



HAL
open science

Age and diet modify acute microhemorrhage outcome in the mouse brain

Maud Pétrault, Thavarak Ouk, Michèle Bastide, Régis Bordet, Olivier Pétrault, Vincent Bérézowski

► To cite this version:

Maud Pétrault, Thavarak Ouk, Michèle Bastide, Régis Bordet, Olivier Pétrault, et al.. Age and diet modify acute microhemorrhage outcome in the mouse brain. *Neurobiology of Aging*, 2021, 98, pp.99 - 107. 10.1016/j.neurobiolaging.2020.10.005 . hal-03493687

HAL Id: hal-03493687

<https://hal.science/hal-03493687>

Submitted on 15 Dec 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Age and diet modify acute microhemorrhage outcome in the mouse brain

Maud Pétrault⁽¹⁾, Thavarak Ouk⁽¹⁾, Michèle Bastide⁽¹⁾, Régis Bordet⁽¹⁾, Olivier Pétrault^(1,2) and
Vincent Bérézowski^(1,2)

Family names are underlined

(1) Univ. Lille, Inserm, CHU-Lille
Lille Neuroscience & Cognition research center
F-59000 Lille, France

(2) UArtois, F-62300, Lens, France

Corresponding author:

Maud Pétrault, PhD

Univ. Lille, Inserm, CHU-Lille,
Lille Neuroscience & Cognition research center,
F-59000 Lille, France
Phone: +33 3 20 44 54 49
Fax: +33 3 20 44 68 63
maud.petrault@univ-lille.fr

1 **Abstract**

2 Cerebral microhemorrhages (CMHs) are considered as asymptomatic lesions, but might
3 impair cognition in non-demented elderly individuals. The aging process includes poor
4 vascular health, enhanced at midlife by metabolic disturbances upon high-fat diet (HFD). The
5 onset of CMHs could thus have more serious consequences in midlife subjects with metabolic
6 disturbances. This hypothesis was tested through the induction of multiple CMHs, using
7 cyclodextrin nanoparticles injection, in mice at midlife (14 months old) or at a younger stage
8 (5 months old) after 12 months or 3 months of normal diet or HFD (40% of animal fat)
9 respectively. When induced at 14 months of age, CMHs were not larger but were more
10 numerous (+25%) in mice on HFD compared with mice on normal diet. They slowed down
11 the locomotor activity significantly but caused neither a change in the working memory nor a
12 difference in the visual recognition memory decline. When induced at 5 months of age,
13 CMHs provoked slighter locomotor and cognitive symptoms, regardless the diet. No
14 spontaneous progression of CMHs towards larger hemorrhages was observed after onset when
15 HFD was prolonged up to midlife. Consistently, no precipitated cognitive decline was
16 observed. Middle-age plus time of metabolic disturbances represent enhanced risk factors for
17 CMH outcome.

18 **Keywords**

19 Cerebral microhemorrhages, cognitive decline, age, high-fat diet, mouse.

20

21

22

23

24

25

26

27

28 **1. Introduction**

29 Cognitive decline is defined as an age-related decrease in cognition. It usually becomes
30 manifest through slight alterations of mnemonic functions such as executive functions (Tomita et
31 al., 2019). These functions deteriorate physiologically along the aging process. Still, age
32 refers to a length of time during which humans under enriched diet can accumulate multiple
33 metabolic risk factors for cerebrovascular disease, possibly leading to precipitated cognitive
34 impairment. Indeed, the presence of metabolic disturbances at midlife has been linked
35 recently to mild cognitive impairment, increasing the risk of vascular pathologies and late-life
36 dementia (Cuspidi et al., 2018; González et al., 2018; Otite et al., 2017; O. Pétrault et al.,
37 2019; Whitmer et al., 2005). Among the consequences of vascular pathologies, post-stroke
38 cognitive impairment is now well documented, but the proper effect of microlesions such as
39 cerebral microhemorrhages (CMHs) is still a subject of debate, since confounded with the
40 expression of concomitant brain disease in the elderly population. The presence of CMHs has
41 yet been associated with age-related cognitive decline in the general population (Ding et al.,
42 2017) and is suggested to contribute to cognitive impairment in non-demented elderly
43 individuals (Paradise et al., 2018). However, the experimental induction of CMHs has been
44 performed in young healthy mice only and was found silent at onset but able to promote slight
45 cognitive alterations only a midlife time after (M. Pétrault et al., 2019). Given the vascular
46 nature of CMHs, their higher incidence or extent relates to poor vascular health as a possible
47 result of both aging and metabolic disturbances. Middle-aged adults with metabolic
48 disturbances might thus be subjected to potentiated effects of CMHs on brain functions at
49 onset, or in the long term through a precipitated cognitive decline with or apart from
50 transformation into symptomatic intracerebral hemorrhage. To test this hypothesis, we have
51 induced a disseminated pattern of CMHs in adult mice of two different ages and times of
52 metabolic disturbances obtained after high-fat diet (HFD, 40% fat) as previously
53 characterized (O. Pétrault et al., 2019). We examined spontaneous locomotion, working
54 memory and visual recognition memory, as well as the extent of the CMH lesions.

55 **2. Experimental procedures**

56 **2.1. Ethical aspects**

57 All experiments were approved by the national Ethical Committee in Animal Experimentation
58 (CEEA n°75, Comité d’Ethique en Experimentation Animale), from the French Ministry for
59 Education and Research (agreement number: n°2016022510527156-V2) and were performed

60 in strict compliance with the European Union Directive 2010/63/EU. Experiments were
61 reported in accordance with the ARRIVE guidelines for reporting experiments involving
62 animals.

63 **2.2. Animals and diet**

64 One hundred and twenty-four adult male C57Bl6/J mice aged 8 weeks at the beginning of the
65 study (Janvier Labs, Le Genest Saint Isle, France) were housed in standard cages. At the start
66 of the study, mice were randomly assigned to either a nutritionally complete, HFD consisting
67 of 40% saturated fat, mostly from lard (HF231, Safe, Augy, France) or normal control diet
68 (ND) consisting of 3 % fat (A04, Safe, Augy, France) throughout the experimental period. All
69 animals had *ad libitum* access to food and water. There were no significant differences in
70 body weight between groups before the experimental diets were given, as mice had been
71 maintained on a regular chow diet.

72 **2.3. Induction of microhemorrhages and time points**

73 Cerebral microhemorrhages were induced by injecting Randomly methylated- β -Cyclodextrins
74 (RAME-CD Sigma-Aldrich, Saint-Quentin Fallavier, France), as previously described (M.
75 Pétrault et al., 2019). Mice were anesthetized with isoflurane (induction: 3%; maintenance:
76 2%) and body temperature was kept constant around 37°C. The carotid arteries were exposed
77 through a midline cervical incision and the common carotid was ligated with a silk suture.
78 The RAME-CD solution (200 mg/ml) was injected in the common carotid via a 29G insulin
79 syringe (0.3g/kg). Then, the suture was carefully removed and the mouse was placed under a
80 red warm light to recover. A maximum pattern of around 20 CMHs per brain was observed at
81 7 days after injection, at both cortical and subcortical levels, and used for the present study.

