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ORIGINAL RESEARCH

# Unveiling the Impact of *Porphyromonas gingivalis*-Associated Periodontitis on Stroke Outcome in Mice

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**BACKGROUND:** Periodontitis is a chronic inflammatory condition with infectious origin that affects the tissues supporting the teeth. Increasing epidemiological evidence suggests that periodontitis is a risk factor for ischemic stroke with associated adverse outcomes. However, the underlying mechanism of this association remains incompletely elucidated.

**METHODS:** We used a C57BL/6J mice model of ischemic stroke induced by transitory occlusion of the middle cerebral artery in the presence or absence of ligature-induced periodontitis using *Porphyromonas gingivalis*-soaked ligatures. Stroke severity was evaluated through infarct volume, sensorimotor deficit, blood–brain barrier (BBB) integrity, and markers of systemic and brain inflammation. The direct effect of *P gingivalis* on BBB endothelial cells was further explored in vitro.

**RESULTS:** Mice with *P gingivalis*-associated periodontitis showed a significant exacerbation of stroke severity: larger infarct volume, more severe sensorimotor deficit, greater BBB disruption, and increased brain neutrophil infiltration compared with sham. Systemic inflammation was also markedly elevated. Intravenous administration of *P gingivalis* alone, without gingival injury, before transitory occlusion of the middle cerebral artery was sufficient to amplify brain inflammation and stroke lesions. In vitro *P gingivalis,* through its gingipain proteases, directly impaired BBB integrity by increasing endothelial permeability and disrupting tight-junction proteins.

**CONCLUSIONS:** Our findings demonstrate that *P gingivalis*-associated periodontitis worsens ischemic stroke outcome both indirectly by enhancing systemic and brain inflammation and directly via BBB disruption. These results highlight periodontitis as a modifiable risk factor and potential therapeutic target for improving stroke prognosis.

**Key Words:** blood–brain barrier ■ inflammation ■ ischemic stroke ■ periodontitis ■ *Porphyromonas gingivalis*

Despite extensive prevention campaigns, available therapeutics, and the management of conventional risk factors such as active smoking, high blood pressure, dyslipidemia, and diabetes, stroke remains a leading cause for neurological disability and significantly contributes to global mortality. Stroke has a profound long-term impact on both mental and physical health.[1] The high prevalence of ischemic stroke suggests the role of often overlooked comorbidities including infectious diseases[2] and oral health conditions.[3] Among these, periodontitis is 1 of the most prevalent oral pathologies.[4] Periodontitis is characterized by oral dysbiosis, which results in gingival inflammation and irreversible destruction of tooth-supporting

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## CLINICAL PERSPECTIVE

### What Is New?

- This study provides the first direct experimental evidence that *Porphyromonas gingivalis*-associated periodontitis aggravates ischemic stroke outcomes by increasing infarct volume, brain edema, and disblood–brain barrier disruption through systemic and ischemic brain inflammation.

### What Are the Clinical Implications?

- Periodontitis, particularly when associated with *P gingivalis*, may represent an unrecognized risk factor for ischemic stroke severity.
- Early detection and management of periodontitis, along with a clinical evaluation of periodontal treatment as an adjuvant strategy, could improve stroke outcomes and support prevention efforts.

### Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **BBB** | blood–brain barrier |
| **hCMEC** | human cerebral microvascular endothelial cell |
| **LIP** | ligature-induced periodontitis |
| **MOI** | multiplicity of infection |
| **tMCAO** | transient middle cerebral artery occlusion |

