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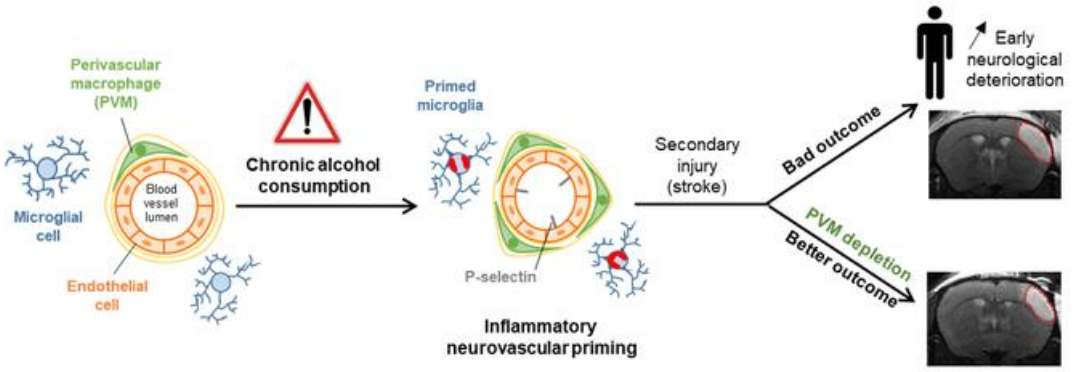
Graphical abstract



Alcohol exposure-induced neurovascular inflammatory priming impacts ischemic stroke and is linked with brain perivascular macrophages

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Running title: Neurovascular inflammatory priming by alcohol

Abstract

Alcohol abuse is a major public health problem worldwide causing a wide range of preventable morbidity and mortality. In this translational study, we show that heavy drinking (HD) (≥6 standard drinks/day) is independently associated to a worse outcome of ischemic stroke patients. To study the underlying mechanisms of this deleterious effect of HD, we performed an extensive analysis of the brain inflammatory responses of mice chronically exposed or not to 10% alcohol before and after ischemic stroke. Inflammatory responses were analyzed at the parenchymal, perivascular and vascular levels by using transcriptomic, immunohistochemical, in vivo two-photon microscopy and molecular MRI analyses. Alcohol-exposed mice show, in the absence of any other insult, a neurovascular inflammatory priming [i.e., an abnormal inflammatory status including an increase in brain perivascular macrophages (PVM)] associated to exacerbated inflammatory responses after a secondary insult (ischemic stroke or LPS challenge). Similar to our clinical data, alcohol-exposed mice showed larger ischemic lesions. We show here that PVM are key players on this aggravating effect of alcohol, since their specific depletion blocks the alcohol-induced aggravation of ischemic lesions. This study opens new therapeutic avenues aiming at blocking alcohol-induced exacerbation of the neurovascular inflammatory responses triggered after ischemic stroke.

Keywords: stroke, inflammatory priming, alcohol, *in vivo* imaging, perivascular macrophages

List of abbreviations used in the text:

NIHSS: National Institute of Health Stroke Scale

mRS: modified Rankin Scale

END: Early Neurological Deterioration

HD: Heavy Drinking

DSM: Diagnostic and Statistical Manual of Mental Disorders

AUD: Alcohol Use Disorder

AUDIT: Alcohol Use Disorders Identification Test

STAI: State-Trait Anxiety Inventory scale

BMI: Body Mass Index

CRP: C-reactive protein

ARRIVE: Animal Research: Reporting of In Vivo Experiments

BAL: Blood Alcohol Levels

MCA: Middle Cerebral Artery

MCAo: Middle Cerebral Artery occlusion

LPS: lipopolysaccharide

MPIO: Micro Particles of Iron Oxide

PMT: Photomultiplier

PVM: Perivascular Macrophages

OR: Odds Ratio

TOAST: Trial of ORG 10172 in Acute Stroke Treatment

TNF: Tumor Necrosis Factor

IL: Interleukin

Introduction

Alcohol abuse is a major public health problem worldwide causing a wide range of preventable morbidity and mortality. In the European Union 89% of men and 82% of women are current drinkers; among them, 15.3% of men and 3.4% of women are heavy drinkers (>6 drinks/day) (1). In the United States, excessive alcohol use is known to kill about 88,000 people each year, and the cost of excessive alcohol use reached $249 billion in 2010 (https://www.cdc.gov/features/costsofdrinking/). Alcohol modifies the risk of stroke: light and moderate alcohol consumption (0-2 drinks/day) are associated with a lower risk of ischemic stroke, whereas higher doses of alcohol are associated with an increased risk (2). Importantly, stroke risk associated with high and heavy drinking in midlife (<75 years) predominates over well-known stroke risk factors like hypertension and diabetes (3). However, the impact of alcohol consumption on stroke outcome is less known. Current clinical studies are controversial and have described either an aggravating effect (4) or no effect of heavy drinking (5) on stroke severity. Preclinical reports have also described either a protective effect of low alcohol consumption on ischemic stroke (6),(7), or larger infarcts in rodents exposed to higher alcohol dose (6),(8). However, the mechanisms mediating this aggravation are not well understood yet. Our previous results on the impact of alcohol consumption on ischemic stroke, obtained in a clinically relevant thromboembolic model of stroke (9),(10), have shown that the aggravating effect of excessive drinking is not due to alcohol-induced changes in hemodynamic parameters (clot formation, stability or sensitivity to fibrinolysis) (8). On the other hand, clinical and preclinical data have shown that alcohol consumption may have an impact on inflammation (11)–(13).

Perivascular macrophages (PVM) are a subpopulation of myeloid cells residing in the central nervous system (CNS). PVM surround brain blood vessels and are located at the perivascular space (14),(15). In physiological conditions, PVM have scavenger functions, revealing a role

of clearing debris from the CNS (16)–(18) and can also present antigens to lymphocytes (19). Recent data implicate PVM in several pathological contexts, including brain infections and immune activation, Alzheimer's disease or multiple sclerosis, suggesting that PVM are a key component of the brain-resident immune system with broad implications for the pathogenesis of major brain diseases (20). PVM can produce reactive oxygen species and cytokines, linking them to the inflammatory response (21). Due to the major role of inflammation in stroke pathobiology and outcome (22), in this translational study we aimed at investigating the role of inflammation in the aggravating effect of chronic alcohol drinking on ischemic stroke.

Results

Heavy drinking (HD) stroke patients show higher stroke baseline severity and worse neurological outcome

A total of 3.645 ischemic stroke patients were included in the retrospective analysis (Figure 1A). HD stroke patients (N=424, 11.6%) (drinking ≥6 drinks/day in the last 5 years) were significantly younger, and more frequently men, presented history of hypertension and higher smoking habits than non HD stroke patients. In addition, HD stroke patients had more severe ischemic strokes, with a higher infarct volume, higher mortality during hospitalization, worse prognosis at 3 months and presented higher levels of markers associated with the inflammatory response. Mortality during hospitalization was significantly increased in HD stroke patients (11.7% vs 9.7%, $p<0.0001$; Table 1).

The National Institute of Health Stroke Scale (NIHSS) scores at admission, 24 hours and 48 hours after stroke onset were significantly higher in HD stroke patients (Figure 1B, Table 1). Interestingly, significantly more HD stroke patients presented early neurological deterioration (END) -defined as the increase of NIHSS in ≥4 in the first 48 hours after admission- (19.2% vs 4.4%; Table 1, Figure 1B).

HD is independently associated with an increased risk of END

Based on the first univariate analysis, END was used as the main variable to analyze the cohort of stroke patients included in this study (Table 2). In this second univariate analysis, a total 3352 patients were included (3146 without END and 206 with END) (293 less patients than for the descriptive analysis since NIHSS data were not recorded or lost between the admission and the 48 hours). As expected, patients with END showed significantly higher hemorrhagic transformation, higher infarct volume and worse prognosis at 3 months. Moreover, patients with END presented higher inflammatory response at admission, characterized by higher

leukocyte numbers as well as C-reactive protein levels. Interestingly, significantly more END patients had HD habits (36.9% vs. 10.2%, p<0.0001; Table 2).

The inflammatory response is associated with the risk of END in HD stroke patients

We then performed multivariate analysis of END adjusted by HD (Model A, Figure 1C, Supplementary Table 1) or by HD and inflammatory markers (Model B, Figure 1D, and Supplementary Table 1). Model A showed that HD is independently associated with an increased risk of END (Odds Ratio (OR) =4.49; 95% CI: 2.94 - 6.86; p<0.0001) (Fig 1B, Supplementary table 1). When inflammatory markers [axillary temperature, leukocyte numbers and C-reactive protein (CRP)] were included in the analysis (Model B, Figure 1D, Supplementary Table 1) the impact of HD on the END OR decreased from 4.49 (obtained on the Model A) to 2.45 (OR=2.45; 95% CI: 1.01 – 5.91; p<0.0001), showing that the inflammatory response is associated with the risk of END in HD stroke patients. Age and gender do not have a relevant impact on END (Supplementary Table 2).

Finally, we studied the effect of HD on infarct volume. After adjusting for those variables that can interfere with the variable "infarct volume" (age, sex, axillary temperature, leukocyte levels, fibrinogen levels, C-reactive protein levels, tPA treatment, thrombectomy, hemorrhagic transformation, NIHSS on admission, END, TOAST (Trial of ORG 10172 in Acute Stroke Treatment) classification and HD; Supplementary Table 3), linear regression analysis of infarct volume showed that the predictor value of HD was 19.66 (B=19.66; 95% CI: 8.03-31.30; p<0.001; Supplementary Table 4).

HD patients without stroke show high levels of inflammatory markers

Data from the independent cohort of HD patients without stroke showed that the levels of circulating monocytes are significantly increased compared to control healthy subjects (0.73 ±

0.27 vs. 0.2 ± 0. 17 respectively, p=0.003) (Table 3). In addition to this, 61.8% of HD showed increased high sensitivity-CRP (hs-CRP) levels (Table 3).

Alcohol exposure induces a neurovascular inflammatory priming in mice

The first set of experiments aimed to analyze the inflammatory status in the brain of mice drinking 10% alcohol for 6 weeks (~5 g/kg/day), in the absence of any other insult (Figure 2A). To study microglial cells, brain samples were stained with Iba1, a constitutive marker of microglial cells, and with the lysosomal marker CD68, present in activated microglial cells (23). We found that whereas the total number of activated microglial ($Iba1^+CD68^+$) cells remained unchanged (Figure 2B-D), the total area of $CD68^+$ staining was significantly increased in alcohol-exposed mice ($p<0.05$ vs. control; Figure 2C, Figure 2E), suggestive of an alcohol-induced increase in microglial activation. Alcohol exposure did not change microglial cell numbers (total number of $Iba1^+$ cells) (Figure 2B, Figure 2F; N=4 mice/group). In terms of microglial morphology, alcohol exposure did not change neither the number of main processes (starting from the soma) (Figure 2C, Figure 2G) nor the mean area of microglial cells (Figure 2C, Figure 2H).

The alcohol-induced increase in microglial activation was demonstrated by an *in vivo* functional measurement of microglial phagocytosis after the injection of nonionic latex beads in the brain cortex of control and alcohol-exposed mice (Figure 2I-L). Eight hours after the intracortical injection of the beads, alcohol-exposed mice showed significantly more phagocyted beads (surrounded by Iba1 positive staining) than control mice (Figure 2I-2K; N=4 mice/group; $p<0.05$ vs. control), demonstrating that microglia in alcohol exposed mice is more prone for phagocytosis. These results show an alcohol-induced microglial priming characterized by a morphological “resting” state accompanied however by an increase in both microglial activation status and phagocytic capacity.