82 Three protocols were carried out in parallel:

83

84 1. The acute effect of CMHs on locomotion and cognition of mice under metabolic disorders
85 was tested. A short-term protocol was designed for the induction of the CMH lesions in the
86 brains of 14-month-old mice (after 12 months of diet) or 5-month-old mice (after 3 months of
87 diet), when metabolic, vascular, and cognitive disturbances were, respectively, well
88 established or just beginning; this was described previously (O. Pétrault et al., 2019) (Fig. 1A
89 and 2A). Mice were randomly divided into 2 groups (n=12-15 per group of 14 month-old
90 mice, and n=7-8 per group of 5 month-old mice), both injected with RAME-CD and fed with
91 ND or HFD. After 7 days, before sacrifice, mice were kept for behavioral studies in order to

92 check for the functional impact of CMHs. Two groups of mice (n=10 per group) were inserted
93 as controls, *i.e.* devoid of RAME-CD injection but fed with ND or HFD.
94

95 2. The long-term effect of CMHs on locomotion and cognition of 5-month-old mice
96 experiencing CMHs when under early metabolic disorders (after 3 months of diet) was tested.
97 A long-term protocol ensured the follow-up study of the effects of CMHs in the brains of
98 mice on ND or HFD (Fig. 4A). Mice were randomly divided into 2 groups (n=10-12 per
99 group) fed with ND or HFD. The CMH lesions were induced after 3 months of diet and
100 behavioral tests were performed at 6-9- and 12-month time points. At the end, mice were
101 sacrificed for histological purposes. Two groups of mice (n=10 per group) were inserted as
102 controls, *i.e.* devoid of RAME-CD injection but fed with ND or HFD.
103

104 3. A metabolic characterization (weight, blood triglycerides and total cholesterol) of each
105 mouse was performed at 3, 6, 9 and 12 months of diet, and visceral adipose tissue was
106 weighted after sacrifice, at 3 and 12 months, as described in (O. Pétrault et al., 2019).

107 **2.4. Behavioral assessments**

108 Mice of each group underwent a battery of tests evaluating spontaneous locomotor activity,
109 visual recognition memory and working memory. These tests were chosen according to the
110 brain areas affected by CMHs, which are those of the Sylvian territory. They were performed
111 every morning between 9 a.m. and 2 p.m. During experiments, the equipment was cleaned
112 with 70% ethanol after each trial so that olfactory cues did not bias the results.

113 - Spontaneous locomotion

114 Spontaneous locomotor activity was assessed in an open field test device, using an infrared
115 actimeter (Bioseb, USA). The apparatus consisted of a square arena (45 x 45 cm) with a black
116 polymethyl methacrylate floor and transparent 34-cm-high polymethyl methacrylate walls.
117 Mice were placed in the center of the arena, and were allowed to explore the arena freely for
118 10 min. Activity was recorded by two rows of infrared photocell sensors and processed with
119 the Actitrack software (Bioseb). The total distance covered (in cm), the duration of inactivity
120 (resting time, in seconds) and the mean speed (cm/sec) of each mouse were measured.

121 - Visual recognition memory

122 The novel object recognition (NOR) test is based on the tendency of mice to explore a novel
123 object rather than a familiar one. Mice were habituated to a square arena (50 x 50 x 25 cm) for
124 10 minutes on the first day. On day 2, mice were exposed to the arena containing two
125 identical objects. In a first phase (“sample phase”), on day 3, mice were presented two novel
126 and identical objects for 15 minutes. Animals returned in their cages. After one hour, they
127 were placed back in the testing arena for 5 minutes (“test phase”), but one of the familiar
128 object had been previously replaced by a novel object. Performance of the mice was video
129 recorded (Ethovision XT, Noldus, Wageningen, The Netherlands). Exploratory behavior was
130 defined as the animal directing its nose toward the object at a distance below 2 cm. Any
131 subject that failed to complete a minimum of 20 seconds of exploration during the sample
132 phase was excluded from the analysis. A discrimination ratio (index) was calculated as the
133 difference between the time spent for the exploration of the novel object minus the time spent
134 for the exploration of the familiar object divided by the sum of both (Antunes and Biala,
135 2012).

136 - Working memory

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

148 **2.5. Determination of the CMH lesions**

149 At the end of the protocols, mice were killed with an overdose of pentobarbital (200 mg/kg,
150 intraperitoneal). Brains were rapidly removed and placed in ice-cold isopentane solution,
151 frozen and coronally dissected into 20 µm-thick slices on cryostat. Then, CMHs were counted
152 blinded to treatment on each brain slice and characterized according to size as described
153 previously (M. Pétrault et al., 2019). Briefly, CMHs that were evident to the eye aided by a 2x

154 magnifying glass, and found in only 1 slice, were defined as type 1 CMHs. When evident to
155 the naked eye and found again in the next slice, CMHs were defined as type 2. When larger
156 and found again in more than 2 slices, CMHs were defined as type 3. A total hemorrhagic
157 score was calculated as the sum of all CMHs found throughout the brain and considered as the
158 CMH lesions extent. Mice with less than 10 CMHs were excluded from the study.

159 **2.6. Statistical analysis**

160 All values were expressed as mean \pm standard error of the mean (SEM). Individual data points
161 are superimposed on boxplots. For behavioral assessment, continuous variables were
162 compared with a two-way ANOVA followed by a post-hoc Tukey's Multiple Comparison
163 Test if variance analysis was significant. For the CMH score, a Pearson's Chi-squared test
164 was realized. In each case, a value of $p < 0.05$ indicated statistical significance.

165 **3. Results**

166 **3.1. Acute functional effect of CMHs in the brain of 14-month-old mice after a 12-** 167 **month diet**

168 At 12 months of diet, mice on HFD endured metabolic disturbances with a significant
169 increase in blood total cholesterol and visceral adipose tissue compared to ND-fed mice
170 (Table A). Significant differences were found in the measured locomotor parameters only
171 between HFD-fed mice with CMHs, compared with mice without CMHs fed with HFD or
172 ND, including a decrease in the travelled distance (-39% vs ND mice, $p=0.002$; -31% vs HFD
173 mice, $p=0.02$), an increased resting time (+92% vs ND mice, $p=0.002$; +68% vs HFD mice,
174 $p=0.005$), and a decrease in the mean walking speed (-38% vs ND mice, $p=0.004$; +31% vs
175 HFD mice, $p=0.02$, Fig. 1B). Concerning cognitive functions, the performances on
176 spontaneous alternation test were not statistically different between all groups ($p < 0.05$, Fig.
177 1C-left panel). On the NOR test, the onset of CMH in mice fed with the same diet did not
178 change the performances significantly. A significant reduction was however found in HFD-
179 fed mice with CMHs compared with mice without CMHs and fed with ND (HFD + CMH -
180 89% vs ND mice, $p=0.014$). A comparable drop in the performances was observed in HFD-
181 fed mice without CMHs compared with ND-fed mice with or without CMHs (HFD -106% vs
182 ND mice, $p=0.0015$; HFD -107% vs ND + CMH mice, $p=0.02$; Fig. 1C-right panel).

