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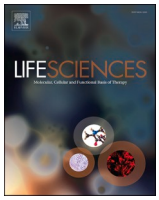

Does concomitant diazepam and ethanol use modulate age-related cognitive decline in mice?

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ABSTRACT

Background: Concomitant use of alcohol and benzodiazepines are described among elderly, raising concerns about their combined impact on memory. We aimed to evaluate the long-term impact of chronic diazepam use associated with ethanol intoxication on memory in aging mice.
Methods: Twelve-month-old male C57BL6 mice were assigned into 4 groups: ethanol (OH), diazepam (DIA), diazepam + ethanol (DOH) and control (CTL). For 16 weeks, ethanol was available ad libitum and diazepam was mixed with food. Behavioral testing, performed during and after treatment cessation included working memory and visual recognition memory assessment. The second session was implemented with spatial reference learning and memory assessment in the Barnes maze test. In vivo magnetic resonance spectroscopy (MRS) acquisitions were performed to quantify hippocampal metabolites during and after cessation treatment.
Results: During treatment, visual recognition memory was significantly different between groups with the DIA group exhibiting the worst performance. MRS acquisition highlighted higher glutamate and choline levels in OH and DOH groups in comparison to CTL and DIA groups. After treatment wash-out, there was no difference between in the different memories evaluated. Only the learning phase of the spatial reference memory test differed significantly with worst performance in OH groups. Three months after treatment cessation, there was no remanent effect of diazepam + ethanol on hippocampal metabolites changes.
Conclusions: We did not evidence additive effect of ethanol and diazepam on memory and hippocampal metabolite levels. The disturbances observed during treatment were no remanent, highlighting the benefits of discontinuing these substances.

1. Introduction

Benzodiazepines are widely used psychotropic drugs. Indicated for the symptomatic treatment of anxiety and insomnia, the guidelines mention that their use should be limited in time [1,2]. However, there are numerous reports of non-compliant prescribing, including longer prescription periods. This extension of the prescription period raises concerns about long-term adverse effects, particularly cognitive impairments [1,2]. Previous observational study [3–5] and meta-analysis [6,7] highlighted a relationship between the long-term use of benzodiazepine and major neurocognitive disorder. However, this association is still being debated due to methodological biases, including a protopathic bias that may explain this association [8–10]. The link found in epidemiological studies may be linked to early use of benzodiazepines to treat anxiety and insomnia, which are prodromes of cognitive disorders, rather than a negative consequence of benzodiazepine use per se. In this way, we previously employed a mice model of diazepam long-term treatment, taking into account both an age-related and a dose-related effect that highlighted a lack of cognitive impairment after long-term treatment discontinuation [11].

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Ethanol acts on several neurotransmission systems in the brain, and shares a common target with benzodiazepines in the facilitation of the GABAergic transmission [12–14], but in a different way than benzodiazepines. More specifically, a previous experimental study suggested that the conformational changes in the $GABA_AR$ produced by ethanol were experimentally separable from the conformational changes produced by benzodiazepines and that both can occur simultaneously to further enhance receptor function [14]. Clinically, ethanol induces cognitive impairments that manifest as a set of alterations in executive function, learning, and memory [15–17]. Chronic ethanol treatment in animals has been found to impair functions related to prefrontal cortex (PFC) [18], and hippocampus [19]. For example, alterations in spatial reference and visual recognition memory [18–22] but also impairments in cognitive flexibility [18] and working memory [23] have been described. However, only few studies have been performed in mature rodents.

As previous studies found that ethanol was frequently consumed in association with benzodiazepine [24,25] and that the prevalence of psychotropic drug and alcohol interactive medicine use rises with older age [26], one may ask about ethanol/benzodiazepines combined effect on brain aging processes. This combination could exacerbate the negative short- and/or long-term neurobiological consequences of these substances taken independently/separately and modify or aggravate the resulting cognitive impairment pattern.

Thus, we aimed to evaluate the long-term impact of ethanol intoxication, associated with chronic benzodiazepine use on memory in an aging mice model.

2. Materials and methods

2.1. Animals

Male C57Bl/6 mice (Elevage Janvier, Le Genest St Isle, France) aged 12 months at the beginning of the experiments were used for this study. We chose to use this age, with behavioral assessment at 15 months and 18 months (Fig. 1), to be in a time frame where the first cognitive signs related to aging may appear. This choice is in line with previous work that has found cognitive alterations at 14 months in C57bl6 mice during normal aging [27].

The mice were housed in transparent cages (five or less per cage) with nested material and were maintained in a climate-controlled room (temperature 19–24 °C; relative humidity: 60–70 %) with a 12-hour light/dark cycle (lights on at 7.00 am). Animals were allowed to acclimate to the laboratory for 10 days before any experimental manipulation.

The national Ethical Committee in Animal Experimentation and the French Ministry of Education and Research gave their approval for these experiments. In addition, the research was carried out in strict compliance with the European Union Directive 2010/63/EU and the ARRIVE guidelines for experiments involving animals.

2.2. Treatment administration

Four experimental groups of mice were set up: (i) a control group with normal diet and access to water ad libitum (CTL, $N = 27$); (ii) a diazepam-supplemented diet group at 30 mg/kg/d with access to water ad libitum (DIA, $N = 19$); (iii) a 16 % ethanol ad libitum group representing the only available beverage source feeding with normal diet (OH, $N = 15$); (iv) a diazepam-supplemented diet group at 30 mg/kg/d with 16 % ethanol ad libitum representing the only available beverage source (DOH, $N = 16$).

Diazepam was chosen among benzodiazepines for its long half-life (32–47 h in humans) likely to increase the risk of long-term cognitive effects [6,28]. The daily dose of benzodiazepine administered to mice was determined by the body surface area-based adjustment method as previously described [29]. 30 mg/kg/d in mice corresponds approximately to the double dose of the maximum recommended human dose (1 mg/kg/d). For the preparation of treatment pellets, diazepam tablets (ARROW Laboratory) were crushed and mixed into the powdered diet (Scientific Animal Food and Engineering (SAFE), A04, France). The normal diet was made up of SAFE pellets.