In this study, we also wanted to examine potential inflammatory effects of alcohol exposure on the brain vasculature (Figure 3A, Figure 3B). For this reason, we analyzed the endothelial levels of the adhesion molecule P-selectin (CD62P marker), responsible of leukocyte tethering and rolling on the vessel wall. At the vascular level, alcohol-exposed mice showed significantly increased P-selectin positive blood vessels (+152% increase; 1.03±0.31 $CD62P^+$ blood vessels/mm^2; Figure 3B, N=4 mice/group; $p<0.05$ vs. control), whereas in control mice very low levels of P-selectin (0.31±0.11 $CD62P^+$ blood vessels/mm^2) were detected, demonstrating an alcohol-induced endothelial activation in the absence of any other brain injury.

To determine the functional impact of the increase in P-selectin expression, we performed *in vivo* two-photon microscopy analyses to measure venular leukocyte adhesion and rolling in mice exposed or not to alcohol, after the intravenous injection of FITC-Dextran (allowing blood vessels visualization) and Rhodamine-6G (staining leukocytes). Alcohol-exposed mice showed significantly more adherent (Figure 3C, Figure 3D) and rolling (Figure 3E, Figure 3F) leukocytes compared to control mice (+317% and +724% increase respectively; N=6 mice/group; $p<0.05$ vs. control).

Concerning mRNA expression of inflammatory markers, alcohol-exposed mice showed a significant increase in TGFβ mRNA levels ($p<0.05$, N=6 mice/group) compared to control mice, that was not accompanied by changes in pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF), IL-6, P-Selectin, TLR4, or VCAM1 (Table 4).

We also studied blood-brain barrier (BBB) integrity after alcohol exposure by using three different methods. Our results showed that chronic alcohol exposure does not seem to alter BBB integrity, since neither Evan's Blue extravasation measured by near-infrared imaging (NIRF) (Supplementary Figure 1A, 1B, N=4 mice/group), positive signals of gadolinium extravasation measured by T1-w MRI (Supplementary Figure 1E, 1F, N=4 mice/group) or

fibrin(ogen) deposits (Supplementary Figure 1I, N=4 mice/group) were found in alcohol-exposed mice.

Concerning the potential alcohol-induced neuronal death, we did not find any fluorojade C positive staining in alcohol-exposed mice (Supplementary Figure 1B, N=4 mice/group).

Alcohol exposure exacerbates brain neurovascular inflammatory reactions after an acute systemic insult

Alcohol-exposed mice showed a global exacerbated inflammatory response in the brain 24 hours after an acute intraperitoneal injection of LPS compared to control mice. At the parenchymal level, alcohol-exposed mice receiving LPS showed a significant increase in (i) total microglial cells ($Iba1^+$ cells) (Figure 4B, Figure 4C; N=5 mice/group; $p<0.05$ vs. control), (ii) activated microglia ($Iba1^+CD68^+$ cells) (Figure 4B, Figure 4D; N=5 mice/group; $p<0.05$ vs. control) and (iii) $CD68^+Iba1^-$ cells that we considered as macrophages (Figure 4B, Figure 4E) compared to LPS-injected control mice.

At the vascular level, P-selectin immunostaining (Figure 4F, Figure 4G) was significantly increased in alcohol-exposed mice receiving LPS compared to control mice (N=5 mice/group; $p<0.05$ vs control). Molecular MRI also showed significantly increased number of P-selectin-coupled $MPIO^+$ blood vessels in alcohol-exposed mice receiving LPS (Figure 4H, Figure 4I; N=5 mice/group; $p<0.05$ vs control). Consequently, venular leukocyte adhesion (Figure 4J, Figure 4K) and rolling (Figure 4L, Figure 4M), measured by intravital two-photon microscopy, were significantly increased in alcohol-exposed mice receiving LPS compared to control mice (N=5 mice/group; $p<0.05$ vs control).

Alcohol exposure aggravates stroke lesions and exacerbates neurovascular inflammatory responses after ischemic stroke in mice.

Alcohol-exposed mice showed significantly larger ischemic lesions than control mice (drinking only water) 24 hours after stroke onset (Figure 5A-C; N=8 mice/group; $p<0.05$ vs control, (8)). These data on lesion volume are part of a previously published figure (8). No hemorrhagic transformation was detected in any mice (data not shown).

Microglial/macrophage reaction after stroke (Figure 5D-K) was exacerbated in the ipsilateral cortex of alcohol-exposed mice, with significant increases in (i) the total number of $Iba1^+$ cells in the peri-infarct area (Figure 5F; N=3 mice/group; $p<0.05$ vs control), (ii) the number of $CD68^+/Iba1^+$ cells in the peri-infarct area and the ischemic core (Figure 5G; N=3 mice/group; $p<0.05$ vs control), and (iii) the area of lysosomal CD68 staining in the ipsilateral hemisphere of alcohol-exposed mice compared to control (Figure 5H; N=3 mice/group; $p<0.05$ vs control). The number of processes starting from the soma was significantly decreased in the ipsilateral cortex of alcohol-exposed mice compared to control mice (Figure 5I; N=3 mice/group; $p<0.05$ vs control), suggestive of an increased microglial phagocytic phenotype induced by alcohol exposure. Whole cell area of $Iba1^+$ cells remained unchanged between groups (Figure 5J; N=3 mice/group). The number of $CD68^+Iba1^-$ cells was significantly increased in the peri-infarct area of alcohol-exposed mice 24 hours after stroke onset (Figure 5K; N=3 mice/group; $p<0.05$ vs control).

Vascular activation was exacerbated in alcohol-exposed mice after stroke (Figure 6A-F). The area of P-selectin in blood vessels was significantly increased in alcohol-exposed mice 24 hours after stroke onset (Figure 6C; N=3 mice/group; $p<0.05$ vs control), although the number of P-$selectin^+$ blood vessels showed no changes between groups (Figure 6B). Similarly, molecular MRI (Figure 6D) showed significantly increased hyposignals corresponding to P-selectin-coupled MPIOs adhering to blood vessels in the ipsilateral cortex of alcohol-exposed mice 24 hours after stroke onset (Figure 6E; N=5-6 mice/group; $p<0.05$ vs control), with no changes in the total number of P-selectin MPIO positive blood vessels (Figure 6F).

Venular leukocyte adhesion (Figure 6H, Figure 6I) and rolling (Figure 6J, Figure 6K) were both significantly increased in alcohol-exposed mice compared to control mice (+317% and +724% increase respectively; N=6 mice/group; $p<0.05$ vs control) 24 hours after stroke. Schemas in Figure 6G show the relative position of the MCA occlusion site and the thinned-skull cranial window were *in vivo* two-photon imaging was performed.

mRNA levels of IL1β, P-selectin and TNF were significantly increased in alcohol-exposed mice in the ipsilateral cortex 24 hours after stroke onset compared to control mice ($p<0.05$, N=6 mice/group; Table 4).

Perivascular macrophage (PVM) depletion prevents the aggravating effect of chronic alcohol consumption on ischemic stroke

In addition to the neurovascular priming profile found in alcohol-exposed mice, we also detected a significant increase in the number of brain perivascular macrophages (PVM) (Figure 7A-C; N=4 mice/group; $p<0.05$ vs control). PVM are a sub-population of resident brain macrophages located at the perivascular space (Supplementary Figures 2A-E). In this study, we have measured only sub-meningeal signals on IHC and *in vivo* imaging experiments to focus on PVM and no other border-associated macrophages. In addition to their specific shape and location surrounding brain blood vessels (Supplementary Figure 2), in naïve conditions PVM can be distinguished from microglia since they are very low positive for Iba1. PVM can be stained with both CD68 and CD206 (Supplementary Figure 2, Figure 7B, 7C). Concerning the origin of the increased number of PVM after alcohol exposure, the longitudinal study of peripheral macrophage accumulation in the brain suggest the absence of a marked peripheral PVM recruitment (Supplementary Figure 3G). Proliferation measurements by Ki67 staining were performed in harvested brains at the end of the alcohol exposure, and have not shown

positive signals of PVM proliferation (Supplementary Figure 3H), perhaps because the measurements have been done at one single time point at the end of the alcohol exposure period.

To determine the specific role of brain PVM on ischemic stroke outcome in both healthy (naïve) and primed mice (chronically exposed to alcohol), we depleted PVM by injecting clodronate (CLO)-encapsulated liposomes to mice exposed or not to 10% ethanol for 6 weeks (see schema in Figure5G) (control mice received PBS-encapsulated liposomes). In the adult brain, the intracerebroventricular (icv) injection of CLO depletes PVM (as well as meningeal macrophages) without affecting microglial cells or peripheral mononuclear cells (21) except for a transient reduction in the number of Kupffer cells in the liver (24). In our study, we have observed that the icv injection of CLO efficiently depletes PVM, but also provokes a series of inflammatory responses *per se*. Our data showed that clodronate acutely induced *per se* an inflammatory response in the ventricle area at 2 days post-injection, which was not visible any more at 6 days by molecular MRI imaging (p-selectin coupled MPIOs) (Supplementary Figure 4A, B; n=4 mice/group). Our IHC data, focused on the injection site (the ventricle area), confirmed the data obtained by MRI and showed the absence of P-selectin positive staining (Supplementary Figure 4C), as well as no neutrophil infiltration ($Ly6G^+$ staining) at that time (Supplementary Figure 4D). Microglia was not affected by the injection of clodronate (Supplementary Figure 4D; n=4 mice/group).

Flow cytometry experiments were performed at 5 days post-injection, to respect the same timing profile than the one used on the stroke study. Our data show that there were no differences in the numbers of $CD45^{low}/CD11b^+$ cells (considered as resting microglia; Supplementary Figure 5B), $CD45^{high}/CD11b^+$ cells (considered as infiltrated macrophages; Supplementary Figure 5C), $CD11c^+/CD11b^+$ cells (considered as dentritic cells; Supplementary Figure 5E) or $Ly6G^+$ cells (considered as granulocytes; Supplementary Figure 5F) between PBS and CLO treated mice. However, the numbers of $CD45^{int}/Cd11b^+$ cells (considered as activated

microglia; Supplementary Figure 5D), $CD3^+$ (considered as total lymphocytes; Supplementary Figure 5G), as well as $CD4^+$ (Supplementary Figure 5H) and $CD8^+$ (Supplementary Figure 5I) lymphocytes were increased in clodronate-treated mice 5 days after CLO injection.

The depletion of PVM was *in vivo* confirmed 24 hours after the icv injection of TRITC-dextran (Figure 7E-J). To assess the harmlessness of CLO injection on microglial cell numbers, we used CX3CR1-GFP+/- mice in which TRITC-dextran-positive PVM can be distinguished *in vivo* from GFP-positive microglial cells, since PVM are very low positive for GFP (Figures 7E, 7G). PVM depletion did not alter microglial cell numbers (GFP+ cells, Figure 7J). The phagocytosis of TRITC-dextran by PVM was confirmed by immunohistochemistry: the TRITC dextran-positive cells were co-stained with CD206 (Figure 7F). PVM depletion was confirmed *in vivo* by 2-photon imaging (Figures 7E, 7H, $p<0.01$ vs PBS) and by IHC analyses (CD206 staining; Figures 7G, 7I, $p<0.01$ vs PBS).