183 **3.2. Acute functional effect of CMHs in the brain of 5 month-old-mice after a 3-** 184 **month diet**

185 At 3 months of diet, mice on HFD were metabolically disturbed, with a significant increase in
186 weight, blood total cholesterol and visceral adipose tissue compared to ND-fed mice (Table
187 A). Regarding spontaneous locomotor parameters, the travelled distance and the mean
188 walking speed increased slightly but without reaching statistical significance in mice with
189 CMHs compared with mice without CMHs (+17% and +18% respectively, $p=0.06$ for both,
190 Fig. 2B). The resting time was yet significantly reduced by CMH onset in both ND- (-53% *vs*
191 HFD mice, $p=0.03$) and HFD-fed mice (-50% *vs* HFD, $p=0.03$). Regarding cognitive
192 functions, the performances on the spontaneous alternance test did not change among groups
193 ($p<0.05$, Fig. 2C-left panel). However, a significant reduction in the performances on the
194 NOR test was observed in mice with CMHs and fed with ND (-44% *vs* ND mice, $p=0.006$) or
195 HFD (-40% *vs* ND mice, $p=0.015$) compared with mice without CMHs and fed with ND (Fig.
196 2C-right panel).

197 **3.3. Acute CMH lesions in the brain of 14 month-old-mice after a 12-month diet**

198 After 12 months of diet, visual CMHs were found in all areas of the Sylvian territory of the
199 brains of all mice injected with RAME-CD (Fig. 3A). In ND-fed mice, a mean number of 20
200 CMHs (19.57 ± 0.91) per brain was observed at 7 days after injection. Microhemorrhages
201 were mostly of small size (type 1). Medium-sized CMHs (type 2) were counted to a
202 maximum of 4. In HFD-fed mice, a mean number of 25 (25.08 ± 1.39) CMHs per brain was
203 counted, mostly of small size (type 1). Medium sized CMHs (type 2) reached a maximum of 5
204 in two brains, and two larger CMHs (type 3) were observed in one of these brains. The
205 number of CMHs induced in HFD-fed mice was significantly higher than in ND-fed mice
206 ($p=0.01$), without a significant modification of the typology of CMHs ($p=0.39$, Fig. 3B).

207 **3.4. Acute CMH lesions in the brain of 5 month-old-mice after a 3-month diet**

208 After 3 months of diet, visual CMHs were found in all areas of the Sylvian territory of the
209 brains of all mice injected with RAME-CD (Fig. 3C). One mouse was excluded of the study
210 with only 9 CMH. In ND-fed and HFD-fed mice, mean numbers of 17 (17.40 ± 0.92) and 19
211 (19.00 ± 0.21) CMHs per brain were observed at 7 days after injection. Microhemorrhages
212 were mostly of small size (type 1). The majority of ND-fed mice had between 1 and 2
213 medium-sized CMHs (type 2), and the majority of HFD-fed mice had between 2 and 3 CMHs
214 of this size. The type 3-CMH was not observed. Neither the number ($p=0.64$) nor typology of
215 CMHs ($p=0.82$) was significantly different between ND- and HFD-fed mice (Fig. 3D).

216 The comparison between the observed CMH lesions at 5 or 14 months of age (3 and 12
217 months of diet respectively) did not show any difference in ND-fed mice ($p=0.92$), whereas
218 there was a significant increase in the number of CMHs in HFD-fed mice after 12 months of
219 diet compared with HFD-fed mice after 3 months of diet ($p=0.02$, Fig. 3E-left panel).
220 However, no significant difference was observed in the typology of CMHs, whatever the
221 group tested ($p=0.90$ and 0.53 respectively, Fig. 3E-right panel).

222 **3.5. Long-term functional effect of CMHs**

223 Throughout the year following the onset of CMHs, the spontaneous locomotor parameters of
224 mice (Fig. 4B) did not change significantly in regard to the travelled distance, the resting time
225 or the mean walking speed. Regarding cognitive functions, the performances on the
226 spontaneous alternance test did not change with time for all groups of mice (Fig. 4C, left
227 panel). The performances on the NOR test of ND-fed mice without CMHs was preserved
228 during the whole protocol, with a stable discrimination NOR index (Fig. 4C, right panel). The
229 induction of CMHs in ND-fed mice reduced these performances from 11 months of age until
230 significance at 14 months of age (-69% vs ND mice, $p=0.05$). The NOR index of HFD-fed
231 mice decreased progressively until 14 months of age (12 months of diet) and became
232 significantly different from ND-fed mice from 11 months of age (9 months of diet, -80% vs
233 ND mice, $p=0.03$). The presence of CMHs in HFD-mice did not precipitate the onset of the
234 decrease of NOR index compared with HFD-mice without CMH. The deficit of visual
235 recognition memory is similar in both HFD groups with a score index significantly reduced in
236 comparison to ND-fed mice without CMHs (-113% vs ND mice, $p=0.001$).

237 **3.6. Progression of the CMH lesions**

238 The mortality observed during the 12-month period did not differ significantly among groups
239 with CMHs, and no hematoma was found in the brain of prematurely died animals, even if the
240 microhemorrhagic score obtained in some of dead mice was high (Fig. S1). The CMHs were
241 induced 3 months after the start of diet, when mice were 5 months old. The CMH count and
242 profile of the survivors was determined 9 months after RAME-CD injection, when mice were
243 eventually 14 months old. (Fig. 5A). In ND-fed and HFD-fed mice, mean numbers of 20
244 (19.57 ± 0.89) and 22 (22.00 ± 1.67) CMHs per brain were observed. Microhemorrhages
245 were mostly of small size (type 1). One to three medium-sized CMHs (type 2) were found in
246 the brains of ND-fed mice, and two to five in the brains of HFD-fed mice. One type 3 CMH
247 was observed in each group. The number of visual CMH events increased slightly upon HFD,

248 but was not significant ($p=0.17$). Likewise, HFD did not modify the typology of CMHs
249 despite a slight increase in the proportion of type 2 CMHs ($p=0.68$, Fig. 5B). The comparison
250 of the numbers of CMHs (Fig. 5C-left panel) and their typology (Fig. 5C-right panel) between
251 onset (i.e. 7 days and 9 months after RAME-CD injection) did not show any significant
252 difference ($p>0.05$).

253 **4. Discussion**

254 The main finding of our study discloses that induced CMHs lesions have differential impact
255 on locomotor and cognitive functions with age and diet, and do not precipitate cognitive
256 decline at midlife.