Ethanol was available ad libitum daily via a bottle of 16 % ethanol representing the only available beverage source according to a chronic alcohol model previously described in the literature [30]. In this model, blood ethanol concentrations were estimated at between 0.8 and 1.6 g/L [30].

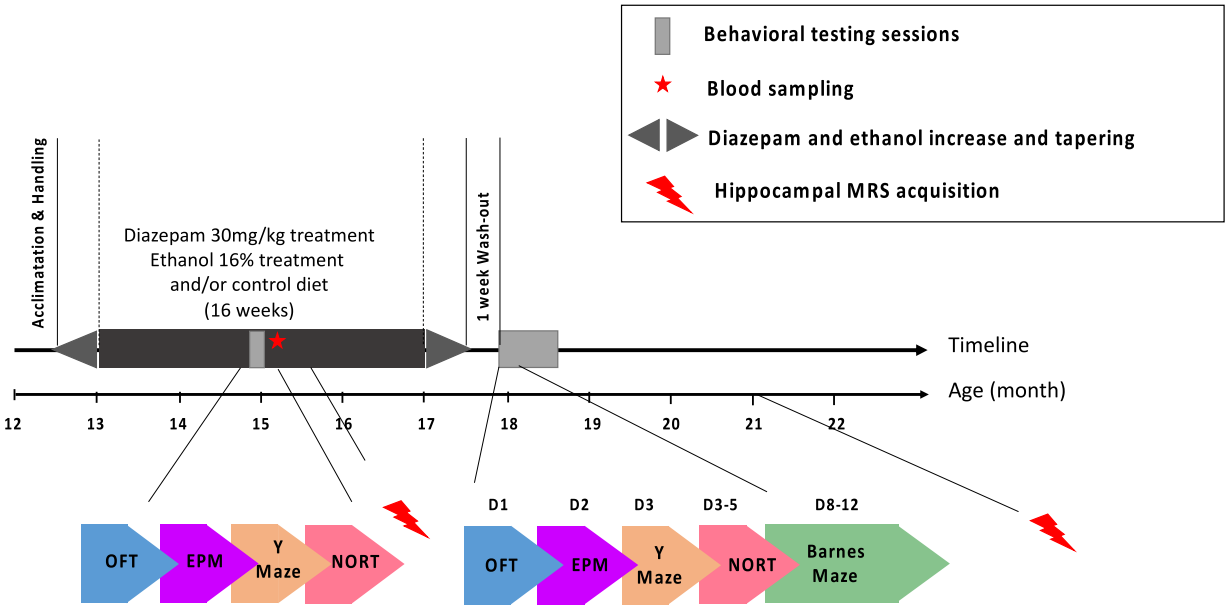

Fig. 1. Study design, timeline and methods of the study.
The top line shows the timing of treatment and behavioral sessions. The bottom line shows the age of mice at the different stages of the experiment. The lower part of the figure indicates the behavioral tests performed for each assessment session and their order. EPM: Elevated plus maze; NORT: Novel Object Recognition Test; OFT: Open-Field Test

Diazepam and ethanol were delivered respectively into the diet and via the beverage source to avoid stress and damage from chronic force feeding. Treatments included first a gradual dose escalation phase; ethanol and diazepam were increased by ¼ dose every 4 days; 4 %, 8 %, 12 %, and 16 % for ethanol, and 7.5 mg, 15 mg, 22.5 mg, and 30 mg for diazepam. Then animals consumed 16 % ethanol and/or 30 mg/kg diazepam for 4 months. At the end of the 4-month treatment, the same scheme was applied inversely to achieve a gradual decrease and avoid a withdrawal syndrome (Fig. 1).

2.3. Experimental design

Fig. 1 shows the experimental design of the study, which includes two sessions of magnetic resonance spectroscopy (MRS) and two sessions of behavioral assessment. The two MRS acquisition sessions were performed on a sample of 10 mice for each treatment group. The first behavioral assessment was performed after 8 weeks of treatment (mid-treatment) to assess the direct effect of the drugs on locomotor activity, anxiety, working memory and visual recognition memory using, in the following order: open-field test (OFT), elevated plus maze (EPM) test, Y maze spontaneous alternation test and novel object recognition (NOR) test. MRS acquisitions in the hippocampus area were performed the week following the one of behavioral testing.

The second behavioral assessment was performed after 16 weeks of treatment, followed by a gradual decline and one-week wash-out period to assess the long-term cognitive effects after diazepam and/or ethanol cessation. At the beginning of this second assessment, the mice were 17 months old. This post-treatment test battery was enriched with the Barnes maze test evaluating the spatial reference memory. The second MRS acquisition in the hippocampal area was performed three months after treatment cessation.

2.4. Behavioral testing

The following behavioral assessments were performed according to previously published protocols [11,31,32]. The OFT was used to assess the spontaneous locomotor activity. The parameters investigated were the total distance traveled (in cm) and the number of rearing. The time spent in the central zone was also recorded, as a marker of anxiety. The EPM test was used to measure the anxiety-like behavior of mice by assessing the percentage of time spent in the open arms. The Y maze spontaneous alternation test was used to assess the working memory. The parameters investigated were the percentage of spontaneous alternations and the total number of entries. The NOR test was used to assess the visual recognition memory. The main outcome was the discrimination index (DI) of novel object. At last, the Barnes maze test was used to explore spatial reference learning and memory. The average daily total escape latency was used to assess spatial reference learning, and the percentage of time spent in the target quadrant during the probe trial was used to assess spatial reference memory performances.

2.5. MRS procedure

Experiments were performed on a 7.0 Tesla Animal Biospec MR Scanner (Bruker, Ettlingen, Germany). Within the MRI bed, isoflurane anesthesia was delivered through a facial mask (isoflurane 1.5–2 % and air 1.5 L/min). Animal vital parameters (blood-oxygen saturation, pulse rate, rectal temperature and respiration rate) were monitored throughout the experiment.

Gradient echo acquisition was first carried out to confirm the placement of the animal in the apparatus. T2-weighted anatomical sequences were then carried out in the axial and coronal planes to position the MRS acquisition voxel (5 * 2 * 1,5 mm^3) in the hippocampus (Fig. 2A) (repetition time (TR)/echo time (TE) = 2500/33 ms, squared field of view = 4 cm, encoded by a squared matrix of 256 * 256 and 16 slices of 0.5 mm).

