). The number of visual CMH events was not different whatever the experimental condition and time point, except for Riv2 where a slight decrease in the total CMHs number was observed at 12 months compared to 7 days ($F_{1,82}$ = 21.932, p = 0.043, Fig. 5A). No significant difference was found in the repartition of CMHs between groups according to Pearson's Chi-squared test (Veh: Pearson's Chi-squared test = 1.14, p = 0.28; War: Pearson's Chi-squared test = 1.14, p = 0.77; Riv1: Pearson's Chi-squared test = 1.09, p = 0.77; Riv2: Pearson's Chi-squared test = 2.11, p = 0.19; Api: Pearson's Chi-squared test = 0.17, p = 1; Dab: Pearson's Chi-squared test = 0.53, p = 0.46,

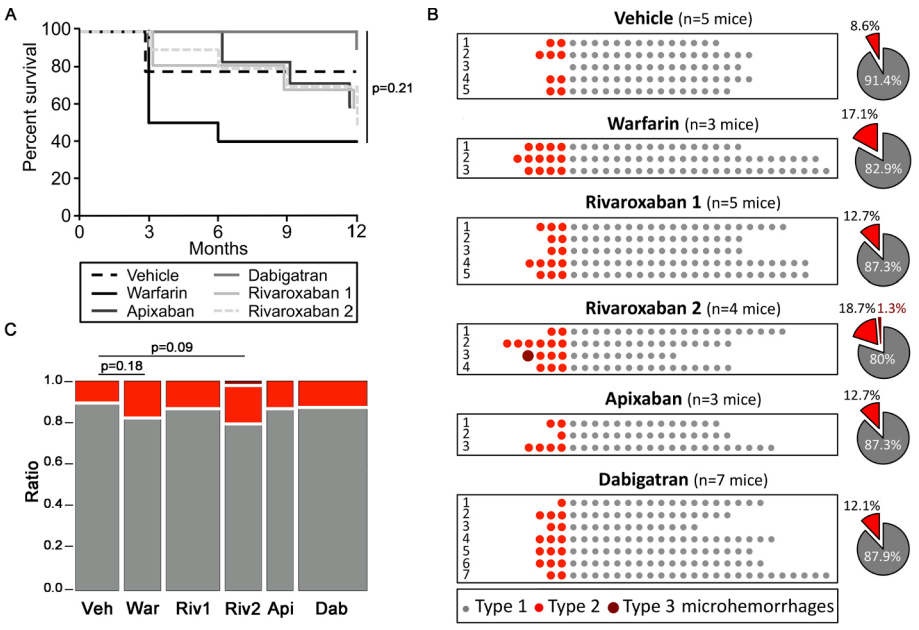


Fig. 4. ***Microbleeding profile of mice in the long term*****. A.** Kaplan-Meyer survival curves (n = 8–12). Mortality did not differ significantly between all groups (Mantel-Cox test, p = 0.21). **B.** Dot plot of cerebral microhemorrhages (CMHs) obtained for each mouse with corresponding proportions (right hand side of the panel). **C.** Mosaic plot of CMHs obtained in each group (n = 3–7). The ratio refers to the percentage of each type of microhemorrhage among all observed CMHs. Repartition of CMHs was not different between all groups according to Pearson's Chi-squared test (p > 0.05).

Fig. 5B). In all experimental conditions, histological observations of brain slices revealed the typical set of CMH morphologies, whatever the brain area examined. The observed CMHs were either positive or negative for hemosiderin staining, at both 7-day (60% positive and 40% negative) and 12-month (38% positive and 62% negative) time points. Lyzed RBCs were found in the brains of mice from each group, at both 7-day (63% of the lesions) and 12-month time points (100% of the lesions), whereas intact RBCs were found in the brains of mice from each group only at the 7-day time point (37% of the lesions) (Fig. 5C). Finally, no infarction was observed during the histological observation of these sections, whatever the group and time point.