257 We first aimed at determining the acute effects of CMHs on midlife (14-month-year old) mice
258 after a 12-month ND or HFD, because the prevalence of the associated metabolic disturbances
259 remains high in middle-aged humans, around 35% of the general population (Aguilar et al.,
260 2015; Bhosale et al., 2019). In our model, a 40% HFD induced a progressive increase in
261 weight, blood lipid profile and visceral adipose tissue (Table A). *Despite being incompletely*
262 *explored, these disturbances can be considered metabolic in nature*, becoming significant from
263 3 months of diet as already reported (O. Pétrault et al., 2019). We define the severity of the
264 induced microhemorrhagic lesions both by the total number of CMHs, reflecting the number
265 of microvascular events, and by the profile (typology) reflecting the extend of blood effusion
266 (M. Pétrault et al., 2019). The increase observed in the number of induced CMHs in midlife
267 mice on HFD compared with mice of the same age on ND suggests the establishment of an
268 enhanced microvascular sensitivity to a mechanical erosion process with HFD. Indeed in our
269 method, RAME-CD induce bleeding through the erosion of the endothelial luminal membrane
270 (M. Pétrault et al., 2019). A greater endothelial sensitivity to blood shear stress can be
271 hypothesized since reported upon various cardiovascular risk factors, although this has to be
272 clarified at the brain microvessels (Borshchev et al., 2019; Granger et al., 2010; Lubrano and
273 Balzan, 2016). This microvascular fragility does not seem to be of critical extent as in
274 structurally diseased vessels, because neither spontaneous lesion nor remodeled vascular wall
275 has been observed in our mice, supporting by our histology investigations (Fig. S2). We rather
276 consider a disturbance of the vascular endothelial cell metabolism and function, as found in
277 the literature and in our previous study (Li et al., 2013; Medeiros et al., 2017; O. Pétrault et
278 al., 2019). This change in the lesions when mice are on HFD was accompanied by a
279 potentiated impairment of the spontaneous locomotion, found not significant in mice on ND.
280 This impairment was reflected by a decreased travelled distance and mean walking speed,

281 associated with an increased resting time. Therefore, in our conditions, middle age alone was
282 not enough to render CMHs symptomatic. Only one year of metabolic disturbances seem to
283 facilitate the emergence of acute locomotor symptoms when CMHs occur. Of course, whether
284 such symptoms would be of enough magnitude to lead to a neurologic complaint in humans is
285 far from evidenced. Conversely, the induction of CMHs neither altered the working memory
286 nor the visual recognition memory in both diet conditions. The impairment of the visual
287 recognition memory already induced by one year of HFD was not aggravated *either*. Whether
288 CMHs induced at midlife could have consequences on mice along the second half of their life
289 span needs further and quite delicate investigations. Other cognitive disturbances could be
290 detected using more sensitive behavioral tests, as Barnes maze. In spite of the reported visual
291 recognition memory impairment in patients in the early course of mild cognitive impairment
292 (Barbeau et al., 2004), an extrapolation to humans needs caution and further studies.

293 The onset of CMHs in young adult (5-month-old) mice after 3 months of HFD, induced
294 similar microhemorrhagic lesions to that of the ND mice, but remained less numerous than the
295 ones observed in HFD mice experiencing acute CMHs at midlife. This confirms that a long
296 time of metabolic disturbances may represent a higher risk of extended CMH number. The
297 CMHs seem to have a slight stimulant effect, in both groups, reflected by a significant
298 decrease in the resting time. This result is consistent with another study carried out in female
299 mice under HFD in which a temporary increase in the locomotor activity was observed and
300 interpreted as an increased anxiety (Krishna et al., 2016). This trouble caused by CMHs was
301 concomitant with an altered visual recognition memory. At this diet duration, we had
302 previously demonstrated that HFD reflects the start of metabolic disturbances and does not
303 alter the brain functions tested here (O. Pétrault et al., 2019). This enabled us in the present
304 study to observe the proper detrimental action of CMHs on cognition. Surprisingly, the acute
305 effect of CMHs on cognition was independent on the diet and even statistically significant
306 only in the ND condition. Since our previous work on younger mice (2 months) revealed
307 completely silent CMHs at onset (M. Pétrault et al., 2019), the age of the present mice (5-
308 months-old) might have been a more potent risk factor of CMHs symptomatology in the acute
309 phase than the start of the metabolic disturbances. This is also surprising since CMHs exerted
310 no effect in our 14-month-old mice on ND. An earlier life period can be more vulnerable to
311 deleterious stimuli affecting mnemonic functions, as reported for HFD itself in some
312 experimental situations (Boitard et al., 2012). These acute symptoms might also be explained

313 by our method which induces a **consistent** average pattern of 20 CMHs in seven days. We
314 thus investigated whether these acute effects are transient or evolving in time.

315 Recently, CMHs have shown long-term effects on cognition (Bergeron et al., 2018; M.
316 Pétrault et al., 2019). In our study, 5-month-old mice that had been fed for 3 months with ND
317 or HFD, were induced CMHs and kept for a prolonged diet during 9 months after CMHs
318 onset, so up to midlife (14 months of age). A prolonged HFD did not significantly influence
319 the development of new CMHs possibly resulting from a rebleeding of previous CMHs or
320 new onset CMHs, although the apparition of a type 3 CMHs was observed in each group at 14
321 months of age compared with the acute CMHs profiles at 5 months of age. However, the
322 comparisons between CMH scores must be considered with caution since from different mice
323 groups. Such a stability observed in the bleedings overtime differs from the large intracerebral
324 hemorrhage situation, where the presence of metabolic disturbances increases the risk of onset
325 and severity (Guo et al., 2016; Park et al., 2013), in link with elevated blood pressure, also
326 reported in a microbleeding context recently (Mitaki et al., 2018; Toth et al., 2015). Although
327 blood pressure was not measured here, we may speculate that our HFD conditions
328 authenticating imbalanced lipid metabolism (Table A) did not have a sufficient effect on
329 microvessel wall remodeling or on local hemostasis to aggravate the CMHs in number or
330 extent. Hence, metabolic disturbances are rather considered as prothrombotic, in particular
331 since hyperplasia and hypertrophy of adipocytes may lead to the overexpression of
332 adipocytokines and plasmatic PAI-1 (McCracken et al., 2018), thus limiting the activity of
333 tissue-type plasminogen activator (tPA) and the extension of the bleeding. The alteration of
334 the spontaneous locomotion induced by CMHs at 5 months of age was not found further,
335 confirming its transient nature. The working memory was not impaired. The visual
336 recognition memory was significantly impacted by the presence of CMHs or HFD only from
337 11 months of age (9 months of diet) as previously described (M. Pétrault et al., 2019; O.
338 Pétrault et al., 2019). Mice combining HFD and CMHs were functionally impaired with the
339 same kinetic, suggesting no synergistic effect, therefore the same underlying
340 pathophysiological mechanism. Otherwise, one might have expected a deficit in the visual
341 recognition memory **at earlier time points, i.e. 8 or 11 months of age**. As our mice did not
342 show a progression of CMH lesions towards larger hemorrhages, the onset of such a 20-CMH
343 burden in average does not seem to behave like a stroke which is a major cause of dementia.
344 Nevertheless, whether the precipitation of cognitive decline relies on the increase in the
345 microlesions number or extent is still under question. Yet in humans, only the accumulation