The specific depletion of PVM by CLO prevented the aggravating effect of chronic alcohol exposure on ischemic lesions (Figure 7K, 7L; $p<0.05$ vs PBS; N=5-6 mice/group), whereas CLO treatment did not modify ischemic lesions in control mice (not exposed to alcohol) (Figure 7K, 7L; N=5-6 mice/group).

In order to explore the mechanisms of the beneficial effect of PVM depletion on stroke in alcohol-exposed mice, we studied the immune responses triggered 24 hours after stroke (N=4 mice/group). We analyzed microglial/macrophage cell numbers ($Iba1^+$ staining, Figures 8A, 8B) and neutrophil infiltration (Figures 8C, 8D) in PBS- and CLO-injected mice (PVM-depleted) exposed or not to alcohol. Microglial/macrophage numbers (Figure 8B), P-selectin protein levels (Figure 8E, Figure 8F) and VCAM-1 protein levels (Figure 8G, Figure 8H) were significantly increased after stroke in PBS-alcohol-exposed mice compared to PBS-control mice (Figures 8A-B; $p<0.05$ vs PBS-treated control mice; N=4 mice/group).

The effects of PVM depletion were different between control and alcohol exposed mice. PVM-depleted control mice showed significantly more microglia/macrophages (Figure 8B, $p<0.05$ vs PBS control; N=4 mice/group), as well as more neutrophils (Figure 8D, $p<0.05$ vs naïve PBS; N=4 mice/group) and increased VCAM-1 protein levels (Figure 8H, $p<0.05$ vs naïve PBS; N=4 mice/group) in the ipsilateral hemisphere compared to PBS-treated control mice. By contrast, in PVM-depleted alcohol-exposed mice microglia/macrophage (Figure 8B) and neutrophil (Figure 8D) infiltration, as well as VCAM levels (Figure 8H) remained unchanged. We only found a significant decrease in P-selectin protein levels between PBS and CLO-treated alcohol-exposed mice (Figure 8E, Figure 8F, $p<0.05$ vs PBS alcohol-exposed mice; N=4 mice/group).

Interestingly, by intravital 2-photon microscopy we observed that the number of adherent leukocytes was significantly decreased in PVM-depleted mice, independently of alcohol exposure (Figures 8I, 8J, 8L, 8M, $p<0.05$ vs PBS-treated mice; N=4 mice/group). This effect was specific of leukocyte adhesion, since no difference was detected in the number of rolling or circulating leukocytes in any of the groups (Figures 8K, 8N).

Discussion

We have performed here a translational study to investigate the effects of chronic alcohol consumption on ischemic stroke outcome. Our clinical results show that heavy drinking (≥6 drinks/day in the last 5 years) is independently associated (i) to early neurological deterioration (END, defined as the increase of NIHSS in ≥4 in the first 48 hours after admission), (ii) to stroke severity baseline, and (iii) to higher infarct volume in stroke patients. The baseline characteristics of the study population, and outcomes at 3 months, were similar to those of large multicenter registries, suggesting good external validity of our results (25).

Our results show that the inflammatory response is associated with the risk of END in heavy drinking (HD) stroke patients. In order to elucidate the role of inflammation on the deleterious effect of heavy drinking after stroke, we performed pre-clinical experiments in mice exposed to 10% ethanol in drinking water during 6 weeks. Our pre-clinical findings show that chronic, alcohol consumption by itself provokes a neurovascular inflammatory priming. The term "priming" is used to describe the propensity of a particular cell type to make an exaggerated response to a secondary stimulus (26) such as intracerebral or systemic LPS injection (27). Microglial priming was first described in the ME7 model of prion disease (28) but has been replicated in other models of chronic neuroinflammatory pathologies including Alzheimer's disease (29) and Parkinson's disease (30).

To our knowledge, this is the first study defining a neurovascular inflammatory priming induced by chronic alcohol consumption. This alcohol-driven priming affects the brain parenchyma as well as the perivascular and vascular compartments. In the parenchyma, it is characterized by (i) an unconventional activation profile of microglial cells, in accordance with results described by Cruz et al. (31); and (ii) an increase in the expression of TGFβ mRNA not accompanied by changes in pro-inflammatory cytokines, thus following a similar profile as in

priming-driving diseases such as prion disease (32). At the brain perivascular compartment, alcohol provokes an increase in the number of perivascular macrophages (PVM). Finally, at the vascular level, alcohol-induced priming is characterized by increased levels of P-selectin at the endothelial surface, accompanied by an increase in the number of adherent and rolling leukocytes in the brain blood vessels.

We demonstrate here that alcohol provokes a brain inflammatory priming in mice by using two different approaches. First, we intracortically injected inert latex beads to control and alcohol-exposed mice. Eight hours after the injection, alcohol-exposed mice showed significantly more phagocyted beads than control mice, thus demonstrating that chronic alcohol exposure makes microglia more prone to phagocytosis. Second, we systemically injected a single dose of LPS to control and alcohol-exposed mice and studied the subsequent parenchymal, perivascular and vascular inflammatory responses 24 hours later. In accordance with our priming hypothesis, mice exposed to alcohol showed increased total microglial and activated microglial cell numbers, increased levels of P-selectin in the brain vasculature, characteristic of increased endothelial activation, as well as significantly increased rolling and adhering leukocyte numbers after the injection of LPS. Previous studies have reported that the exposure of C57BL/6 mice to 10 daily doses of ethanol followed by a LPS or Poly I:C challenge resulted in a sustained increase of proinflammatory cytokines in the brain compared with control-challenged animals (33),(34).

In accordance with our inflammatory priming hypothesis, our data show that inflammatory responses at the parenchymal, perivascular and vascular levels are exacerbated in alcohol-exposed mice also after stroke. More precisely, microglial numbers and phagocytic capacity, brain perivascular macrophages (PVM)/infiltrated macrophages numbers, P-selectin levels at the brain endothelium, as well as leukocyte rolling and adhesion to brain vasculature are all exacerbated in alcohol-exposed mice after stroke. Inflammatory responses participate in the

progression of ischemic lesions (35), but the causality or consequence relationship between the exacerbation of inflammatory responses and the increased lesion volume is difficult to determine. For this reason, our data on the inflammatory status prior to the ischemic injury are crucial and show that, even in the absence of stroke, heavy drinking alters the inflammatory status in both humans and mice. It has been proposed that inflammatory priming could have significant implications for acute sterile inflammatory insults such as stroke and traumatic brain injury occurring on a background of aging or neurodegeneration (26). Our data show that chronic heavy drinking triggers a similar generalized heightened inflammatory sensitivity than in the aforementioned models of neurodegenerative diseases, and that alcohol-induced inflammatory priming has extremely deleterious consequences in stroke, not only in mice, but also in humans.

Importantly, we show here that PVM are determinant for the exacerbation of ischemic lesions, since their specific depletion by clodronate blocks the aggravating effect of chronic alcohol exposure on stroke. Interestingly, the beneficial effect of PVM depletion on lesion volume is exclusive of alcohol-exposed mice, in which PVM numbers are significantly increased. Indeed, PVM accumulation at the perivascular space seems to be deleterious for ischemic stroke outcome, as it has been described in other neurological pathologies such as multiple sclerosis or Alzheimer's disease (for review, see (20)). It is also possible that alcohol exposure provokes not only an increase in PVM numbers, but also a shift of PVM phenotype and function from a scavenger, "buffer" cell (16),(17) to a pro-inflammatory, ROS-producing cell in "primed" conditions (36). In accordance to this hypothesis, it has recently been described that homeostatic subsets of CNS endogenous tissue macrophages are able to quickly change their phenotypes and generate context- and time-dependent subsets (37). An additional question that deserves future studies is whether PVM could drive microglial priming during chronic alcohol exposure,

or, instead, if microglial cells are responsible for the accumulation of PVM at the perivascular spaces and the activation of brain endothelial cells.

Concerning the hypothesis of a role of PVM on mediating the stroke-induced inflammatory responses, our data show that, in naïve mice, PVM do modulate stroke-induced inflammatory responses such as microglial activation/macrophage infiltration, neutrophil infiltration, endothelial activation and leukocyte rolling/adhesion *in vivo*. It is important to take into account the effects of CLO *per se* found in our study, especially concerning lymphoid cells, which could also modify the post-stroke inflammatory responses. Surprisingly, these altered inflammatory responses are not associated with changes in the final lesion volume between PBS and CLO-treated naïve mice. These data are in accordance with the results reported by Pedragosa et al. (2018) (38), in which modifications of granulocyte infiltration in PVM-depleted mice are observed, but are not accompanied by changes in final lesion volume in a model of ischemia/reperfusion. These results agree with previous studies showing no impact of leukocyte recruitment on ischemic stroke volume (39),(40). Further studies are thus needed to better understand the links between final lesion volume and leukocyte infiltration at the ischemic area.

Our data suggest that the previous inflammatory status is critical for the ischemic stroke outcome. In the context of alcohol-induced inflammatory priming, PVM are linked to the aggravating effects of alcohol on stroke lesion volumes, since PVM-depleted mice show smaller lesion volumes. This aggravating effect of alcohol in lesion volume is associated to an increased inflammatory response in which PVM seem to play a critical role, since PVM-depleted mice show an attenuated inflammatory response compared to alcohol-exposed mice with intact PVM. One possible hypothesis to explain this differential role of PVM upon naïve conditions and alcohol exposure could be a phenotypic change in PVM during alcohol exposure, as it has been described for other neuroinflammatory conditions (37), that could be crucial in case of a secondary CNS injury such as ischemic stroke.

Although research on PVM has intensified in recent times, we still have many unanswered questions concerning PVM phenotype and origin in pathological contexts. Also, since CLO depletes both meningeal macrophages and PVM, the observed effects cannot be attributed exclusively to PVM. In any case, PVM seem to be potential therapeutic targets for limiting the aggravating effects of risk factors on stroke outcome.

A potential limitation of this study is that we cannot exclude an impact of alcohol withdrawal on the outcome worsening of HD stroke patients and mice. Alcohol-withdrawn neurons are more sensitive to excitotoxic injuries (41) characteristic of early phases of ischemic stroke. Indeed, we cannot exclude that this mechanism also participates to the aggravation of stroke lesions observed in our study, making the exacerbated inflammatory responses observed after stroke the consequence and not the cause of the aggravated stroke outcome. However, our preclinical data on intracortical latex beads-injected mice and on systemic LPS-injected mice, which were performed in mice not subjected to alcohol withdrawal, demonstrate that the exacerbated inflammatory response to both latex beads and acute LPS is present even in the absence of alcohol withdrawal. Other mechanisms that could contribute to the aggravation of stroke severity could be an endothelial dysfunction induced by chronic alcohol consumption (42),(43), which could have an impact by itself on microglial priming and PVM accumulation.

Interestingly, our new data and the recently published data on the beneficial effect of low-dose alcohol consumption on inflammation following transient focal cerebral ischemia in rats (7) suggest that, similarly to the biphasic effects of alcohol on the risk of ischemic stroke (2), alcohol consumption also could have a biphasic effect on the consequences of ischemic stroke. These data thus help to better understand the apparently controversial clinical results found on the impact of alcohol consumption on stroke (4),(5).