MRS acquisition and postprocessing were performed as previously described [33]. Creatine peaks was used as an internal reference to estimate metabolite quantity. The integrated area under the curve was used for quantification. The quality of the spectrum allowed evaluation of the signal for the following metabolites: (i) Glu (glutamate, 2.35 ppm), (ii) Gln (glutamine, 2.43 ppm), (iii) NAA (N-Acetyl-Aspartate, 2.02 ppm), a marker related to neuronal activity and integrity, (iv) tCr (total creatine, 3.03 and 3.9 ppm) considered a stable metabolite and

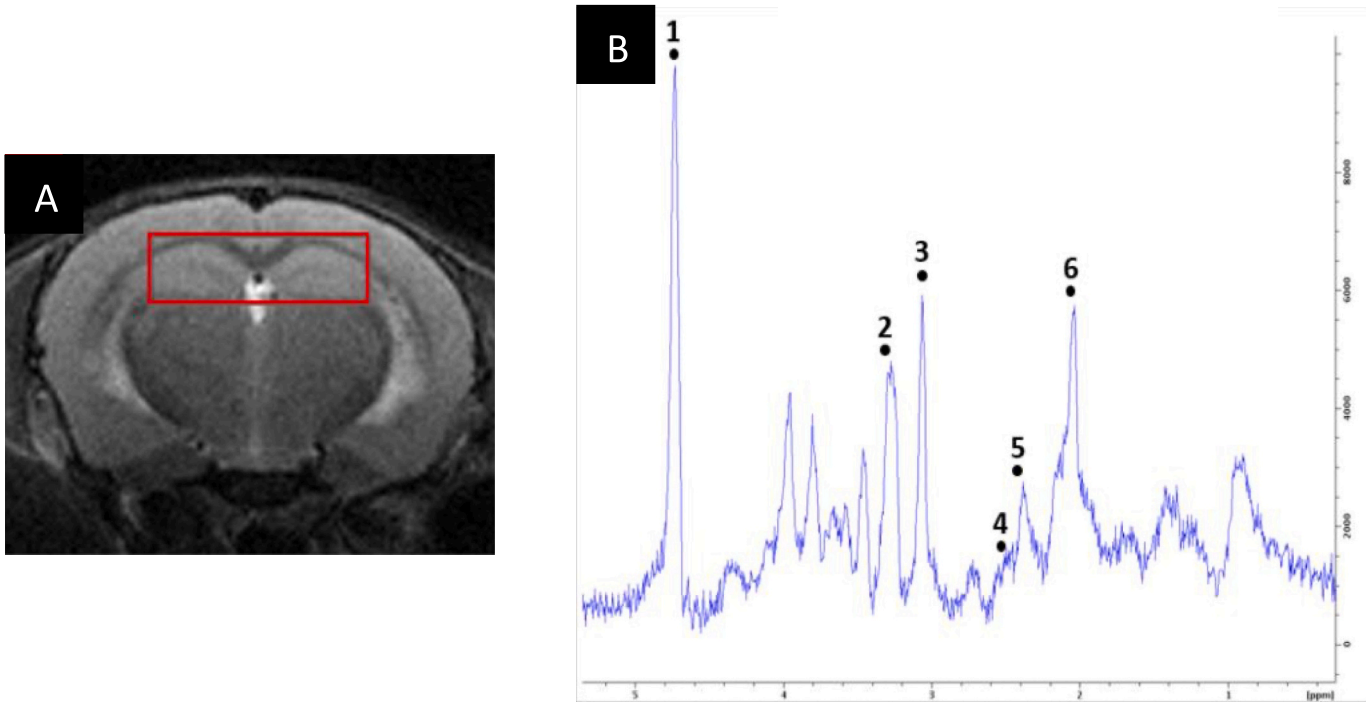

Fig. 2. A) Voxel localization in the hippocampal area B) Example of spectra obtained in MRS acquisition with pic of water (1), choline (2), creatine (3), glutamine (4), glutamate (5), N-Acetyl-Aspartate (6).

commonly used as a reference concentration, and (v) Cho (choline, 3.2 ppm), whose levels are generally associated with alterations in membrane composition [34] (Fig. 2B). Postprocessing of the MRS data was performed using JMRUI 5.2 software [35,36] while the AMARES algorithm (Advanced Method for Accurate, Robust, and Efficient Spectral fitting) was used for quantification of the main metabolites [37].

2.6. Statistical analysis

Categorical variables were presented as frequencies and percentages. Quantitative variables were reported as mean (standard deviation, SD) or median (interquartile range, IQR) for non-normally distributed data. The normality of the distributions was evaluated both graphically and with the Shapiro-Wilk test. We compared mid-term quantitative variables among the 4 experimental groups using one-way ANOVA, followed by pairwise post-hoc comparisons with linear contrasts and Bonferroni correction. Variables that did not follow a normal distribution were log-transformed. The same statistical analyses (ANOVA and post-hoc) were applied to compare long-term quantitative variables between groups. Additionally, we assessed the within-mice variability in spatial reference learning across the 4 groups using a linear mixed model, incorporating time, group, and time * group interaction as fixed effects. When a significant interaction between time and groups was detected, post-hoc comparisons between groups were conducted using linear contrasts