346 of CMHs above 2% per year (Poels et al., 2011) evokes a potential progression towards
347 cognitive decline, underlying a cerebrovascular or neurodegenerative disease. The potential
348 mechanisms leading to functional impairment may include an alteration of the neurovascular
349 coupling, targeting either the vascular endothelial cells or the smooth muscle cells, leading to
350 an interruption in the retrograde propagation of neurovascular coupling (Iadecola, 2017). As
351 our method and conditions target the endothelium, CMHs or metabolic disorders could
352 therefore interfere with this spread and thus limit vascular responses which could impact the
353 cognitive performance in the long term (Iadecola, 2017; Mogi and Horiuchi, 2011).
354 Neuroinflammation can also be implied in both stresses, as CMHs (Ahn et al., 2018) and
355 metabolic disorders are known to induce inflammation *via* the activation of astrocytes and
356 microglia and the release of cytokines (van Dijk et al., 2015; Van Dyken and Lacoste, 2018).
357 More work including a diet or pharmacological modulation might help determine the
358 mechanisms involved. But before that, technically challenging investigations on the very
359 long-term consequences of early or midlife CMHs onset are needed to define their role on
360 late-life dementia in link with metabolic disturbances. Indeed, very long-term experiments
361 including mice with lesioned brains often result in a high spontaneous mortality rate (Selim et
362 al., 2018).

363 Taken together, our results suggest that contrary to hemorrhagic stroke, the onset of CMHs in
364 the young adult stage can be slightly and transiently symptomatic but neither evolves towards
365 larger lesions nor a precipitated impairment of the visual recognition memory after a
366 prolonged HFD up to midlife. However, the onset of CMHs at midlife induces significant
367 locomotor symptoms only under HFD. Research on the consequences of CMHs should
368 consider that even within the midlife range, age plus duration of diet-induced metabolic
369 disturbances enhance the outcome of CMHs.

370 **5. Acknowledgements**

371 We warmly thank Prof Anne Muhr-Tailleux for her great technical assistance in blood assays
372 (U1011-European Genomic Institute, Lille) and Dr Charlotte Laloux (41-UMS 2014
373 functional testing core facility) for her great technical advice and access to the Lille Animal
374 Facility.

375 This work was supported by the French Ministry for Research and Higher Education and the
376 VasCog Research Network, Lille, France.

377 **6. Disclosures:**

378 None.

379 7. References

- 380 Aguilar, M., Bhuket, T., Torres, S., Liu, B., Wong, R.J., 2015. Prevalence of the Metabolic
381 Syndrome in the United States, 2003-2012. *JAMA* 313, 1973–1974.
382 <https://doi.org/10.1001/jama.2015.4260>
- 383 Ahn, S.J., Anrather, J., Nishimura, N., Schaffer, C.B., 2018. Diverse Inflammatory Response
384 After Cerebral Microbleeds Includes Coordinated Microglial Migration and
385 Proliferation. *Stroke* STROKEAHA.117.020461.
386 <https://doi.org/10.1161/STROKEAHA.117.020461>
- 387 Antunes, M., Biala, G., 2012. The novel object recognition memory: neurobiology, test
388 procedure, and its modifications. *Cogn Process* 13, 93–110.
389 <https://doi.org/10.1007/s10339-011-0430-z>
- 390 Barbeau, E., Didic, M., Tramon, E., Felician, O., Joubert, S., Sontheimer, A., Ceccaldi, M.,
391 Poncet, M., 2004. Evaluation of visual recognition memory in MCI patients.
392 *Neurology* 62, 1317–1322.
- 393 Bergeron, S., Chen, Y., Auger, F., Deguil, J., Durieux, N., Skrobala, E., Barus, R., Potey, C.,
394 Cordonnier, C., Pasquier, F., Ravasi, L., Bordet, R., Gautier, S., 2018. Role of cortical
395 microbleeds in cognitive impairment: In vivo behavioral and imaging characterization
396 of a novel murine model. *J Cereb Blood Flow Metab* 0271678X17752765.
397 <https://doi.org/10.1177/0271678X17752765>
- 398 Bhosale, V.V., Singh, S., Srivastava, M., Pathak, P., Prakash, S., Sonkar, S., Misra, A.K.,
399 Misra, R., Ghatak, A., 2019. A case control study of clinical and biochemical
400 parameters of metabolic syndrome with special attention among young and middle
401 aged population. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews* 13,
402 2653–2659. <https://doi.org/10.1016/j.dsx.2019.07.031>
- 403 Boitard, C., Etchamendy, N., Sauvants, J., Aubert, A., Tronel, S., Marighetto, A., Layé, S.,
404 Ferreira, G., 2012. Juvenile, but not adult exposure to high-fat diet impairs relational
405 memory and hippocampal neurogenesis in mice. *Hippocampus* 22, 2095–2100.
406 <https://doi.org/10.1002/hipo.22032>
- 407 Borshchev, Y.Yu., Uspensky, Y.P., Galagudza, M.M., 2019. Pathogenetic pathways of
408 cognitive dysfunction and dementia in metabolic syndrome. *Life Sciences* 237,
409 116932. <https://doi.org/10.1016/j.lfs.2019.116932>
- 410 Cuspidi, C., Sala, C., Tadic, M., Gherbesi, E., Grassi, G., Mancia, G., 2018. Association of
411 metabolic syndrome with carotid thickening and plaque in the general population: A
412 meta-analysis. *The Journal of Clinical Hypertension* 20, 4–10.
413 <https://doi.org/10.1111/jch.13138>
- 414 Ding, J., Sigurðsson, S., Jónsson, P.V., Eiriksdóttir, G., Meirelles, O., Kjartansson, O., Lopez,
415 O.L., van Buchem, M.A., Gudnason, V., Launer, L.J., 2017. Space and location of
416 cerebral microbleeds, cognitive decline, and dementia in the community. *Neurology*
417 88, 2089–2097. <https://doi.org/10.1212/WNL.0000000000003983>
- 418 González, H.M., Tarraf, W., Harrison, K., Windham, B.G., Tingle, J., Alonso, A., Griswold,
419 M., Heiss, G., Knopman, D., Mosley, T.H., 2018. Midlife cardiovascular health and
420 20-year cognitive decline: Atherosclerosis Risk in Communities Study results.
421 *Alzheimer's & Dementia* 14, 579–589. <https://doi.org/10.1016/j.jalz.2017.11.002>
- 422 Granger, D.N., Rodrigues, S.F., Yildirim, A., Senchenkova, E.Y., 2010. Microvascular
423 responses to cardiovascular risk factors. *Microcirculation* 17, 192–205.
424 <https://doi.org/10.1111/j.1549-8719.2009.00015.x>