3.4. Effect of cerebral microhemorrhages on locomotor and cognitive functions

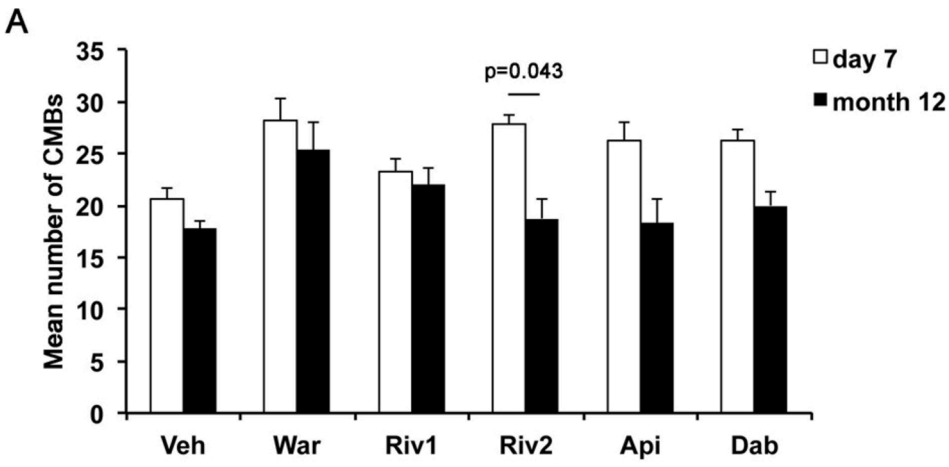
At onset, CMHs did not show any impact on the locomotor function in mice, whatever the group tested, in regard to the distance traveled ($F_{6,41} = 0.44$, $p = 0.84$), resting time ($F_{6,41} = 0.61$, $p = 0.72$) and number of rearings ($F_{6,41} = 0.27$, $p = 0.95$, Fig. 6A). Furthermore, no alteration of the working memory or visual recognition memory was observed ($F_{6,40} = 0.76$, $p = 0.37$; $F_{6,36} = 0.39$, $p = 0.17$ respectively, Fig. 6B). Throughout the year following the onset of CMHs, the locomotor function did not change significantly until 9 months, when the distance traveled by DOAC-treated mice was significantly lower than in the one traveled by mice of the nl group ($F_{6,231} = 33.46$, Api = $p < 0.01$, Dab = $p < 0.001$, Riv1 = $p < 0.05$, Riv2 = $p < 0.001$; Fig. 7A). Moreover, the resting time was increased ($F_{6,231} = 50.58$, Api = $p < 0.001$, Dab = $p < 0.01$, Riv1 = $p < 0.01$, Riv2 = $p < 0.001$; Fig. 7A), and the number of rearings was decreased ($F_{6,231} = 23.99$, Api = $p < 0.05$, Dab = $p < 0.01$, Riv1 = $p < 0.05$, Riv2 = $p < 0.01$; Fig. 7A). Regarding cognitive function, the performance on spontaneous alternation test was not statistically different between all groups ($F_{6,209} = 0.61$, $p = 0.66$; Fig. 7B). On the Novel Object Recognition test, a reduction was found in the performance of all mice compared to nl mice at 9 months ($F_{6,160} = 18.18$, Veh = $p < 0.001$, War = $p < 0.05$, Api = $p < 0.01$, Dab = $p < 0.01$, Riv1 = $p < 0.001$, Riv2 = $p < 0.05$), that remained significant at 12 months for Veh-, War- and Riv1-treated mice (Veh = $p < 0.05$, War = $p < 0.05$, Riv1 = $p < 0.001$). Moreover, no statistical difference was noticed between anticoagulant-treated and vehicle-treated animals ($p > 0.05$). All mice, including warfarin survivor mice, were able to perform the pre-validation tests, except for the Riv2 mice at 12 months.