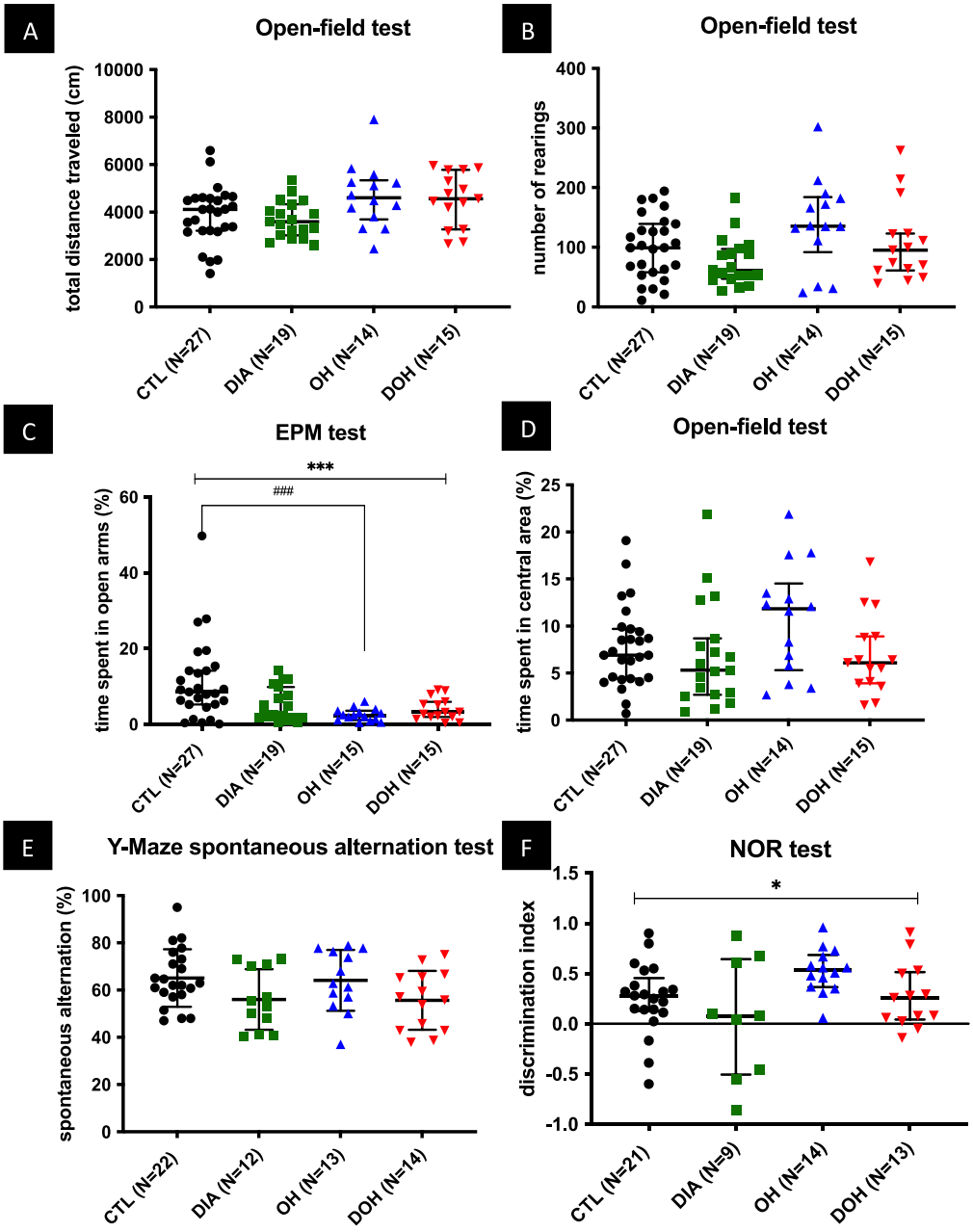

Fig. 3. Effect of 2 months of treatment with 30 mg/kg/d diazepam (DIA), 16 % ethanol (OH), 30 mg/kg/d diazepam + 16 % ethanol (DOH) or control conditions (CTL) on: A) the total distance traveled in the Open-Field Test (OFT), B) the number of rearing in the OFT, C) the percentage of time spent in open arms in the Elevated Plus Maze (EPM) test, D) the time spent in the central zone in the OFT, E) the percentage of spontaneous alternations in the spontaneous alternation test and F) the discrimination index during the Novel Object Recognition (NOR) test. Results are expressed as mean ± SD for the OFT and spontaneous alternation test, and as median (IQR) for the EPM and NOR tests.
Differences between groups are represented by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and *post-hoc* pairwise comparisons by $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

with Bonferroni correction. Statistical tests were performed at a two-tailed α level of 0.05. The data were analyzed using SAS software, version 9.4 (SAS Institute, Cary, NC).

Variables with a non-normal distribution were log-transformed. The same analyses (ANOVA and post-hoc) were done to compare long term quantitative variables between groups. We further compared the intra-mice variability in spatial reference learning between the 4 groups by using a linear mixed model including time, groups and time * groups interaction as fixed effects. In case of a significant interaction between time and groups, a post-hoc comparison between each group was done using linear contrast after Bonferroni correction. Statistical testing was done at the two-tailed α level of 0.05. Data were analyzed using the SAS software package, release 9.4 (SAS Institute, Cary, NC).

3. Results

3.1. Behavioral consequences assessed mid-way through treatment.

3.1.1. Spontaneous locomotor activity assessment in the OFT

The total distances traveled by mice were not significantly changed by the diazepam and/or ethanol treatment, $F(3,71) = 2.66$; $p = 0.055$. The number of rearings was not different between groups, $F(3,71) = 1.95$; $p = 0.13$ (Fig. 3).

3.1.2. Anxiety-like behavior assessment in the EPM and OFT

The percentage of time spent in the open arms was significantly different between groups, $F(3,72) = 6.06$; $p < 0.001$ with a difference between the OH group, median 2.4 % (1.2; 3.6) versus the CTL group 8.6 % (5.3; 14.2), $t(72) = 3.94$; $p < 0.001$ in *post-hoc* analysis, suggesting a greater anxiety-like behavior in the OH group. The analysis of the time spent in the central zone in the OFT was not significantly different between the groups, $F(3,71) = 1.95$, $p = 0.13$ (Fig. 3).

3.1.3. Working memory assessment in the Y-maze spontaneous alternation test

Respectively 15.4 %, 36.8 %, 13.3 % and 12.5 % of animals in the CTL, DIA, OH and DOH groups were excluded because they did not reach the minimum number of total entries. This exclusion rate was not significantly different between groups ($p = 0.25$). The percentage of spontaneous alternations did not reach significant difference between groups, with 65.1 ± 12.2 %, 56.1 ± 12.8 %, 64.1 ± 12.9 % and 55.7 ± 12.5 %, for the CTL, DIA, OH and DOH groups, $F(3,57) = 2.5$; $p = 0.068$ (Fig. 3).