In conclusion, we show that chronic alcohol consumption provokes by itself a neurovascular inflammatory priming in mice and inflammatory responses in HD patients. We experimentally

demonstrate that this priming, involving notably PVM, drives the exacerbation of the damages provoked by a secondary insult such as an ischemic stroke. Our study opens new avenues for the study of strategies targeting the alcohol-induced inflammatory priming prior to any neurological insult, in order to prevent a worsened neurological outcome in heavy drinkers.

Materials and methods

Stroke cohort study population and patient characteristics

We retrospectively analyzed a cohort of 3.645 ischemic stroke patients from the stroke registry of the Stroke Unit of the Neurology Department of the University Clinical Hospital of Santiago de Compostela (Spain) included from January 2010 to December 2016. Acute management (diagnostic and treatment) of patients with stroke was performed according the protocol described by the European Stroke Organization.

Stroke outcome variables

To evaluate the influence of chronic alcohol consumption on the outcome of stroke patients, the following primary outcomes variables were considered: i) neurological stroke severity determined by the National Institute of Health Stroke Scale (NIHSS) at admission, 24 and 48 hours, ii) early neurological deterioration (END) defined as increase of NIHSS in ≥4 in the first 48 hours after admission, iii) infarct volume determined by TC scan between the 4th and 7th day after admission, iv) degree of disability at 3 months assessed by modified Rankin Scale (mRS), and v) percentage of patients with good outcome at 3 months (mRS ≤ 2).

To determine the association between chronic alcohol consumption and inflammatory response, the following biological variables were included in the analysis: 1) leukocyte numbers, 2) fibrinogen, 3) C-reactive protein and 4) sedimentation rate. Axillary temperature >37.5 ºC at admission was also considered as a marker of inflammation.

History of arterial hypertension was considered when the blood pressure was >140/90 mmHg at least two different days before stroke onset, if the patient was diagnosed for hypertension or when the patient was under antihypertensive treatment. History of diabetes disease was defined as serum glucose levels ≥7.0 mmol/L, if the patient was diagnosed of diabetes or when the patients was under diabetic medication. Smoking patients were defined as those patients who

presented smoking habits in the last 5 years. Heavy drinking (HD) habits were defined as a daily alcohol consumption ≥6 drinks/day in the last 5 years.

Stroke cohort statistical analyses are detailed in the Supplementary materials and methods section.

Heavy drinking (HD) patients without stroke

We retrospectively analyzed data from an independent cohort including 34 patients with a DSM-V (Diagnostic and Statistical Manual of Mental Disorders - 5th edition) diagnostic of severe Alcohol Use Disorder (AUD) (heavy drinking patients, HD) and 21 healthy control subjects. All the participants were informed about the study, approved by the local ethics committee (CPP Nord Ouest III, no. IDRCB: 2011-A00495-36), and provided their written informed consent before their inclusion.

Blood samples were collected from fasted participants, either at inclusion (control subjects) or the day after admission to hospital (HD patients). Immune cell counts (leukocytes, neutrophils, eosinophils, basophils, lymphocytes and monocytes) were measured in all participants. C-reactive protein (CRP) levels were measured only in HD patients.

Additional information about this cohort and statistical analyses are detailed in the Supplementary materials and methods section.

Experimental study design

The goal of this study was to investigate the impact of heavy drinking on stroke outcome and describe the underlying mechanisms in an experimental model of ischemic stroke in mice.

Animals were randomized to treatment groups, and all analyses were performed by investigators blinded to group allocation. Unblinding was performed after completion of statistical analysis. All animal experiments were performed and reported in accordance with the

Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (http://www.nc3rs.org.uk).

Animals

Two months-old male Swiss mice (35-45g) (Centre Universitaire de Ressources Biologiques, Normandy University, Caen, France) were housed at 21° C in a 12 h light/dark cycle with food and water (control group) or a 10% (v/v) alcohol solution (alcohol group) with *ad libitum* free access for 6 weeks. All mice were checked daily for fluid consumption, health and abnormal behavior. The average daily liquid intake and weight gain were similar between both groups (~6 ml of liquid intake/mouse/day and a weight gain of ~6g between the beginning and the end of the alcohol exposure period (final weight ~40g in both groups). Blood alcohol levels (BAL) were measured at the pharmacology unit of Caen University Hospital in mice at the end of the 6 weeks of alcohol exposure (N=10 mice). At the moment of the blood extraction, only 3 out of 10 mice showed positive BAL values (0.39, 0.54 and 0.63 g/L), the rest of the mice showed non-detectable values (<0.1 g/L).

For intravital two-photon imaging of perivascular macrophages (PVM), male C57/BL6J CX3CR1-GFP$^{+/-}$ mice were used (N=6 mice/group).

All the procedures needing anesthesia of the mice were performed by an initial exposure to 5% isoflurane followed by a maintaining phase of 1.5-2% isoflurane 30%O_2/70%N_2O.

Thromboembolic Focal Cerebral Ischemia

We used the *in situ* thromboembolic stroke model consisting in the injection of thrombin directly into the middle cerebral artery as described before (5). In order to mimic clinical conditions, alcohol solutions were changed by water after stroke onset and until killing. Additional information is provided in the Supplementary materials and methods section.

Intraperitoneal lipopolysaccharide (LPS) injection

A subset of control and alcohol-exposed mice (N=5-6 mice per group) were intraperitoneally injected with a single dose of the bacterial endotoxin LPS (1 mg/kg) (Sigma-Aldrich, France) (6) and underwent two-photon imaging and molecular MRI 24 hours after the injection of LPS. Alcohol-exposed mice kept free access to the alcohol solution after LPS injection and until killing.

***In vivo* microglial phagocytic capacity measurement**

The protocol consisted in the injection of 1 µl of nonionic latex beads in the brain cortex and was modified from Hughes et al. (7). Details are provided in the supplementary materials and methods section.

Vascular adhesion molecular imaging

Micro-sized particles of iron oxide (MPIOs) (diameter 1.08 mm) (Invitrogen) covalently conjugated to purified polyclonal goat anti-mouse antibodies for P-selectin (R&D Systems, clone AF737) were prepared as previously described (8). Details are provided in the supplementary materials and methods section.

In vivo detection of blood-brain barrier (BBB) leakage

Three dimensional T1 FLASH sequences (spatial resolution 70mm*70mm; TE/TR 4.46/15; 3 averages; 4 min 2 sec) were used before and 15 min after the iv injection of 200µl of a solution containing 50µl of Gadolinium chelate (DOTAREM) diluted in saline, as previously described (9). Additional information is provided in the supplementary materials and methods section.

Near-infrared detection of BBB leakage

NIRF imaging experiments were performed using a PhotonIMAGER (Biospace, Paris, France), as previously described (10). Additional information is provided in the supplementary materials and methods section.

Depletion of perivascular macrophages (PVM)

Ten µl of PBS-liposomes (PBS group) or clodronate-encapsulated liposomes (CLO group) were injected in the left lateral ventricle. Additional information is provided in the supplementary materials and methods section. In order to minimize the pro-inflammatory effects of CLO *per se*, the icv injection of CLO was performed 5 days before the stroke.

***In vivo* macrophage labelling for the follow-up study of macrophage accumulation in the brain**

Two hundred microliters of 2 mg Fe/kg of nude Micro-sized Particles of Iron Oxide (MPIOs) were injected iv to naïve mice and T2*-w acquisitions were performed at different times after its injection to detect peripheral macrophage accumulation at the brain perivascular spaces. Additional details are provided in the supplementary materials and methods section.

Intravital two-photon microscopy

Intravital two-photon microscopy was performed through a thin-skull cranial window to measure leukocyte rolling and adhesion as well as PVM visualization. Details are provided in the supplementary materials and methods section.

Flow cytometry analysis

Five days after the icv injection of PBS or CLO, mice were deeply anesthetized with isoflurane 5% and intracardially perfused with 1X PBS (Sigma-Aldrich). Cell isolation from brains (without the cerebellum) and flow cytometry acquisition were performed as previously described (51) to obtain two separate panels for myeloid cells or lymphoid cells (see Supplementary table 6) . Data were analyzed with the FlowJo 7.6.5 software (TreeStar Inc.). Data are expressed as total cell count for each sample.

Quantitative PCR analyses

Details on the methods for quantitative PCR analyses are provided in the supplementary materials and methods section.

Immunohistochemistry

Epifluorescence and confocal microscopy analyses were performed in brain sections and isolated brain vessels. Details on the protocol and methods are provided in the supplementary materials and methods section.

Statistical analyses on pre-clinical data

Results are the mean ± SEM. Statistical analyses were performed by the Mann-Whitney U test using the Statview software.

Study approval

Stroke patient registry was approved by the Ethics Committee of Galicia (CEIC) (Spain). Signed informed consent was obtained from patients or a relative before study inclusion.

Experimental studies were approved by the French ministry of education and research (Project #3748; Center agreement #D14118001).

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Author contributions

- Study design, coordination of the study: MR
- Stroke model surgeries: MR
- Histological and transcriptional analyses, *in vivo* functional analyses of microglial phagocytosis: AD, DL
- Molecular MRI: AD, DL, AQ and EL
- Two-photon microscopy: AD and MR
- Analyses of immunohistochemical samples: AD, DL and MN
- Flow cytometry analyses: MG, MR
- Analyses of data from the ALCOBRAIN cohort of HD patients: AL and ALP
- Analyses of data and writing of results from the stroke cohort of patients: FC and JC
- Supervision of the study: DV

- Manuscript writing: MR with the revision and approval of all authors

Conflict of interest

The authors have declared that no conflict of interest exists.

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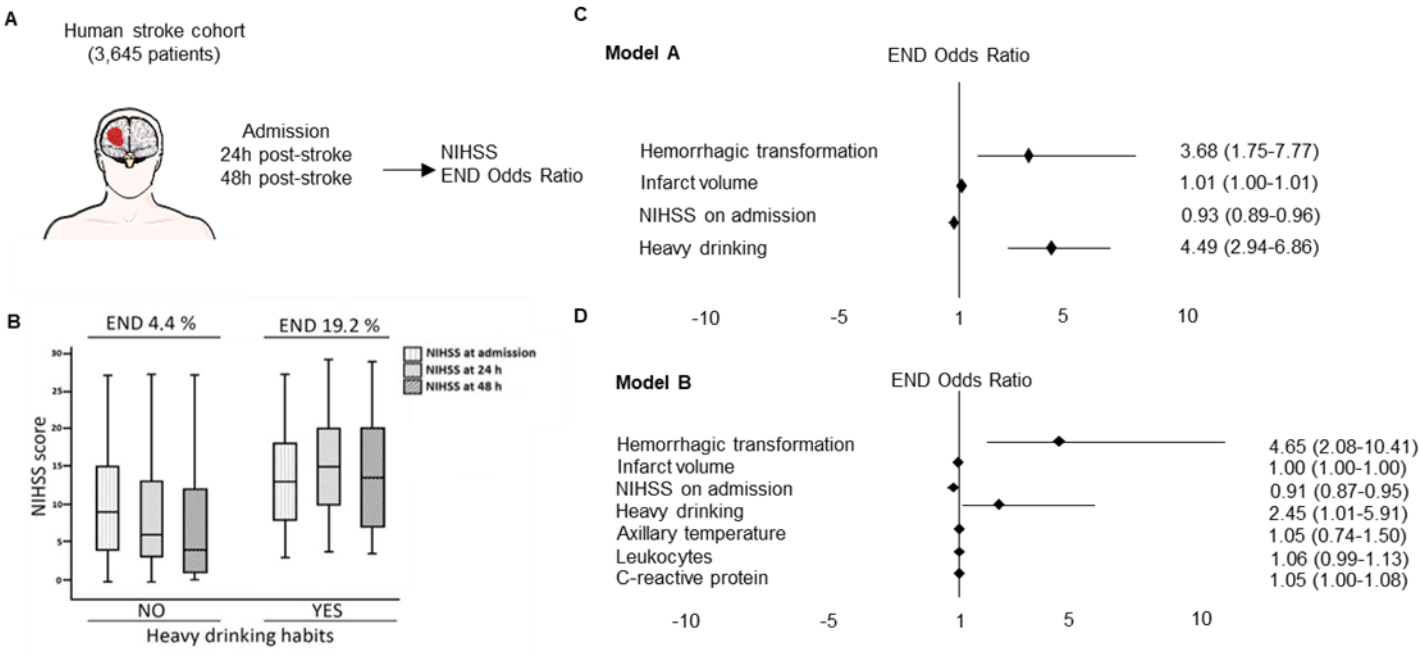