- 425 Guo, X., Li, H., Zhang, Z., Li, S., Zhang, L., Zhang, J., Han, G., 2016. Hyperglycemia and
426 Mortality Risk in Patients with Primary Intracerebral Hemorrhage: A Meta-Analysis.
427 *Molecular Neurobiology* 53, 2269–2275. <https://doi.org/10.1007/s12035-015-9184-4>
- 428 Hidaka, N., Suemaru, K., Li, B., Araki, H., 2008. Effects of Repeated Electroconvulsive
429 Seizures on Spontaneous Alternation Behavior and Locomotor Activity in Rats.
430 *Biological and Pharmaceutical Bulletin* 31, 1928–1932.
431 <https://doi.org/10.1248/bpb.31.1928>
- 432 Iadecola, C., 2017. The Neurovascular Unit Coming of Age: A Journey through
433 Neurovascular Coupling in Health and Disease. *Neuron* 96, 17–42.
434 <https://doi.org/10.1016/j.neuron.2017.07.030>
- 435 Krishna, S., Lin, Z., de La Serre, C.B., Wagner, J.J., Harn, D.H., Pepples, L.M., Djani, D.M.,
436 Weber, M.T., Srivastava, L., Filipov, N.M., 2016. Time-dependent behavioral,
437 neurochemical, and metabolic dysregulation in female C57BL/6 mice caused by
438 chronic high-fat diet intake. *Physiology & Behavior* 157, 196–208.
439 <https://doi.org/10.1016/j.physbeh.2016.02.007>
- 440 Li, W., Prakash, R., Chawla, D., Du, W., Didion, S.P., Filosa, J.A., Zhang, Q., Brann, D.W.,
441 Lima, V.V., Tostes, R.C., Ergul, A., 2013. Early effects of high-fat diet on
442 neurovascular function and focal ischemic brain injury. *Am. J. Physiol. Regul. Integr.*
443 *Comp. Physiol.* 304, R1001-1008. <https://doi.org/10.1152/ajpregu.00523.2012>
- 444 Lubrano, V., Balzan, S., 2016. Roles of LOX-1 in microvascular dysfunction. *Microvascular*
445 *Research* 105, 132–140. <https://doi.org/10.1016/j.mvr.2016.02.006>
- 446 McCracken, E., Monaghan, M., Sreenivasan, S., 2018. Pathophysiology of the metabolic
447 syndrome. *Clinics in Dermatology* 36, 14–20.
448 <https://doi.org/10.1016/j.clindermatol.2017.09.004>
- 449 Medeiros, R.F., Gaique, T.G., Bento-Bernardes, T., Kindlovits, R., Gomes, T.M.B., Motta,
450 N.A.V., Brito, F.C., Fernandes-Santos, C., Oliveira, K.J., Nóbrega, A.C.L., 2017.
451 Arginine and aerobic training prevent endothelial and metabolic alterations in rats at
452 high risk for the development of the metabolic syndrome. *British Journal of Nutrition*
453 118, 1–10. <https://doi.org/10.1017/S0007114517001702>
- 454 Mitaki, S., Takayoshi, H., Nakagawa, T., Nagai, A., Oguro, H., Yamaguchi, S., 2018.
455 Metabolic syndrome is associated with incidence of deep cerebral microbleeds. *PLOS*
456 *ONE* 13, e0194182. <https://doi.org/10.1371/journal.pone.0194182>
- 457 Mogi, M., Horiuchi, M., 2011. Neurovascular Coupling in Cognitive Impairment Associated
458 With Diabetes Mellitus. *Circulation Journal* 75, 1042–1048.
459 <https://doi.org/10.1253/circj.CJ-11-0121>
- 460 Otite, F.O., Liaw, N., Khandelwal, P., Malik, A.M., Romano, J.G., Rundek, T., Sacco, R.L.,
461 Chaturvedi, S., 2017. Increasing prevalence of vascular risk factors in patients with
462 stroke: A call to action. *Neurology* 89, 1985–1994.
463 <https://doi.org/10.1212/WNL.0000000000004617>
- 464 Paradise, M., Seruga, A., Crawford, J.D., Chaganti, J., Thalamuthu, A., Kochan, N.A.,
465 Brodaty, H., Wen, W., Sachdev, P.S., 2018. The relationship of cerebral microbleeds
466 to cognition and incident dementia in non-demented older individuals. *Brain Imaging*
467 *and Behavior* 1–12. <https://doi.org/10.1007/s11682-018-9883-3>
- 468 Park, Y.K., Yi, H.-J., Lee, Y.J., Cho, H., Chun, H.-J., Oh, S.-J., 2013. The relationship
469 between metabolic syndrome (MetS) and spontaneous intracerebral hemorrhage
470 (ICH). *Neurological Sciences* 34, 1523–1528. <https://doi.org/10.1007/s10072-012-1272-x>
- 471
- 472 Pétrault, M., Ouk, T., Pétrault, O., Bastide, M., Bordet, R., Bérézowski, V., 2019. Safety of
473 oral anticoagulants on experimental brain microbleeding and cognition.
474 *Neuropharmacology* 155, 162–172. <https://doi.org/10.1016/j.neuropharm.2019.05.030>

- 475 Pétrault, O., Pétrault, M., Ouk, T., Bordet, R., Bérézowski, V., Bastide, M., 2019. Visceral
476 adiposity links cerebrovascular dysfunction to cognitive impairment in middle-aged
477 mice. *Neurobiol. Dis.* 130, 104536. <https://doi.org/10.1016/j.nbd.2019.104536>
- 478 Poels, M.M.F., Ikram, M.A., van der Lugt, A., Hofman, A., Krestin, G.P., Breteler, M.M.B.,
479 Vernooij, M.W., 2011. Incidence of Cerebral Microbleeds in the General Population:
480 The Rotterdam Scan Study. *Stroke* 42, 656–661.
481 <https://doi.org/10.1161/STROKEAHA.110.607184>
- 482 Selim M, Hanley D, Broderick J for the Hemorrhagic Stroke Academia Industry (HEADS)
483 Roundtable Participants., 2018. Basic and Translational Research in Intracerebral
484 Hemorrhage: Limitations, Priorities, and Recommendations. *Stroke* 49, 1308–1314.
485 <https://doi.org/10.1161/STROKEAHA.117.019539>
- 486 Tomita, Y., Tanaka, S., Takahashi, S., Takeuchi, N., 2019. Detecting cognitive decline in
487 community-dwelling older adults using simple cognitive and motor performance tests.
488 *Geriatrics & Gerontology International* n/a. <https://doi.org/10.1111/ggi.13863>
- 489 Toth, P., Tarantini, S., Springo, Z., Tucsek, Z., Gautam, T., Giles, C.B., Wren, J.D., Koller,
490 A., Sonntag, W.E., Csiszar, A., Ungvari, Z., 2015. Aging exacerbates hypertension-
491 induced cerebral microhemorrhages in mice: role of resveratrol treatment in
492 vasoprotection. *Aging Cell* 14, 400–408. <https://doi.org/10.1111/accel.12315>
- 493 van Dijk, G., van Heijningen, S., Reijne, A.C., Nyakas, C., van der Zee, E.A., Eisel, U.L.M.,
494 2015. Integrative neurobiology of metabolic diseases, neuroinflammation, and
495 neurodegeneration. *Front Neurosci* 9, 173. <https://doi.org/10.3389/fnins.2015.00173>
- 496 Van Dyken, P., Lacoste, B., 2018. Impact of Metabolic Syndrome on Neuroinflammation and
497 the Blood–Brain Barrier. *Frontiers in Neuroscience* 12, 930.
498 <https://doi.org/10.3389/fnins.2018.00930>
- 499 Whitmer, R.A., Sidney, S., Selby, J., Johnston, S.C., Yaffe, K., 2005. Midlife cardiovascular
500 risk factors and risk of dementia in late life. *Neurology* 64, 277–281.
501 <https://doi.org/10.1212/01.WNL.0000149519.47454.F2>
- 502