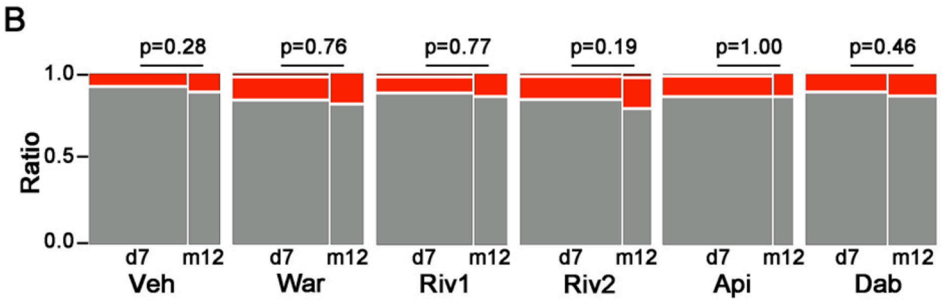
4. Discussion

The main finding of our study is that the onset of a CMH burden under apixaban, rivaroxaban or dabigatran neither transforms into ICH, nor precipitate cognitive impairment.

Whether anticoagulation promotes ICH through an aggravation of occurring CMHs in humans remains unknown. Addressing this issue might be rendered difficult in clinical studies as the integrity of the brain tissue and vasculature may influence the rate and severity of bleedings to a variable extent depending on the disease setting. Our new method induced a disseminated pattern of different sized CMHs, which occurred in both cortical and subcortical areas of the brain. This pattern is verified to a certain extent by post-mortem findings in patients experiencing cerebral amyloid angiopathy but also in healthy elderly human subjects (De Reuck et al., 2011). Since performed in healthy mice, this method enabled us to assess the effect of the sole anticoagulation, without potential input of diseased vessel wall or parenchyma. Expectedly, CMHs remained silent as no functional alteration was observed 7 days after the injection of RAME-CD. Since CMHs appeared from day 3 to day 7, we assume that our mice underwent a diffused erosion of the endothelium wall along all the brain vasculature, causing delayed and non-synchronous bleedings, as suggested by the presence of both intact and lyzed erythrocytes on brain slices. Although potentially affecting large vessels as well as microvessels, the occurrence of microscopic hemorrhages suggests their microvascular origin. The obtainment of such microscopic hemorrhages urged us to determine the burden through a visual assessment, which considers the number of consecutive slices where each CMH is still observable. Although quite challenging if applied to such microscopic lesions, the lack of volumetric measurement through histomorphometric analysis represents a limitation of our method.

The risk of ICH for healthy elderly people under warfarin is regarded as critical, stressing anticoagulation decisions. The replacement of vitamin K antagonists by DOACs as an initial prescription in community medicine is still under study, and no consensus has yet been reached. An increasing number of patients are administered DOACs in virtue of the absence of routine monitoring, but specialists still warn about medical interactions and bleeding risk. In our study, the three tested DOACs did not provoke significant mortality during the short period of CMH onset, and failed to transform the CMHs into a