Figure 1. Heavy drinking (HD) aggravates ischemic stroke baseline severity and outcome, and increases early neurological deterioration (END) risk. Inflammatory markers are independently associated to the alcohol-induced increased risk of END. A) Schema of the study. B) Representation of neurological stroke severity determined by the National Institute of Health Stroke Scale (NIHSS) at admission, 24 and 48 hours in stroke patients with and without heavy drinking habits. Early neurological deterioration (END) is defined as an increase of NIHSS in ≥4 points in the first 48 hours after admission. C) Forest plot of the multivariate analysis including variables predicting END (n=206) such as hemorrhagic transformation, infarct volume, NIHSS on admission and heavy drinking habits (Model A). D) Forest plot of the multivariate analysis of the variables included in the Model A and inflammatory biomarkers (axillary temperature, number of leukocytes levels and C-reactive protein levels) (Model B).

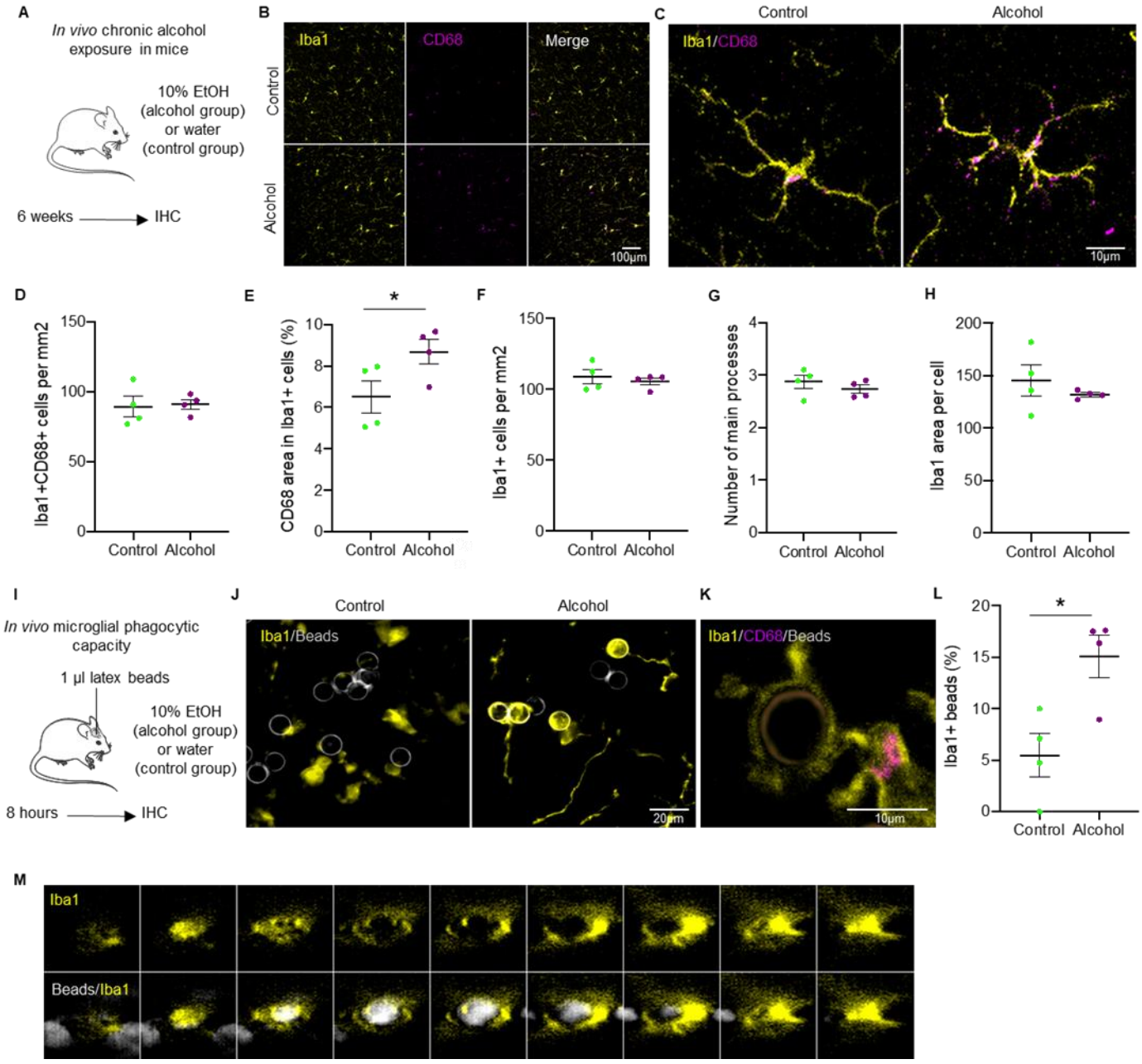

Figure 2. Alcohol exposure provokes a microglial priming in mice. A) Experimental design to study the effects of chronic alcohol exposure on microglia. B) Representative photomicrographs of microglial cells stained with Iba1 and CD68. Scale bar: 100µm. C) High magnification- representative photomicrographs of microglial cells in control and alcohol-exposed mice. D) Quantification of Iba1 and CD68 double-positive cells. E) Quantification of CD68 area on $Iba1^{+}$ microglial cells. F) Quantification of $Iba1^{+}$ cells. G) Quantification of the number of processes starting from the soma. H) Quantification of the area of microglial cells.

N=4 mice per group. I) Experimental design to *in vivo* study the microglial phagocytic capacity: 1 µl of latex beads were injected in the cortex of control and alcohol-exposed mice. Eight hours after, phagocytosed latex beads were quantified by immunohistochemical analyses. J) Representative photomicrographs of $Iba1^+$ microglial cells and latex beads. K) Detail of a latex bead phagocytosed by a microglial cell in an alcohol-exposed mouse. Note the lysosomal activation (CD68, red) at the apex of microglial process. L) Quantification of phagocytosed latex beads/total number of beads. N=4 mice per group, *$p<0.05$ vs Control, Mann-Whitney test. Scale bar: 20µm. M) Sequential confocal photomicrographs of a phagocytosed latex bead.

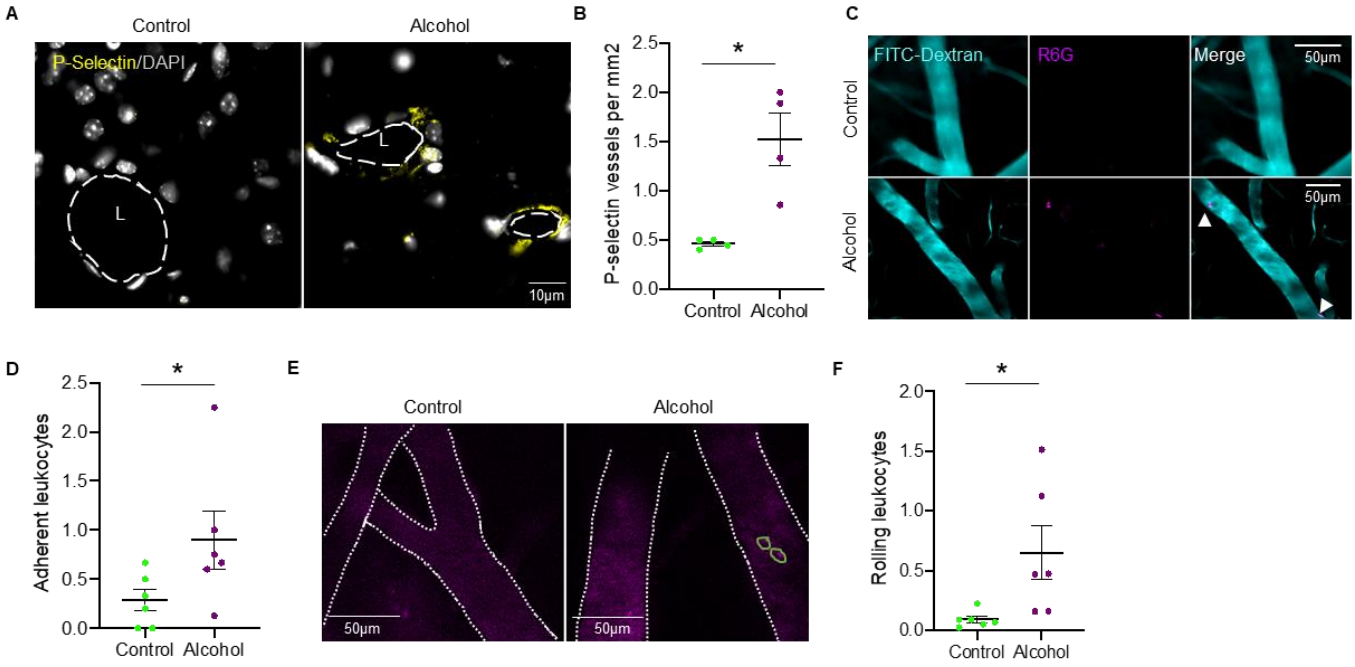

Figure 3: **Alcohol exposure provokes endothelial activation in the brain.** A) Representative photomicrographs of P-selectin positive vessels in control and alcohol-exposed mice (note the absence of positive staining in control mice). Dotted lines represent the lumen (L) of the blood vessel. Scale bar: 20µm. B) Quantification of P-selectin signal in the brain cortex of control and alcohol-exposed mice. N=4 mice per group. C) Compilation of *in vivo* time-lapse images obtained by two-photon microscopy showing Rhodamine 6G $(R6G)^+$ leukocyte adhesion (arrows) in control and alcohol-exposed mice (note the absence of $R6G^+$ cells in control mice). Scale bar: 50µm. D) Quantification of adherent leukocytes. E) Representative time-lapse images of leukocyte rolling (green circles). Scale bar: 50µm. F) Quantification of rolling leukocytes per second. N=4 mice per group. $P<0.05$ vs control, Mann-Whitney test.