3.1.4. Visual recognition memory assessment in the NOR test

A significant difference in exclusion rates was observed, with 22.2 %, 52.6 %, 6.7 % and 18.7 % of animals across the CTL, DIA, OH and DOH groups that did not sufficiently explore the objects during the acquisition phase ($p = 0.021$). The discrimination index was different between groups with median 0.28 (0.13; 0.45), 0.08 (−0.50; 0.64), 0.54 (0.37, 0.69) and 0.26 (0.04; 0.51), $F(3;53) = 3.21$; $p = 0.018$ for the CTL, DIA, OH and DOH respectively, with $0.076 < p < 1$ after *post-hoc* statistical correction (Fig. 3). Both results suggest a poorer performance in the DIA group in visual recognition memory.

3.2. Long-term behavioral consequences after treatment withdrawal

3.2.1. Spontaneous locomotor activity assessment in the OFT

The total distances traveled by mice were not significantly different between groups, $F(3,70) = 2.01$; $p = 0.12$. Conversely, the number of rearings was significantly different between groups with a median of 50.0 (28.0; 80.0), 142.5 (84.0; 188.0), 53.0 (31.0; 103.0), and 76.5 (50.5; 109.0) for the CTL, DIA, OH, and DOH groups respectively, $F(3,70) = 8.02$; $p < 0.001$. *Post-hoc* analysis found a higher number of rearing in the DIA group in comparison to the CTL, OH and DOH groups, respectively $t(70) = 4.83$; $p < 0.001$; $t(70) = 3.03$; $p = 0.002$ and $t(70) = 2.22$, $p = 0.015$ (Fig. 4).

3.2.2. Anxiety-like behavior assessment in the EPM and OFT

The percentage of time spent in the open arms of the EPM was significantly different between groups, with medians of 5.0 % (2.1; 12.0), 9.2 % (3.9; 14.3), 3.4 % (2.8; 5.6) and 2.5 % (1.4; 5.9) for the CTL, DIA, OH and DOH respectively, $F(3,72) = 4.18$; $p = 0.009$. *Post-hoc* analysis found significant differences between DIA and OH groups, $t(72) = 2.61$; $p = 0.035$ and between the DIA and DOH groups, $t(72) = 3.11$; $p = 0.016$ (Fig. 4).

The time spent in the central zone in the OFT was significantly different between groups with medians of 7.5 % (2.7; 11.3), 16.1 % (12.5; 18.8), 6.3 % (4.1; 12.8) and 11.9 % (5.7; 19.1) for the CTL, DIA, OH and DOH groups respectively, $T(3,70) = 5.94$; $p < 0.001$. *Post-hoc* pair-wise analysis found significant differences between CTL and DIA groups, $t(70) = 4.00$; $p < 0.001$ and between DIA and OH groups, $t(70) = 2.73$; $p = 0.042$ (Fig. 4).

Taking together, the increase of rearing and the higher time spent in the central zone in the OFT suggest a benzodiazepine withdrawal syndrome in the DIA group.

3.2.3. Working memory assessment in the Y-maze spontaneous alternation test

Respectively, 7.8 %, 10.5 %, 6.7 %, and 37.5 % of mice in the CTL, DIA, OH, and DOH groups were excluded because they did not meet the minimum number of entries. This exclusion rate was not significantly different between the groups ($p = 0.057$). The percentage of spontaneous alternations was not significantly different between groups, with means of 66.1 ± 11.6 %, 65.9 ± 9.0 %, 61.3 ± 10.6 %, and 66.4 ± 6.9 % for the CTL, DIA, OH, and DOH groups, respectively, $F(3,61)$; $p = 0.49$ (Fig. 4).

3.2.4. Visual recognition memory assessment in the NOR test

Exclusion rates were significantly different between groups, with 25.9 %, 0 %, 53.3 % and 37.5 % of animals across the CTL, DIA, OH and DOH groups that did not sufficiently explore the objects during the acquisition phase ($p = 0.021$). No differences were seen between groups on the discrimination index, median 0.23 (−0.0057; 0.36), 0.32 (−0.19; 0.58), 0.43 (0.29; 0.79) and 0.28 (−0.11; 0.58) for the CTL, DIA, OH and DOH groups respectively, $p = 0.39$ (Fig. 4).

3.2.5. Spatial reference learning and memory assessment in the Barnes maze test

Assessment of spatial learning in the Barnes maze showed a significant decrease in the latency to escape during maze acquisition in all groups, indicative of intact learning. A difference in learning was found between groups ($p = 0.0055$), with higher total latencies in the OH groups compared to the CTL group, especially in the DOH group, suggesting a potential additive effect of these substances (Fig. 5A). The comparison of the percentage of time spent in the target quadrant found no differences between the treated and untreated groups, mean 44.9 ± 22.2 %, 47.3 ± 23.7 %, 43.9 ± 30.3 % and 52.5 ± 18.2 for the CTL, DIA, OH and DOH groups, respectively, $F(3,71) = 0.45$; $p = 0.72$ (Fig. 5), indicating no impairment of spatial reference memory after discontinuation of long-term treatments.

3.3. Impact on hippocampal metabolites of chronic diazepam and/or ethanol exposure

3.3.1. Impact after 8 weeks of treatment

3.3.1.1. Glutamate. The glutamate levels were significantly different between groups with a median of 0.2 (0.19; 0.2), 0.21 (0.19; 0.24), 0.41 (0.39; 0.44), and 0.46 (0.43; 0.48) for CTL, DIA, OH, and DOH groups respectively, $t(3) = 30.12$; $p < 0.001$. *Post-hoc* analyses notably found a