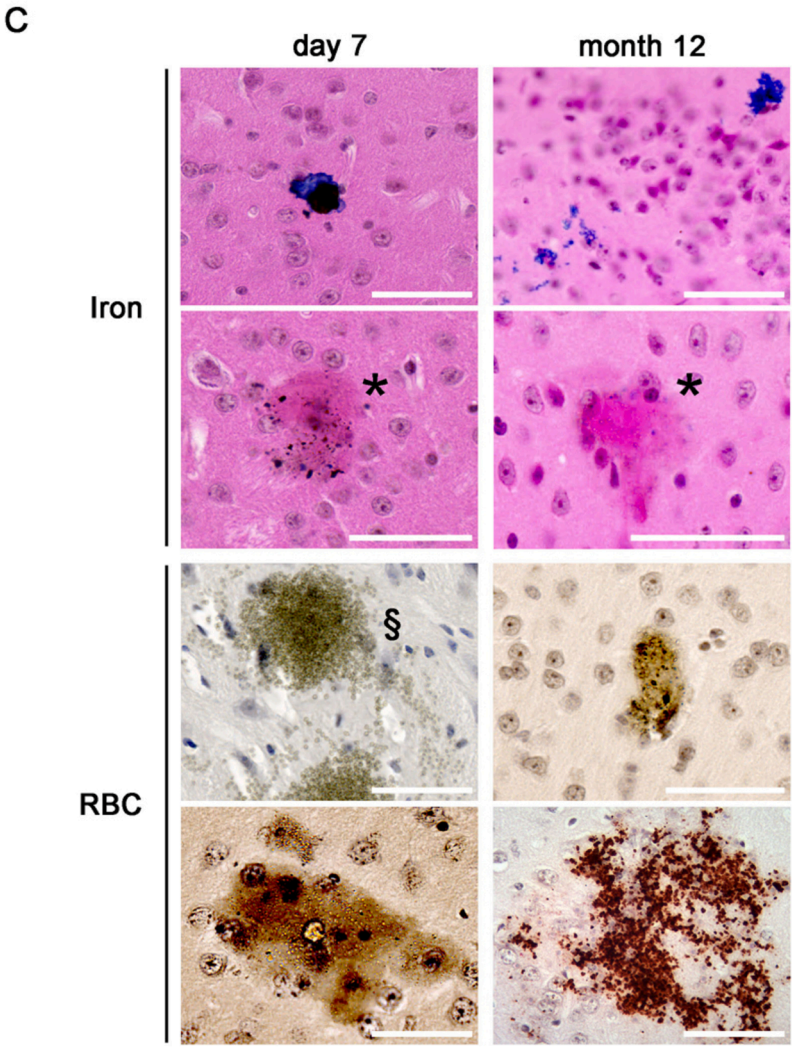


Fig. 5. ***Comparison of short- and long-term mean numbers and repartition of cerebral microhemorrhage (CMH) events, and histological analysis.*** **A.** Mean numbers of CMHs at 7 days and 12 months. Groups treated at CMH onset with rivaroxaban twice a day (Riv2) showed significantly different mean numbers of CMHs at 7 days versus 12 months (Two-Way ANOVA followed by a Tukey's post-hoc test, $p = 0.043$). **B.** Mosaic plot of CMHs obtained in each group and time point (n = 3–15). No significant difference was found in the repartition of CMHs between groups according to Pearson's Chi-squared test ($p > 0.05$). The ratio refers to the percentage of each type of microhemorrhage among all observed CMHs. **C.** Representative brain slices after Perl's Prussian blue staining of iron deposits (Iron, top side of the panel), and diaminobenzimide staining of red blood cells (RBC) peroxydase, (bottom side of the panel). *, absence of Prussian Blue-positive staining. §, intact RBCs. Scale bars = 50 μm.