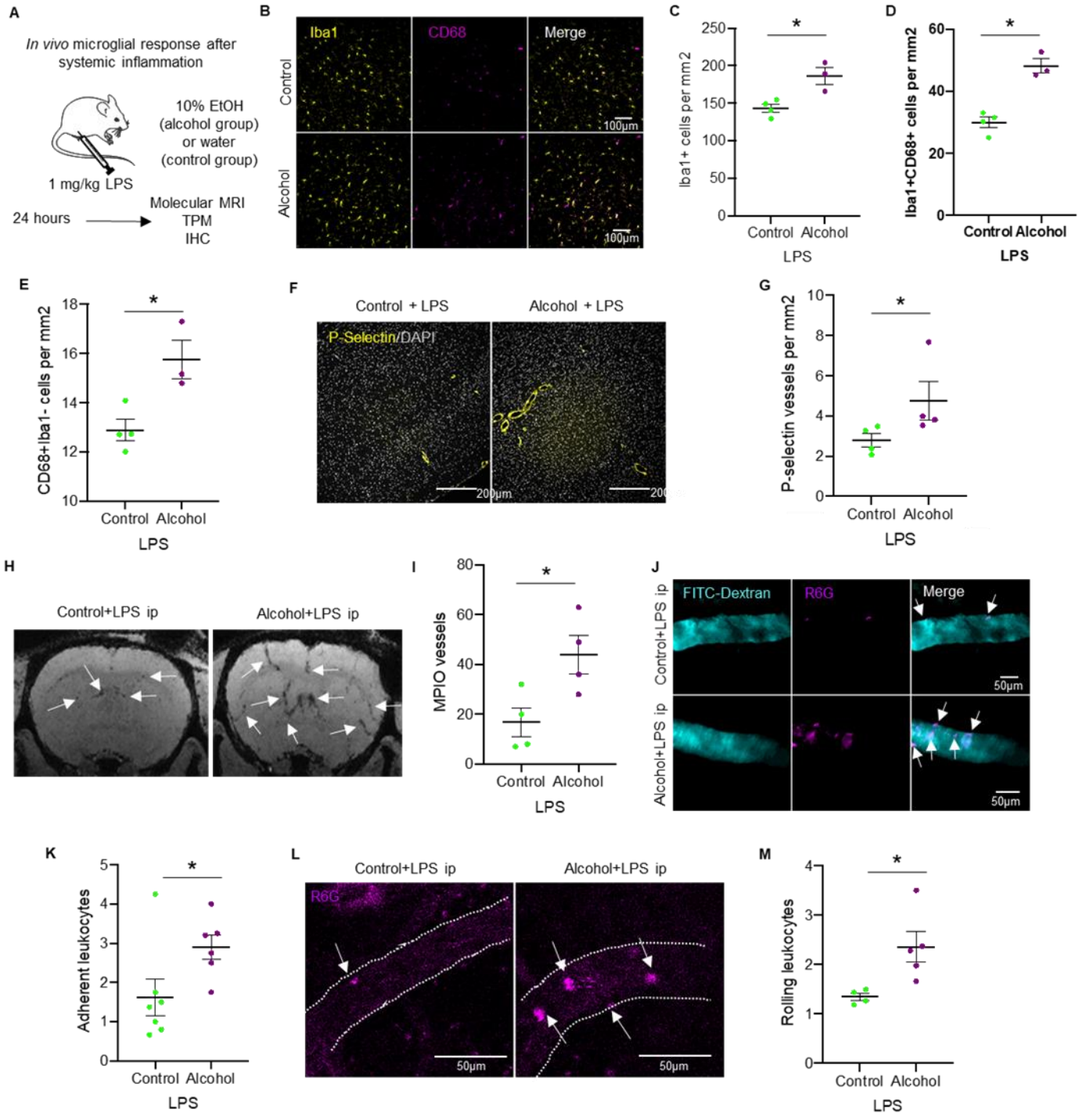

Figure 4: Alcohol exposure exacerbates brain neurovascular inflammatory reactions after an acute systemic insult in mice. A) Experimental design to study whether chronic alcohol exposure provokes exacerbated inflammatory responses after a secondary injury (peripheral acute LPS injection). B) Representative photomicrographs of the brain cortex of control and alcohol-exposed mice 24 hours after the acute systemic injection of LPS. Scale bar: 100µm.

N=5-6 mice per group. C) Quantification of total microglia ($Iba1^+$ cells). D) Quantification of activated microglia ($Iba1^+/CD68^+$ cells). Scale bar: 100µm. E) Quantification of macrophages ($CD68^+/Iba1^-$ cells). F) Representative photomicrographs of P-selectin staining in control and alcohol-exposed mice 24h after the acute systemic injection of LPS. G) Quantification of the number of P-$selectin^+$ blood vessels. H) Representative T2*-weighted images showing P-selectin-coupled MPIO *in vivo* accumulation in the brain of control and alcohol-exposed mice after the acute injection of LPS. I) Quantification of $MPIO^+$ blood vessels. J) Compilation of time-lapse images showing representative *in vivo* leukocyte adhesion (arrows) obtained by two-photon microscopy. Scale bar: 50µm. K) Quantification of leukocyte adhesion. L) Representative time-lapse images of *in vivo* leukocyte rolling (see also Movies S1 and S2). Scale bar: 50µm. M) Quantification of rolling leukocytes per second. $P<0.05$ vs control, Mann-Whitney test.

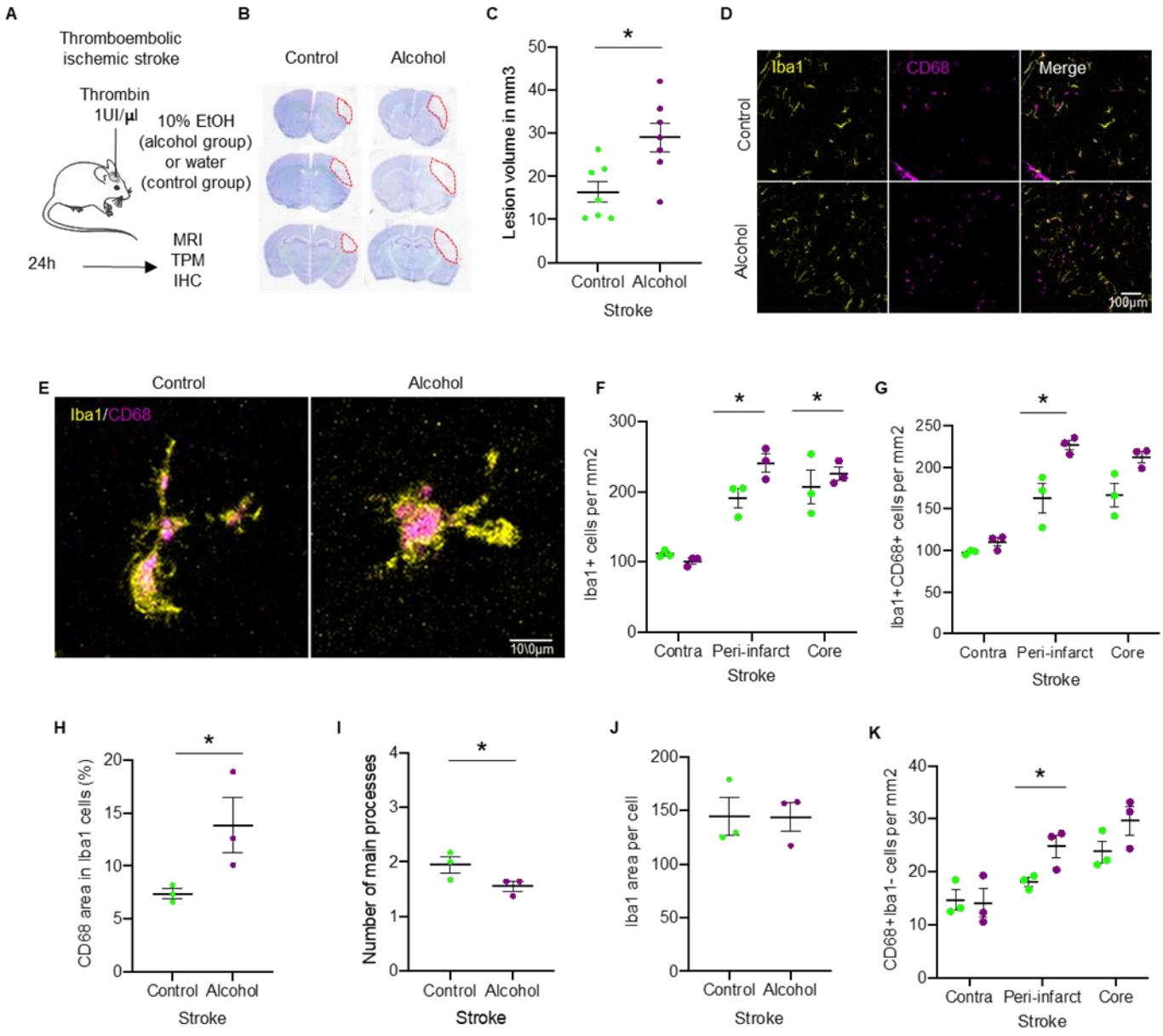

Figure 5. Alcohol exposure increases lesion volume and parenchymal inflammatory responses after ischemic stroke in mice. A) Experimental design to study whether chronic alcohol exposure worsens ischemic stroke outcome. B) Representative brain lesions and C) corresponding quantifications in control and alcohol-exposed mice 24 hours after stroke onset (N=8 mice/group; $p<0.05$ vs control mice). D) Representative photomicrographs of microglial cells and infiltrated macrophages in the ipsilateral cortex 24 hours after stroke onset. Scale bar: 100µm. N=4 mice per group. E) High magnification- photomicrographs of microglial cells at

the peri-infarct area. Scale bar: 20µm. F) Quantification of $Iba1^+$ cells. G) Quantification of $Iba1^+/CD68^+$. H) Quantification of CD68 area in $Iba1^+$ cells. I) Quantification of the number of processes starting from the soma. J) Quantification of the mean whole cell area of $Iba1^+$ cells. K) Quantification of $CD68^+/Iba1^-$ cells.