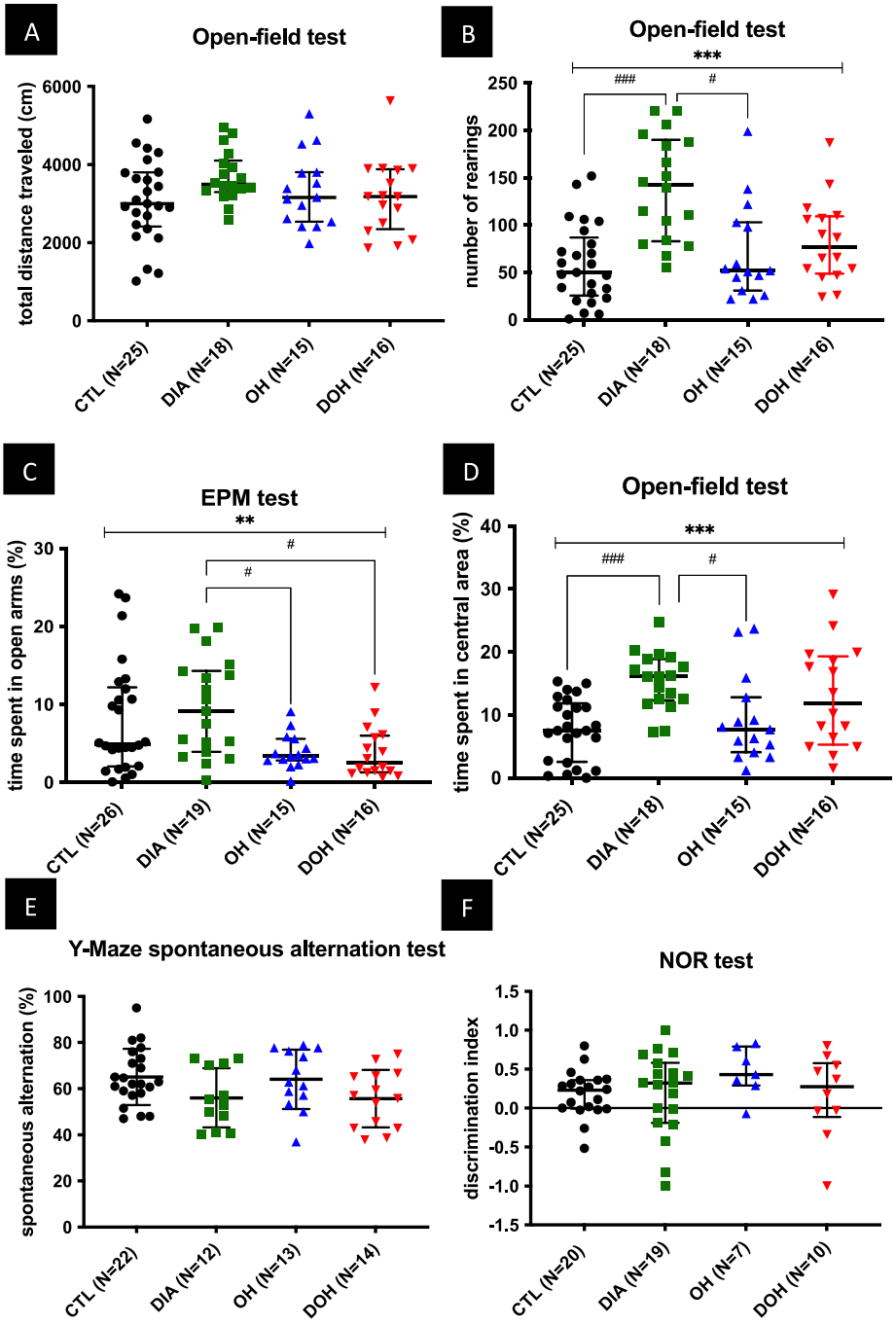

Fig. 4. Long-term effects of 30 mg/kg/d diazepam (DIA), 16 % ethanol (OH), 30 mg/kg/d diazepam + 16 % ethanol (DOH) or control conditions (CTL) after treatment cessation on: A) the total distance traveled in the Open-Field Test (OFT), B) the number of rearing in the OFT, C) the percentage of time spent in open arms in the EPM test, D) the time spent in the central zone in the OFT, E) the percentage of spontaneous alternations in the spontaneous alternation test, F) the discrimination index during the NOR test. Results are expressed as mean ± SD for the OFT and spontaneous alternation test, and as median (IQR) for the EPM and NOR tests. Overall differences between groups are represented by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and *post-hoc* pairwise comparisons by $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

higher level in the OH group compared to the CTL and DIA groups (t(1) = 14.29; $p = 0.001$ and t(1) = 13.72; p = 0.001, respectively), and a higher level in the DOH group compared to the CTL and DIA groups (t (1) = 13.5; p = 0.001 and t(1) = 12.91; $p = 0.002$) (Fig. 6).

3.3.1.2. Glutamine. The glutamine levels were significantly different between groups with a median of 0.36 (0.36; 0.37), 0.37 (0.35; 0.37), 0.30 (0.29; 0.31), and 0.40 (0.33; 0.45) for the CTL, DIA, OH, and DOH groups respectively, t(3) = 18.46; $p < 0.001$. *Post-hoc* analyses found a lower level in the OH group compared to the CTL, DIA and DOH groups (t(1) = 14.29; p < 0.001, t(1) = 13.17; p = 0.002 and t(1) = 7.26; $p = 0.042$, respectively) (Fig. 6).

3.3.1.3. NAA. The NAA levels were significantly different between groups with a median of 0.66 (0.65; 0.7), 0.71 (0.69; 0.73), 0.59 (0.54; 0.67) and 0.71 (0.69; 0.73) for the CTL, DIA, OH and DOH groups respectively, t(3) = 7.88; $p = 0.049$. *Post-hoc* analyses did not find significant differences between groups (Fig. 6).