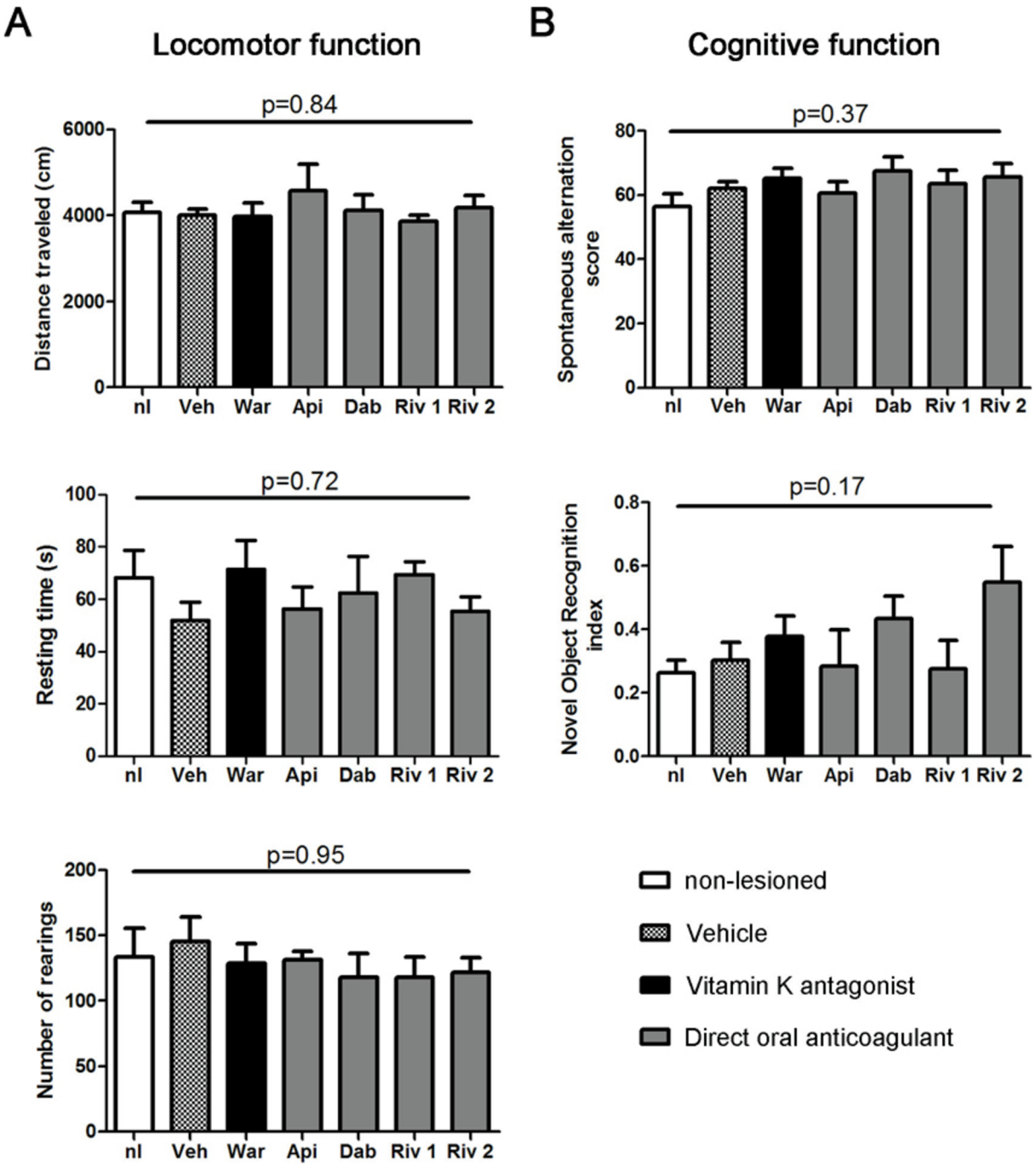


Fig. 6. ***Short-term effects of cerebral microhemorrhages on mouse locomotor and cognitive functions***. **A.** Spontaneous locomotor behavior. No significant difference was obtained between groups: distance traveled (in cm; p = 0.84), resting time (in s; p = 0.72) and number of rearings (p = 0.95). **B.** Working memory (% alternation) and visual recognition memory (novel object recognition (NOR) index). No difference was found in the performance on both tests, between groups according to one-way ANOVA followed by a Tukey's post-hoc test (Spontaneous alternation test: p = 0.37; NOR test: p = 0.17). n = 6–10.

symptomatic hematoma. These results are in line with a recent study, which reported that even a 3-month anticoagulation with dabigatran in the APP23 mouse model of amyloid angiopathy did not transform spontaneous CMHs into a large ICH, contrary to warfarin (Marinescu et al., 2017b). In accordance, warfarin provoked ICH in our mice, which never displayed neither type-3 CMHs nor hematomas when not under anticoagulants. As CMHs are induced in our mice, our results support the hypothesis of a warfarin-mediated aggravation of CMHs into ICH, as well as the suggestion of using DOACs as an alternative to vitamin K antagonists, raised in virtue of an about 50% lower risk of ICH observed in atrial fibrillation cohorts (Wilson et al., 2015).