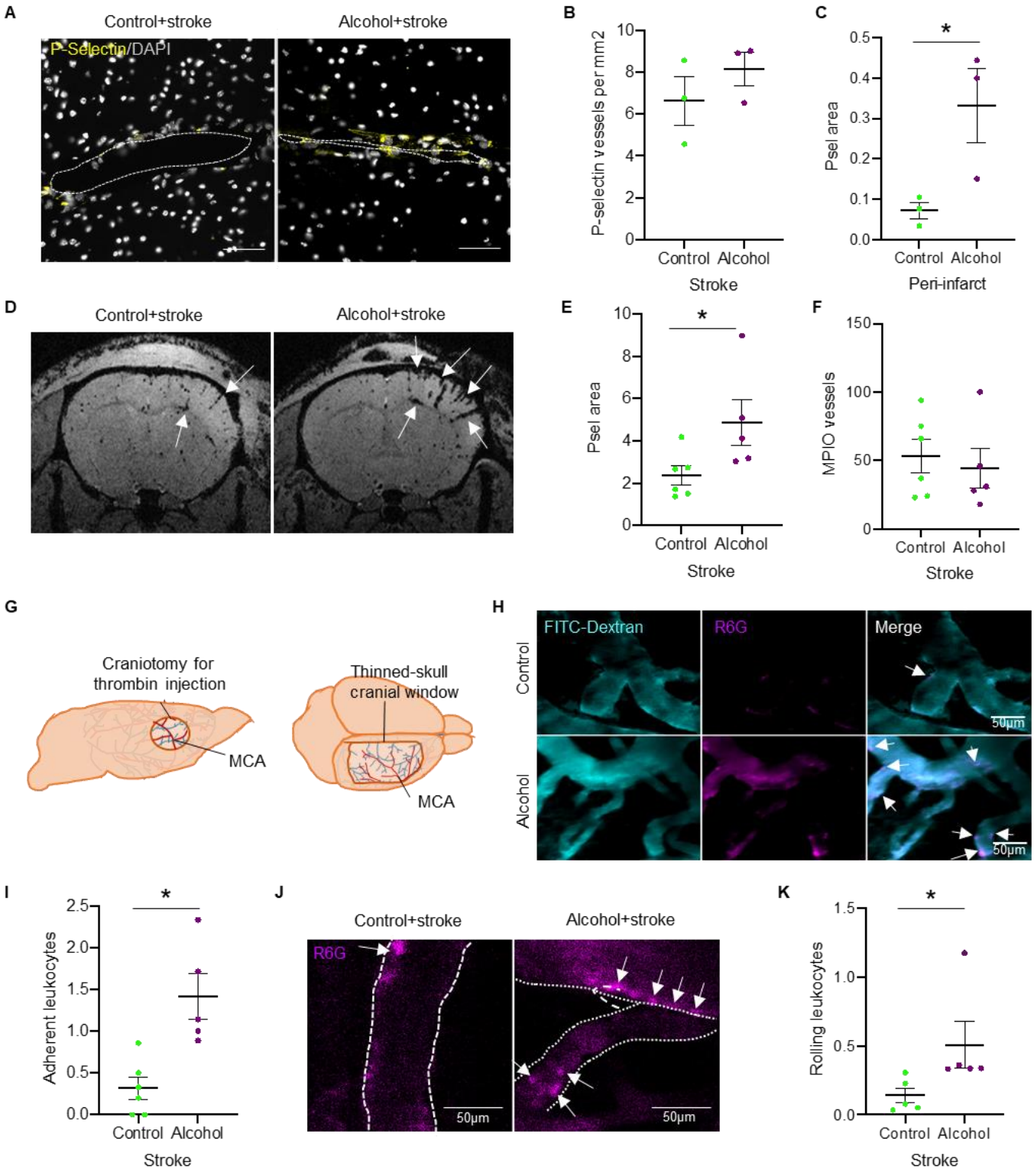

Figure 6. Alcohol exposure increases vascular inflammatory responses after ischemic stroke in mice. A) Representative photomicrographs of P-selectin immunostaining in control and alcohol-exposed mice 24h after stroke onset. Scale bar: 50µm. B) Quantification of the

number of P-selectin^{+} vessels. C) Quantification of P-selectin^{+} immunostaining area. D) Representative T2*-weighted images of *in vivo* P-selectin molecular imaging. Arrows show MPIO^{+} blood vessels (N=5-6 mice/group). E) Quantification of MPIO^{+} signal. F) Quantification of MPIO^{+} blood vessels. G) Schematic view of the craniotomy performed for thrombin injection, which leads to the occlusion of the MCA and the thinned-skull window for intravital two-photon imaging. H) Compilation of representative time-lapse images showing *in vivo* leukocyte adhesion (arrows) obtained by two-photon microscopy. Scale bar: 50µm. I) Quantification of leukocyte adhesion. J) Representative time-lapse images of *in vivo* leukocyte rolling. Scale bar: 50µm. K) Quantification of leukocyte rolling (see also Movies S3 and S4). N=4 mice per group. *P<0.05 vs control, Mann-Whitney test.

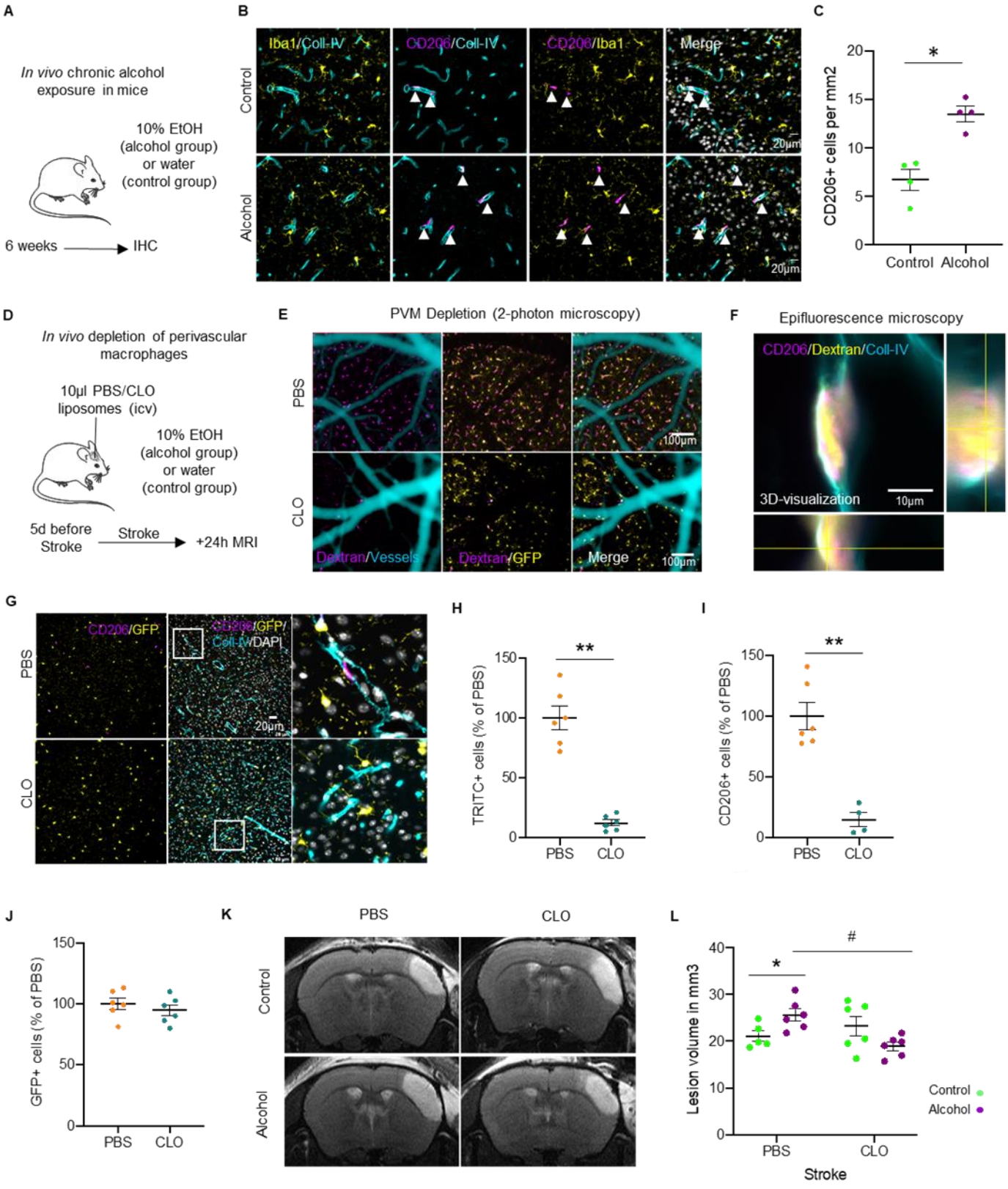

Figure 7. Perivascular macrophages (PVM) mediate the aggravating effect of chronic alcohol consumption on ischemic stroke in mice. A) Experimental design to study the effects

of chronic alcohol exposure on PVM. B) Representative photomicrographs of PVM $CD206^{+}Iba1^{-}$ (arrows) cells in control and alcohol-exposed mice. C) Quantification of PVM ($CD206^{+}$ cells). D) Experimental design to deplete PVM in control and alcohol-exposed mice. E) Representative *in vivo* Z stack of $CX3CR1^{GFP+/-}$ PBS/CLO-injected mice. Blood vessels are visualized through the intravenous injection of FITC-Dextran. Twenty-four hours after the icv injection of TRITC-Dextran, PVM can be *in vivo* visualized (note the absence of TRITC-Dextran signals in CLO-treated mice). Microglia is positive for GFP in $CX3CR1^{GFP+/-}$ mice. Scale bar: 100µm. F) Colocalization of CD206 and phagocyted TRITC-dextran in PVM (epifluorescence microscopy): 3D visualization of a double positive TRITC-dextran and CD206 PVM for. Scale bar: 10 µm. G) Representative photomicrographs of GFP^{+} microglial cells and $CD206^{+}$ PVMs in PBS- and CLO-treated mice. Scale bar: 20 µm. Quantification of PVM depletion by $TRITC^{+}$ cell counting (H) and $CD206^{+}$ counting (I). J) The number of microglial cells remained unchanged after CLO treatment. K) Representative T2-weighted MRI images showing ischemic lesions in PBS- and CLO-treated naïve and alcohol-exposed mice 24 hours after stroke onset. E) Quantification of lesion volumes. N=5-6 mice per group; $p<0.05$ vs PBS, Mann-Whitney test.