503 **Figure captions**

504 **Figure 1: Acute effects of cerebral microhemorrhages on locomotor and cognitive**
505 **functions of 14 month-old mouse (12 months of diet).** **A.** Short-term-test protocol for the
506 acute effects of the CMH lesions induced at 14 month-old (after 12 months of diet, n=12-15
507 per group). **B.** Spontaneous locomotor behavior of mice. A significant decrease in the
508 travelled distance (left, in cm) and mean walking speed (right, in cm/s) was observed in HFD-
509 fed mice with CMHs, accompanied with a significant increase in the resting time (in second).
510 **C.** Working memory (spontaneous alternation index) and visual recognition memory (novel
511 object recognition index) were examined through the spontaneous alternation test and the
512 novel object recognition (NOR) test respectively. No difference was found in the performance
513 on the spontaneous alternation test. Several mice were excluded of the test according to the
514 exclusion criteria (ND-CMH = 1; HFD = 1; HFD-CMH = 6). A significant difference was
515 found in the performance on the NOR test in HFD-fed mice compared with ND-fed mice with
516 or without CMHs. HFD-fed mice with CMHs were significantly different from ND-fed mice.

517 Several mice were excluded of the test according to the exclusion criteria (ND-CMH = 1;
518 HFD = 1; HFD-CMH = 6). Data are shown as mean \pm sem, individual data points are
519 superimposed on boxplots. Statistical analysis was performed using a two-way ANOVA
520 followed by a Tukey's post-hoc test. * $p < 0.05$, ** $p < 0.01$. ND: Normal Diet. ND+CMH:
521 Normal Diet with CMHs. HFD: High-Fat Diet. HFD+CMH: High-Fat Diet with CMHs.

522 **Figure 2: Acute effects of cerebral microhemorrhages on locomotor and cognitive**
523 **functions of 5 month-old mouse (3 months of diet).** **A.** Short-term test protocol for the acute
524 effects of the CMH lesions induced at 5 month-old (after 3 months of diet, $n=7-8$ per group).
525 **B.** Spontaneous locomotor behavior of mice. No significant differences were observed in the
526 travelled distance (left, in cm) and mean walking speed (on the right, in cm/s) between
527 groups. A significant decrease in the resting time (in second) was observed among mice with
528 CMHs (ND+CMH and HFD+CMH). **C.** Working memory (spontaneous alternation index)
529 and visual recognition memory (novel object recognition index) were examined through the
530 spontaneous alternation test and the novel object recognition (NOR) test respectively. No
531 difference was found in the performance on the spontaneous alternation test between groups.
532 A significant difference was found in the performance on the NOR test in mice with CMHs
533 (ND+CMHs and HFD+CMHs) compared to ND mice. Data are shown as mean \pm sem,
534 individual data points are superimposed on boxplots. Statistical analysis was performed using
535 a two-way ANOVA followed by a Tukey's post-hoc test. * $p < 0.05$, ** $p < 0.01$. ND: Normal
536 Diet. ND+CMH: Normal Diet with CMHs. HFD: High-Fat Diet. HFD+CMH: High-Fat Diet
537 with CMHs.

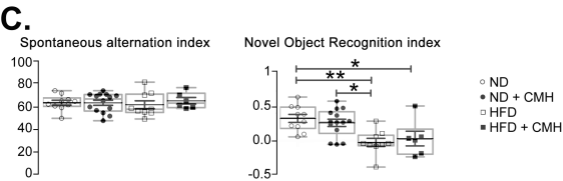
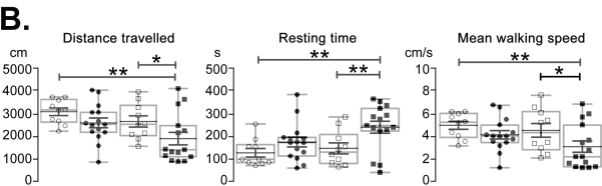
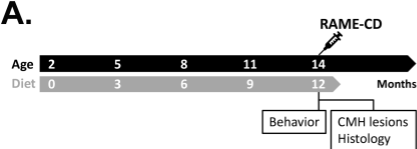
538 **Figure 3: Acute microbleedings of 14 month-old mice (12 months of diet) and 5 month-old**
539 **mice (3 months of diet).** **A.** Representative view of cerebral microhemorrhages (CMHs) in
540 mice after 12 months of normal diet (ND) or high-fat diet (HFD), and the dot plots of CMHs
541 for each mouse ($n=12-15$ per group). **B.** Mean number of CMHs at onset after 12 months of
542 diet. A significant increase was found in HFD-fed mice compared with ND-fed mice (Student
543 test, $p=0.01$). Mosaic plot of CMHs in each group. The ratio refers to the percentage of each
544 type of microhemorrhage among all the observed CMHs. These CMH profiles were not
545 different between both groups according to Pearson's Chi-squared test ($p=0.39$). **C.**
546 Representative view of CMHs in mice at 5 months of age, after 3 months of ND or HFD, and
547 the dot plots of the CMHs for each mouse ($n=6-7$ per group). **D.** Mean number of CMHs at
548 onset after 3 months of diet. No significant difference was obtained between groups (Student
549 test, $p=0.64$). Mosaic plot of CMHs in each group. These CMH profiles were not different