Although warfarin anticoagulation is known as associated to a greater number of CMHs in patients, whether anticoagulation promotes CMHs in humans is still unresolved. Whereas neither dabigatran nor warfarin is reported to increase the number of spontaneous CMHs in APP23 mice (Marinescu et al., 2017b), we observed a significant aggravation of the CMH burden (with a major contribution of type 2 CMHs) in the brains of our mice that survived under warfarin, and apixaban or rivaroxaban (twice a day). Our results suggest the impact of some but not all anticoagulants at onset, which seems, among DOACs, relevant to anti-X inhibitory mechanism. In this regard, while the bleeding enhancing effects of warfarin may be attributable to its potent

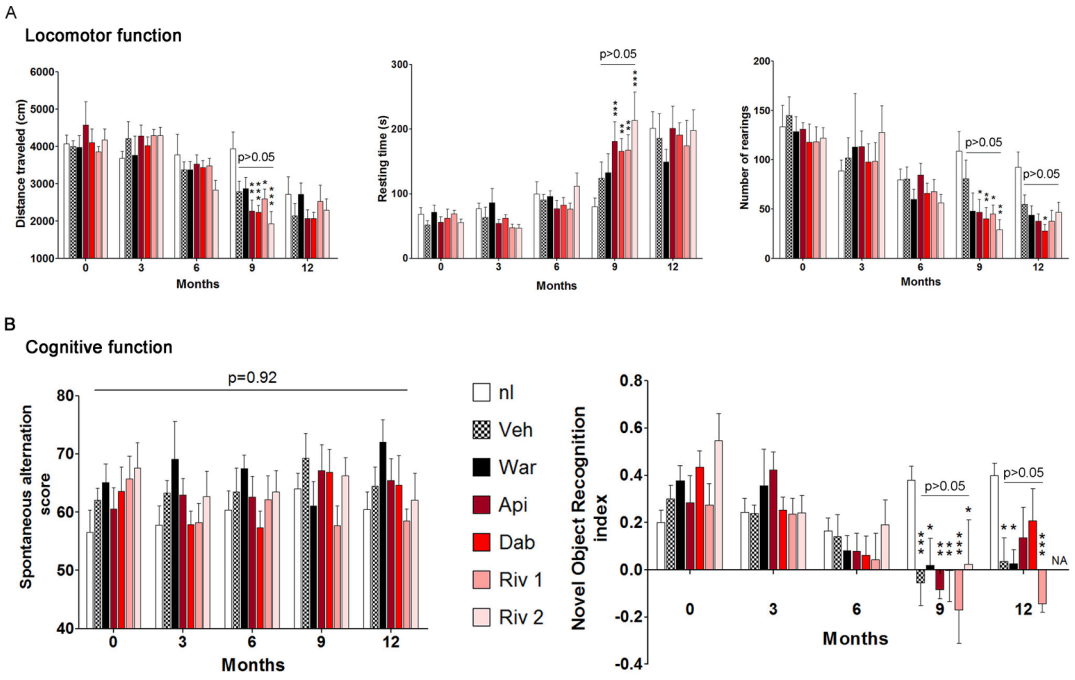


Fig. 7. ***Long-term effects of cerebral microhemorrhages on mouse locomotor and cognitive functions*. A.** Spontaneous locomotor behavior. A significant decrease in the distance traveled was found at 9 months between DOACs-treated mice and nl mice, accompanied by a significant increase in the resting time and a significant decrease in the number of rearings according to two-way ANOVA followed by a Tukey's post-hoc test. **B.** Working memory (% alternation) and visual recognition memory (index) are examined through spontaneous alternation test and novel object recognition (NOR) test respectively. No difference was found in the performance on spontaneous alternance test between all groups according to one-way ANOVA followed by a Tukey's post-hoc test ($p = 0.92$). A significant difference was found in the performance on NOR test compared to nl mice at 9 months and 12 months, according to two-way ANOVA followed by a Tukey's post-hoc test. None of the Riv2 mice was able to perform the pre-validation phase of the NOR test at this time of the protocol. n = 3–12. ***p < 0.001, **p < 0.01, *p < 0.05 *vs* nl.