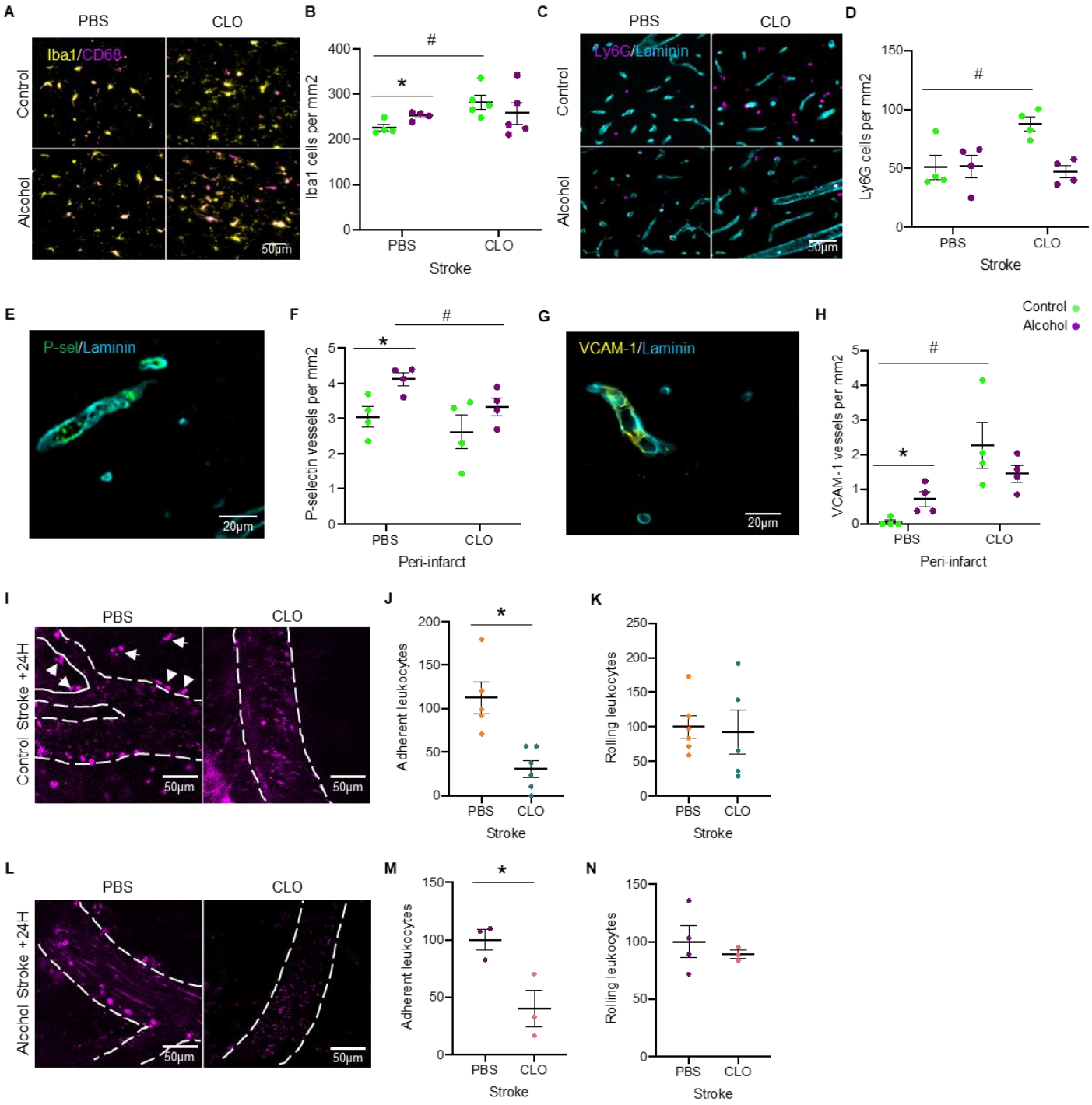

Figure 8. PVM modulate inflammatory responses to stroke in naïve mice, but not in alcohol-exposed mice. A) Representative photomicrographs of different subsets of microglia/macrophages 24 hours after stroke onset at the core of the lesion. B) Quantification of Iba1+ cells. C) Representative photomicrographs of Ly6G+ neutrophils at the core of the lesion. D) Quantification of neutrophil numbers. E) Representative photomicrographs of P-

selectin staining 24 hours after stroke onset. F) Quantification of P-selectin positive blood vessels. G) Representative photomicrograph of VCAM-1 positive blood vessel 24 hours after stroke onset. H) Quantification of VCAM-1 positive blood vessels. I) Representative photomicrographs obtained by 2-photon microscopy of leukocytes (in magenta, Rhodamine 6G) in PBS- and CLO-treated mice (not exposed to alcohol) 24 hours after stroke onset. J) Quantification of adherent leukocytes. K) Quantification of circulating/rolling leukocytes. L) Representative photomicrographs obtained by 2-photon microscopy of leukocytes (in magenta, Rhodamine 6G) in PBS- and CLO-treated mice (exposed to alcohol) 24 hours after stroke onset. M) Quantification of adherent leukocytes. N) Quantification of circulating/rolling leukocytes. N=4 mice per group, $p<0.05$ vs PBS, Mann-Whitney test.

Table 1. Univariate analysis of stroke patients with and without heavy drinking (HD) habits (>6 standard drinks/day). Analyses were retrospectively performed from data of the stroke registry at the Stroke Unit of the University Clinical Hospital of Santiago de Compostela (Spain).

	Heavy drinking		
	No n = 3221	Yes n = 424	
Age (years)	72.60 ± 14.06	66.79 ± 11.87	< 0.0001
Men (%)	50.5	85.8	< 0.0001
History of hypertension (%)	64.6	52.4	< 0.0001
History of diabetes (%)	24.7	22.6	0.368
History of smoking (%)	12.5	56.1	< 0.0001
History of dyslipemia (%)	34.0	32.3	0.513
History of ischemic heart disease (%)	11.5	11.3	0.491
Axillary temperature at admission (ºC)	36.3 ± 0.6	36.6 ± 0.6	< 0.0001
Leukocytes on admission ($x10^3$/mL)	8.8 ± 3.1	9.9 ± 3.2	0.135
Fibrinogen on admission (mg/dL)	416.9 ± 97.6	439.5 ± 87.1	< 0.0001
C-reactive protein on admission (mg/L)	2.7 ± 4.2	3.5 ± 3.1	< 0.0001
Sedimentation rate (mm)	21.9 ± 23.6	24.1 ± 23.5	0.05
tPA treatment (%)	22.9	21.0	0.206

Trombectomy (%)	3.2	1.4	0.025
Hemorrhagic transformation (%)	8.8	10.5	0.147
Infarct volume (mL), N=2121	40.1 ± 70.4	46.9 ± 64.8	0.021
NIHSS on admission, N=3645	9 [5,15]	14 [11,19]	< 0.0001
NIHSS at 24 h, N=3277	6 [3,10]	18 [9,20]	< 0.0001
NIHSS at 48 h, N=3352	4 [1,10]	14 [7,20]	< 0.0001
END (%), N=3352	4.4	19.2	< 0.0001
mRS at 3 months, N=2935	2 [1,4]	3 [2,4]	< 0.0001
Poor outcome at 3 months (%), N=2935	53.6	65.1	< 0.0001
TOAST			0.286
Atherothrombotic (%)	23.2	26.7	
Cardioembolic (%)	37.1	32.8	
Small vessel disease (%)	7.3	6.1	
Indeterminate (%)	31.2	33.3	
Others (%)	1.1	1.2	
Mortality during hospitalization (%)	9.1	11.7	<0.0001

tPA, tissue-type Plasminogen Activator; NIHSS, National Institute of Health Stroke Scale; mRS, modified Rankin Scale; END, Early Neurological Deterioration (defined as the increase of NIHSS in ≥4 in the first 48 hours after admission); TOAST, Trial of ORG 10172 in Acute Stroke Treatment classification.

Table 2: Univariate analysis of stroke patients with and without early neurological deterioration (END).

	Early neurological deterioration		
	No n = 3146	**Yes** n = 206	
Age (years)	72.1 ± 13.4	73.8 ± 11.9	0.977
Men (%)	54.7	59.2	0.219
History of hypertension (%)	62.8	62.6	0.509
History of diabetes (%)	24.5	23.3	0.384
History of smoking (%)	17.4	20.2	0.193
History of dyslipemia (%)	33.8	35.4	0.339
History of ischemic heart disease (%)	11.3	14.1	0.135
Axillary temperature at admission (ºC)	36.3 ± 0.6	36.4 ± 0.8	0.005
Leukocytes on admission ($x10^3$/mL)	8.9 ± 3.3	10.4 ± 4.2	0.019
Fibrinogen on admission (mg/dL)	426.7 ± 102.8	422.8 ± 120.5	0.255
C-reactive protein on admission (mg/L)	3.2 ± 4.3	4.6 ± 4.9	< 0.0001
Sedimentation rate (mm)	27.7 ± 25.6	28.5 ± 26.1	0.695
tPA treatment (%)	23.5	22.3	0.389
Trombectomy (%)	2.7	3.9	0.210

Hemorrhagic transformation (%)	9.4	17.1	< 0.0001
Infarct volume (mL)	50.3 ± 77.1	107.7 ± 107.8	< 0.0001
NIHSS at admission	12 [7,17]	13 [8,15]	0.017
mRS at 3 months	3 [1,4]	5 [3,6]	< 0.0001
Poor outcome at 3 months (%)	51.0	81.2	< 0.0001
TOAST			0.613
Atherothrombotic (%)	24.3	25.2	
Cardioembolic (%)	35.8	38.3	
Small vessel disease (%)	7.2	4.4	
Indeterminate (%)	31.6	31.1	
Others (%)	1.1	1.0	
Heavy drinking (%)	10.2	36.9	< 0.0001

Table 3. Heavy drinkers have higher levels of inflammatory markers, in the absence of any other injury. Demographic and clinical characteristics, leukocyte cell counting and C-reactive Protein (CRP) levels in an independent cohort of healthy control participants and heavy drinkers recruited in the Addiction Unit of the University Hospital of Caen (Normandy, France) while they were receiving withdrawal treatment as inpatients. Data are expressed as mean+/- Standard Deviation; parenthesis data correspond to interval.

	Control subjects N=21	**Heavy drinkers N=34**	**P**
Age	43.8 ± 7.4 (29-55)	45.7 ± 9.2 (33-66)	0.29
Women/Men Ratio	5/21	9/34	0.83
BMI (*2 missing data)	25.3 ± 4.4* (19.5-35.5)	23.9 ± 4.4 (17.3-39.8)	0.32
AUDIT	2.9 ± 1.6 (0-6)	28.9 ± 7.7 (4-40)	<0.001
Leucocytes (g/L)	6.4 ± 1.7 (3.7-9.7)	7.0 ± 2.1 (3.5-12.3)	0.24
Neutrophils (g/L)	3.6 ± 1.4 (1.7-7.2)	4.1 ± 1.7 (1.7-8.6)	0.28
Eosinophils (g/L)	0.17 ± 0.08 (0.04-0.31)	0.18 ± 0.13 (0-0.53)	0.80
Basophils (g/L)	0.03 ± 0.02 (0.01-0.07)	0.04 ± 0.02 (0.01-0.1)	0.09
Lymphocytes (g/L)	1.95 ± 0.72 (1.03-3.85)	1.93 ± 0.75 (0.93-3.88)	0.96

Monocytes (g/L)	0.52 ± 0.17 (0.27-0.99)	0.73 ± 0.27 (0.11-1.42)	0.003
C Reactive protein >3 mg/L		61.8% (21/34)	

BMI, Body Mass Index; AUDIT, Alcohol Use Disorders Identification Test.

Table 4. Alcohol exposure alters the gene expression of inflammatory markers in the brain of mice, both in the absence of any other insult and after ischemic stroke. Gene expression was calculated using two housekeeping genes. The increase of the inflammatory markers expression was exacerbated in alcohol-exposed mice after thromboembolic stroke (basal: n=4 mice per group, MCAo+24h: n=5 mice per group; *p<0.05 vs Control basal), Mann-Whitney test.

			IL1b	IL6	P-Selectin	TGFb	TLR4	TNF	VCAM1
Basal		C	0.19 ± 0.05	0.67 ± 0.28	1.89 ± 1.38	0.69 ± 0.11	0.80 ± 0.20	0.44 ± 0.18	0.78 ± 0.15
		A	0.54 ± 0.22	1.25 ± 0.33	0.44 ± 0.14	**1.00 ± 0.14***	1.27 ± 0.24	0.19 ± 0.01	1.02 ± 0.21
MCAo +24h	Contro	C	0.14 ± 0.03	0.25 ± 0.07	0.22 ± 0.06	0.99 ± 0.20	0.77 ± 0.18	0.06 ± 0.02	0.97 ± 0.24
		A	0.15 ± 0.05	0.11 ± 0.03	0.13 ± 0.07	0.82 ± 0.14	0.64 ± 0.12	0.08 ± 0.00	0.73 ± 0.13
	Ipsi	C	3.01 ± 0.99	14.00 ± 4.04	9.32 ± 1.93	1.90 ± 0.17	1.77 ± 0.28	1.45 ± 0.23	1.39 ± 0.21
		A	**5.51 ± 0.94***	20.54 ± 3.11	**17.52 ± 2.21***	2.17 ± 0.26	2.31 ± 0.21	**2.51 ± 0.38***	1.29 ± 0.22