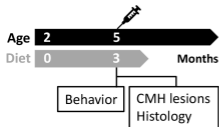
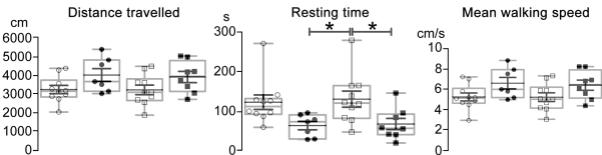
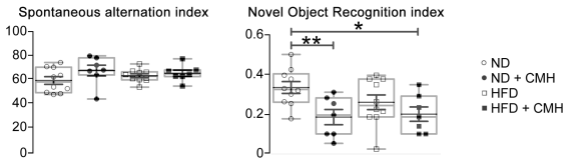
550 between both groups according to Pearson's Chi-squared test ($p=0.82$). **E.** Time comparison
551 of acute microbleedings at 5 month-old (3 months of diet) or 14 month-old (12 months of
552 diet). The mean numbers of CMHs were not significantly different between both ND-fed mice
553 groups ($p=0.92$), but significantly increased in HFD-fed mice at 14 months of age (after 12
554 months of diet) compared with 5 months of age (after 3 months of diet) according to two-way
555 ANOVA followed by a Tukey's post-hoc test ($p=0.02$). The CMH profiles were not different
556 among ND-fed mice ($p=0.90$) and HFD-fed mice ($p=0.53$) according to the Pearson's Chi-
557 squared test. Data are shown as mean \pm sem, individual data points are superimposed on
558 boxplots. ND: Normal Diet. HFD: High-Fat Diet.

559 **Figure 4: Long-term effects of cerebral microhemorrhages on mouse locomotor and**
560 **cognitive functions.** **A.** Long-term test protocol for the follow-up of the effects of early
561 CMHs in the brain of mice after 12 months of diet ($n=10-12$ per group). **B.** Spontaneous
562 locomotor behavior, reflected by the travelled distance (in cm), the resting time (in second)
563 and the mean walking speed (in cm/s). No significant difference was observed between
564 groups. **C.** Working memory (spontaneous alternation index) and visual recognition memory
565 (novel object recognition index) were examined through the spontaneous alternation test and
566 the novel object recognition (NOR) test respectively. No difference was found in the
567 performance on spontaneous alternation test between groups. Several mice were excluded of
568 the test according to the exclusion criteria ($n=1-2$ per group and time). Visual recognition
569 memory of ND-fed mice was preserved during the protocol, as given by the stable
570 discrimination NOR index. The induction of CMHs in ND-fed mice reduced these
571 performances until significance from 14 months of age (12 months of diet). The NOR index
572 of HFD-fed mice decreased progressively until 14 months of age and became significantly
573 different from ND-fed mice after 11 months of age (9 months of diet). The presence of CMHs
574 in HFD-fed mice resulted in a significantly reduced NOR index at 11 months of age compared
575 with ND control group. Several mice were excluded of the test according to the exclusion
576 criteria ($n=1-2$ per group and time). Data are shown as mean \pm sem, individual data points are
577 superimposed on boxplots. Statistical analysis was performed using a two-way ANOVA
578 followed by a Tukey's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ND: Normal Diet.
579 ND+CMH: Normal Diet with CMHs. HFD: High-Fat Diet. HFD+CMH: High-Fat Diet with
580 CMHs.

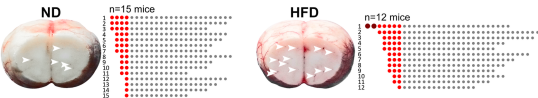
581 **Figure 5: Cerebral microhemorrhage profiles of 14 months-old mice on prolonged diet, 9**
582 **months after onset.** **A.** Representative view of cerebral microhemorrhages (CMHs) in 14

583 months-old mice on prolonged normal diet (ND) or high-fat diet (HFD), 9 months after onset,
584 and the dot plots of CMHs for each mouse (n=6-7 per group). **B.** Mean number of CMHs 9
585 months after onset. No significant difference was found in HFD-fed mice compared with ND-
586 fed mice (Student test, $p=0.17$). Mosaic plot of CMHs in each group. The ratio refers to the
587 percentage of each type of microhemorrhage among all observed CMHs. These CMH profiles
588 were not different between both groups according to the Pearson's Chi-squared test ($p=0.68$).
589 **C.** Time comparison of CMHs numbers and profiles between onset in 5 months-old mice after
590 3 months of diet, and 9 months after (14 months of age, 12 months of diet). The mean number
591 of CMHs was not significantly different in ND-fed mice ($p=0.93$) and in HFD-fed mice
592 ($p=0.66$) according to two-way ANOVA followed by a Tukey's post-hoc test. The CMH
593 profiles were not different among ND-fed mice ($p=0.69$) and HFD-fed mice ($p=0.55$)
594 according to the Pearson's Chi-squared test. Data are shown as mean \pm sem, individual data
595 points are superimposed on boxplots. ND: Normal Diet. HFD: High-Fat Diet.

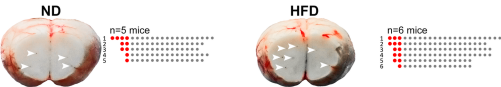


A.**RAME-CD****B.****C.**

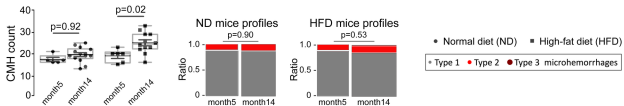
A. Acute CMH lesions of 14 month-old mice



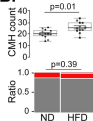
C. Acute CMH lesions of 5 month-old mice



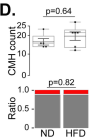
E. Time comparison of CMH numbers and profiles

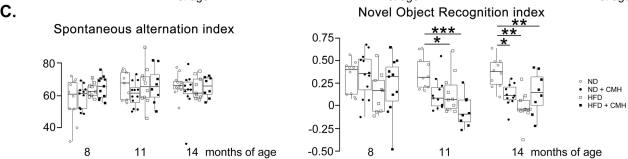
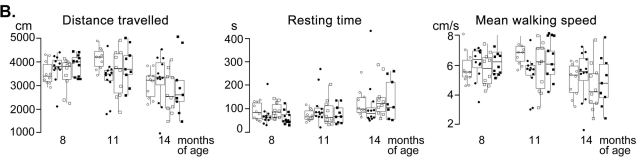
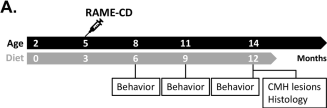


B.

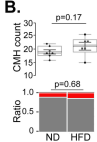
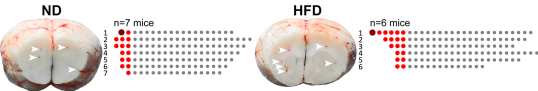


D.

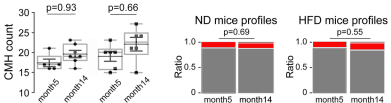




A. Late CMH lesions of 14 month-old mice (9 months after onset)



C. Time comparison of CMH numbers and profiles



• Normal diet (ND) ■ High-fat diet (HFD)

• Type 1 • Type 2 • Type 3 microhemorrhages