(targeting four coagulation factors) and long lasting anticoagulatory action, the less pronounced effects of DOACs are thus more difficult to explain. Since coagulation tests are reported as inconsistent in subjects under DOACs, we measured anti-Xa and anti-IIa activities in the blood of mice to validate their anticoagulatory status (Adcock and Gosselin, 2015). In particular, the aggravation of the CMH burden between mice under rivaroxaban and vehicle control mice reached statistical significance only when mice were treated twice a day. This drug is prescribed once a day in community medicine, but twice a day in hospital medicine, as a treatment of specific diseases such as venous thromboembolic disease (Delavenne et al., 2014). This result points to the risk that might comprise an increased dose of DOACs.

The risk for patients with more than 10 CMHs of developing premature cognitive impairment is of growing interest (Paradise et al., 2018). In line with this point, our findings have prompted us to stop anticoagulation in order to investigate the long-term effects of the sole baseline or enhanced CMH burdens.

Despite cessation of anticoagulation, CMHs induced various percentages of premature death between the mice groups, ranging from 11% to 58%, except for the non-lesioned control mice. Although no hematoma was found in the brains collected from mice cadavers, possible peripheral hemorrhage cannot be ruled out as a potential explanation for mortality. Indeed, studies lasting more than 3 months in mice with lesioned brains remain a challenge, since resulting in high spontaneous mortality rate, with limited information on the cause of death (Participants∗, 2018). The age-related reduction of locomotor function observed in the non-lesioned mice, has been precipitated in survivor mice treated with vehicle or anticoagulants during CMHs onset. However, this did not affect the performance of these mice on spontaneous alternation test, suggesting a preserved working memory, as well as the validation phase of the NOR test, since even warfarin survivor mice were able to perform the tests. Surprisingly, both mice with baseline- or anticoagulant-enhanced CMH burdens underwent a progressive decline of their performance on the second phase of NOR test, reaching statistical significance at 9 months after onset, i.e. at middle-age since mice were 12 months old at that time point. This result indicates an impaired visual recognition memory, which is reported as altered early in the course of patients with mild cognitive impairment (Barbeau et al., 2004). Of course, one cannot rule out possible effect of CMHs on other brain functions. These results are all the more important, as neither spontaneous hemorrhagic transformation nor accumulation of CMHs have been observed with time. The latter observation needs to be however considered with caution, since the mice used for the long-term study could not be assessed for their CMH burden at onset. The resolution of some CMHs and the appearance of new ones could thus be missed. It is tempting to speculate that ICH did not occur in our mice because of the cessation of anticoagulation, although the accumulation of CMHs is known to increase the risk of ICH in various populations that are not necessarily anticoagulated (Wilson et al., 2015). In our mice, the structure and thus hemostatic regulatory function of the cerebral vasculature may have not been enough compromised to undergo a spontaneous ICH in the long term, or to result in a significant increase in the number of CMHs as found in population-based studies (2% per annum)(Poels et al., 2011). On the other hand, a significant reduction in the number of CMHs at the 12-month time point could have also been expected, which is not supported by our results, even in the non-anticoagulated mice. This suggests the absence of influence of age in our mice, and a slow lesion resolution process, which could have been missed in our long-term study (since our mice were 15 months old, thus close to middle age) but might be observable at a much later age.

The absence of precipitated cognitive impairment in previously anticoagulated mice that have experienced an enhanced CMH burden is in favor of short-term oral anticoagulation when needed in subjects at risk of presenting CMHs. In line, these results would suggest not prompting MRI screening in this population. This is moreover