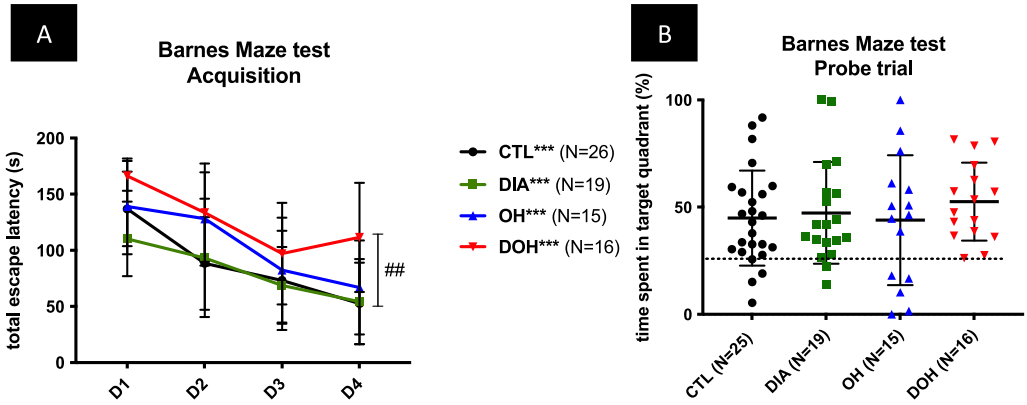

Fig. 5. Long-term effects of treatment with 30 mg/kg/d diazepam (DIA), 16 % ethanol (OH), 30 mg/kg/d diazepam +16 % ethanol (DOH) or control conditions (CTL) on: A) the mean total escape latency during the learning session in the Barnes Maze test and B) the time spent in target quadrant during the probe trial of the Barnes Maze test. Results are expressed as mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ for the intragroup comparisons and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ for the intergroup comparisons.

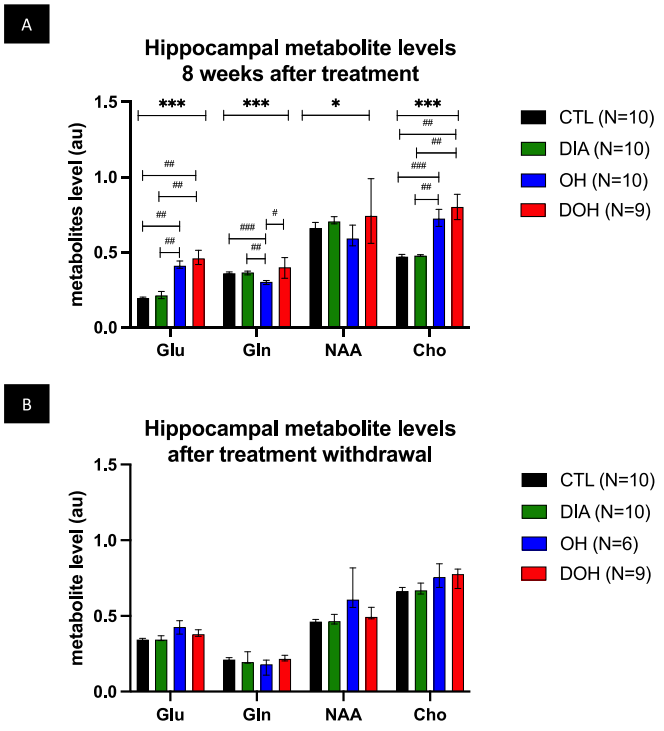

Fig. 6. Effects of treatment with 30 mg/kg/d diazepam (DIA), 16 % ethanol (OH), 30 mg/kg/d diazepam +16 % ethanol (DOH) or control conditions (CTL) on hippocampal metabolite levels assessed: A) two months after treatment beginning and B) 3 months after treatment cessation. Results are expressed as median ± IQR. Overall differences between treatment groups for each metabolite are represented by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and post hoc pairwise comparisons of groups by $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$. Glu: glutamate; Gln: glutamine; NAA: N-Acetyl-Aspartate; Cho: choline.

3.3.1.4. Choline. The choline levels were significantly different between groups with a median of 0.47 (0.47; 0.49), 0.48 (0.47; 0.48), 0.72 (0.68; 0.77), and 0.8 (0.73; 0.83) for the CTL, DIA, OH, and DOH groups respectively, $t(3) = 29.58$; $p < 0.001$. *Post-hoc* analyses found a higher level in the OH group compared to the CTL and DIA groups ($t(1) = 14.29$; $p < 0.001$ and $t(1) = 14.29$; $p = 0.001$, respectively) and a higher level in the DOH group compared to the CTL and DIA groups ($t(1) = 13.50$; $p = 0.001$ and $t(1) = 13.50$; $p = 0.001$, respectively) (Fig. 6).

3.3.2. Delayed impact on hippocampal metabolites three months after treatment cessation

The loss of half of the data from the OH group due to methodological issues led to the exclusion of the group from the statistical analyses. There was no significant difference between the CTL, DIA and DOH groups regarding the levels of the different metabolites three months after the long-term treatment cessation (Fig. 6).

4. Discussion

In this study, we aimed to evaluate the impact of chronic ethanol and diazepam intake in aging mice with the hypotheses of an additive effect of the combination of these two substances on cognition and hippocampal metabolites. We have chosen to focus on the hippocampal region because of its central role in learning and memory processes [38]. In parallel, we evaluated different types of hippocampal-dependent and -independent memories in order to evaluate the effect of alcohol and benzodiazepines on different memory functions sensitive to these two substances [38].

Overall, we found during treatment an impairment of working memory and visual recognition memory in the diazepam group, albeit non-significant for the spontaneous alternation test. MRS evaluation found variations in hippocampal metabolites levels with ethanol consumption, with no additive effect of diazepam intake. After treatment wash-out, there was no difference between groups concerning the different type of memories evaluated except for the learning phase of spatial reference memory with an impaired learning in the OH groups. A disappearance of the differences of metabolites levels in MRS was observed.

The increased anxiety-like behavior in mice in the ethanol group during treatment is consistent with those reported in other preclinical models [39] as well as in chronic alcohol consumers [40]. Indeed, if alcohol can initially be taken for anxiolytic purposes, we know that chronic consumption can cause or even increase anxiety [41] and/or depressive symptoms [42]. The absence of significant anxiety in the group diazepam + ethanol indicated that the association with diazepam may counteract the anxiety generated by ethanol. This is consistent with the clinical use of the anxiolytic drug diazepam in the prevention and treatment of alcohol withdrawal [28].