supported by the recommendation of proceeding with anticoagulation in these subjects despite detection of CMHs (Smith et al., 2017). However, as CMHs were able to induce a cognitive impairment *per se* in non-anticoagulated mice also, our results would urge the need for CMH detection, in case such a high and disseminated pattern would be underlain, thus enabling the initiation of preventive clinical care. Whereas ischemic stroke and ICH are known to precipitate cognitive decline in humans and in rodents (Brainin et al., 2015; Shih et al., 2018), no preclinical study reports a functional impact for disseminated CMHs in wild-type rodents. A recent work including behavioral assessments has been based on the induction, through collagenase injection, of a single bleed of larger dimension compared to ours, but of reduced size compared to a symptomatic hematoma (Bergeron et al., 2018). The authors observed the first functional disturbances started at 90 days after bleeding, and argued in favor of a volume of the single hemorrhage that corresponded to the entire CMH burden as estimated in humans. In our model, CMHs have arisen in both cortical and subcortical zones. This could explain why, contrary to a model of single CMH, we observed functional disturbances from 9 months after onset. Further work is required to understand the numerous processes that may lead to such impairment, a task rendered difficult in our model since the disseminated nature of the CMH burden might prevent the study of a specified brain area and functional consequence. Liu and colleagues (Liu et al., 2018) have put forward the idea that the presence of multiples microlesions may interfere with neuronal connections in both cortical and subcortical zones that support brain functions. The evaluation of this hypothesis needs to overcome, in the rodent brain, the challenging diffusion tensor magnetic resonance imaging (DTI) technique. As well, neuroinflammation has been proposed as a contributor to cognitive decline, but with no clear causal relationship (Ahn et al., 2018). Our histopathological examinations have demonstrated the asynchronous onset of CMHs through the presence of both lyzed and intact erythrocytes in the same brains after 7 days. However, no intact erythrocytes were observed at 12 months, pointing to the absence of acute CMHs at this time point. This needs further clarification since this histological examination at 12 months was performed in different groups of mice, which burden at onset could not be determined. As well, focal iron staining disclosed hemosiderin deposits suggestive of more matured bleeds with potential harmful effects along the year following the onset of CMHs (Toth et al., 2015; Veluw et al., 2016, 2017). Surprisingly, a lower proportion of iron-positive CMHs compared to iron-negative CMHs was found at 12 months after onset, whereas a majority of iron-positive CMHs was found at onset. This inconsistency would firstly suggest the occurrence of new CMHs between 7 days and 12 months, and observed as sub-acute CMHs at 12 months. This remains however speculative since the CMH burden of our 12-month old mice could not be determined at onset. In addition, histopathological studies are cross-sectional and do not allow assessing the order of the bleeding events, as reported in humans (van Veluw et al., 2016). Another hypothesis, supported by the major proportion of iron-positive CMHs at onset, would involve a long-lasting clearance of hemosiderin. Further work is needed to determine whether this greater proportion of iron-negative CMHs in the long term discloses only sub-acute CMHs or involves a process of clearance of hemosiderin by macrophages (so-called siderophages), a cell type of therapeutic interest in intracranial bleedings (Bulters et al., 2018).

5. Summary and limitations

To conclude, the onset of a CMH burden in a non-diseased brain upon anticoagulation with apixaban, rivaroxaban or dabigatran, does not represent a risk of symptomatic bleeding, neither an increased risk for mortality nor precipitated cognitive impairment. Our study holds still two significant limitations on its lesional part. First, our visual assessment of the CMH burden defines the latter as a number of events of estimated severity, but does not account for an accurate measurement of blood load in the brain, which could be given by volumetric data after histomorphometric analysis, or MRI for macro-bleedings. Second, since performed from the brains of sacrificed animals, our method ruled out the determination of the early-onset CMH burden of the mice assessed after one year. Therefore, CMH burdens were assessed in two different cohorts of mice, one at onset, and the other one a year later, and the comparison between both precluded us from conclusive results about the accumulation or resolution of CMH lesions in the long term. Nevertheless, our results regarding the input of the four oral anticoagulants tested here suggest that future clinical studies should include and compare CMH patients treated with various oral anticoagulants in search of differential effects potentially supporting the replacement of warfarin by DOACs as an initial prescription. In addition, our observations argue that the presence of a high number and disseminated pattern of CMHs represents a risk for premature cognitive impairment that suggests considering CMHs for early detection and preventive memory care apart from anticoagulation decisions.

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