The cognitive assessment did not reveal a significative difference between the groups in working memory performances, thus the assumed additive effect on cognition during treatment was not observed. However, the results were close to the significance level ($p = 0.068$). Moreover, among the included mice, the diazepam and the diazepam + ethanol groups seemed to show the worst performance suggesting a diazepam dependent effect on working memory. Similarly, exclusion

rates were high in the visual recognition memory test with a significantly higher rate in the diazepam group (52.6 %). In this test, visual recognition memory performance was significantly different between groups. Although *post-hoc* analyses did not identify pair-wise differences, performance was worse in the diazepam group. Furthermore, the distribution of memory performance of diazepam-treated mice around a median of 0 (corresponding to the chance level) indicates impaired recognition memory abilities in this group. Taken together, these results could be in favor of an impact of diazepam on cognitive functions. The failure to detect a significant effect on spontaneous alternation test could reflect the behavioral variability in the task or a lack of sensitivity of the task itself to detect effects. Also, the high exclusion rates of the animals which predominates in the diazepam group may have limited the evidence of a treatment-related effect due to a lower statistical power. These data raise questions about the motivation of the mice, which were 15 months old at the time of the evaluation, to perform tests based solely on exploratory behavior, without external reinforcement. The high number of exclusions reported within the "spontaneous" cognitive tests, also observed in the control group around 20 % of the population, raises the problem of a variable level of motivation within the middle-aged mouse population. The most important exclusions were found in the diazepam group, which also raises the question of an increase of the lack of motivation by this molecule, the hypothesis of a sedative effect having been ruled out by the measure of spontaneous locomotor activity.

In MRS, significant differences in hippocampal metabolite levels between groups were observed after 8 weeks of treatment. These changes occurred in the ethanol and ethanol + diazepam groups since the metabolite levels in the diazepam group did not differ from the control group regardless of the metabolite analyzed. The evaluation of the glutamate-glutamine system showed a higher level of glutamate in both ethanol- and ethanol + diazepam treated groups associated with a lower level of glutamine only in the ethanol- group compared to the other groups. The increase in glutamate in the hippocampal region is consistent with previous preclinical data and is thought to reflect neuroadaptive changes occurring after chronic alcohol intoxication [43]. The lower level of glutamine, a precursor of glutamate and GABA, in the ethanol-only group in comparison to the other groups could result from an adaptive phenomenon secondary to the increase in glutamate that may be modulated by the combination with diazepam acting exclusively on the GABAergic system. The level of NAA, an indicator of neural integrity, lowered in the ethanol group compared to the diazepam group could reflect neuronal damage. Indeed, in the literature, the level of NAA is most often found stable or lowered during chronic ethanol consumption in rodents and humans [44,45]. The lack of a significant difference in NAA level in the diazepam + ethanol group compared to the other groups may be in favor of a protective effect of diazepam or an opposite effect on NAA level as reported with other psychotropic drugs, although this remains controversial [46]. Choline levels were significantly higher in the ethanol group, which was previously suspected to be related to an excessive membrane turnover or inflammation [47]. Our data are consistent with clinical studies finding a positive correlation between choline levels in the frontal cortex and alcohol consumption [48], and preclinical studies in rodents finding an increase in choline in the basal ganglia [47], thalamus [45] and an increase in phosphocholine in the hippocampus [43]. However, data in the literature regarding the variation in choline levels during ethanol intoxication are heterogeneous in rodents [33,43,49,50].

In the post-treatment evaluation, the greater number of rearing in the diazepam group may reflect a higher exploratory and locomotor activity. Also, the greater time spent in the central zone of the open-field test was in favor of a less anxiety-like or disturbed behavior. Such behavioral disorder raised the hypothesis of a benzodiazepine withdrawal syndrome. These results may be related to a too rapid discontinuation procedure despite our efforts to gradually reduce the doses of diazepam administered before its cessation. Regarding cognitive functions, the assessment of working memory and visual recognition did not show any difference between the groups after treatment cessation. Indeed, although exclusion rates were still relatively high within groups, the number of mice excluded in the diazepam group decreased drastically compared to mid-treatment assessment and memory performance was no longer different between groups. The acquisition phase in the Barnes maze showed a significant difference between the groups in learning. The impairment seemed to concern the ethanol-treated groups with longer acquisition latencies than the other groups, especially in the diazepam + ethanol group, suggesting a potential additive effect on learning. On the other hand, the evaluation of spatial reference memory did not show any difference between the groups. The use of more discriminating and rewarding tests, such as the Touchscreen test, a touchscreen-based automated system inspired from the human neuropsychological test automated battery (CANTAB) [51], could have provided more precise information about a mild cognitive impairment which could be suggested in our study in the ethanol groups by the differential learning in the Barnes maze. Adding tests assessing motivation could also be considered.

After 3 months treatment discontinuation, there was no difference in metabolites concentration levels between the control, diazepam and diazepam + ethanol groups. This results are in favor of a reversible impact of ethanol consumption under our environmental conditions, which is consistent with previous studies finding a disappearance of ethanol-related changes after a period of abstinence [52–54]. However, the loss of half of the data from the ethanol group due to poor spectral resolution during acquisition, leading to its exclusion from statistical analysis, limits data interpretation. Some limits inherent to the current MRS procedures also need to be acknowledged: (i) SRM acquisitions cannot distinguish between intracellular and extracellular metabolites in the acquired data, (ii) the use of isoflurane is a potential confounding factor, as isoflurane also acts on GABA receptors [55].

To our knowledge, our study is the first one to evaluate the combined effect of a long-term diazepam and ethanol intoxication in aging mice using both behavioral assessment and in vivo MRS in the hippocampal area. Overall, this work did not demonstrate an additive effect of long-term ethanol and diazepam intake on the different memories evaluated and in MRS spectroscopy during and after treatment cessation. We also found that the effects of diazepam or ethanol observed during treatment were no remanent after treatment cessation underlining the value of treatment discontinuation in clinical practice.

CRediT authorship contribution statement

Louise Carton: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Camille Landmann:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Florent Auger:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Nicolas Durieux:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis. **Charlotte Laloux:** Writing – review & editing, Supervision, Software, Methodology, Formal analysis. **Maéva Kyheng:** Validation, Software, Methodology, Formal analysis. **Maud Petrault:** Writing – review & editing, Supervision, Methodology, Investigation. **Kelly Timmerman:** Writing – review & editing, Supervision, Methodology, Investigation. **Camille Potey:** Writing – review & editing, Validation. **Sandrine Bergeron:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis. **Julie Deguil:** Writing – review & editing, Validation, Supervision, Software, Methodology, Formal analysis, Conceptualization. **Régis Bordet:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors have no conflict of interest to disclose.

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