tissues, ultimately resulting in tooth loss. Importantly, periodontitis is considered as an independent risk factor for various conditions, including rheumatoid arthritis, neurodegenerative diseases, cardiovascular diseases, and notably ischemic stroke.[5] Epidemiological studies have consistently highlighted the association between periodontitis and ischemic stroke. Pussinen et al[6] demonstrated indirectly that exposure to periodontal pathogens, specifically to *Porphyromonas gingivalis*, 1 of the most relevant periodontal diseases in humans[7] measured by the plasma concentration of antibodies specific to *P gingivalis*, a keystone pathogen of periodontitis,[8] was associated with an increased predisposition to ischemic stroke occurrence. Furthermore, a study published in 2004 reported a substantial increase (4.34-fold) in ischemic stroke risk associated with periodontal disease.[9] A systematic review of the literature and meta-analysis have further reported that severe periodontitis in patients with ischemic stroke was associated with recurrent vascular events[10] and increased mortality.[11] Despite the established associations between periodontitis and stroke, the precise mechanism underlying its contribution to ischemic stroke pathophysiology remains poorly understood,[12] with gaps in preclinical research that fail to fully elucidate the specific both indirect and direct effect of *P gingivalis,* including its role in systemic inflammation and bacterial dissemination in the bloodstream. Previous studies[13–16] have provided valuable insights but remain incomplete in the exploration of critical pathways.

In this study, we aimed to address these gaps by using an integrated experimental approach. Specifically, we will investigate the impact of *P gingivalis* in the context of chronic periodontitis using a clinically relevant ligature model with live bacteria alongside an intravenous injection model to explore *P gingivalis* as a blood-circulating pathogen. By combining these models, we aimed to comprehensively dissect the effects of periodontitis and *P gingivalis* on the pathological processes and outcomes of ischemic stroke in mice subjected to transient middle cerebral artery occlusion (tMCAO).

## METHODS

The authors declare that the data that support the findings of this study are available from the corresponding author upon reasonable request.

All supporting data and the detailed methods are provided in Data S1. A brief description on the main methodology is presented below.

Ten-week-old male C57BL6/J mice were randomly assigned to 4 experimental groups. The first group underwent ligature-induced periodontitis using a ligature soaked in *P gingivalis* (LIP-Pg),[17] followed 15 days later by tMCAO.[18] The second group served as the control and received no ligatures and bacterial infection followed by tMCAO. To control for stroke-independent effects of periodontitis, a third group underwent LIP-Pg induction but received sham surgery instead of tMCAO. Mice were euthanized either 2 or 7 days after stroke or sham procedures for assessment of infarct volume, sensorimotor deficits (Table S1),[19] blood–brain barrier (BBB) integrity, inflammation, and immune cell profiling, including peripheral blood cell counts and brain-infiltrating leukocytes analyzed by flow cytometry (Tables S2 and S3, respectively).

In a second set of experiments, the impact of systemic infection with *P gingivalis* was studied independently of ligature effect by intravenous injection of *P gingivalis* before stroke induction.

All experimental procedures followed were conducted in accordance with institutional guidelines (European Communities Council guideline [2010/63/EU]) and received approval from the French Ministry of Education (APAFIS number 2020032719365322 and number 201810171005384). This study adheres to

the Animal Research: Reporting of In Vivo Experiments guidelines.[20] The impact of *P gingivalis* on BBB disruption and junctional protein expression, analyzed by WES simple Western platform (Table S4), was investigated using human cerebral microvascular endothelial cells (hCMEC)/D3 cells as an in vitro model of BBB.[21]

## Statistical Analysis

Sample size per group was calculated using the G*Power version 3.1 software. Statistical analyses were performed with GraphPad Prism at a significance level of 95% ($P<0.05$).

# RESULTS

## Impact of *P gingivalis*-Associated Periodontitis on Infarct Volume, BBB Rupture, and Stroke Outcome

In the first set of experiments, we investigated the impact of periodontitis on stroke outcome. To achieve this, we induced stroke using a 1-hour tMCAO in mice that were previously exposed to ligature-induced periodontitis with *P gingivalis* (soaked on a ligature) for 15 days as well as in a control group that did not undergo periodontitis. Mice were euthanized during the acute phase of stroke (2 days poststroke) to study brain inflammation and tissue remodeling, or at a later time point (7 days poststroke) to study sensorimotor deficits (Figure 1A).

## LIP-Pg-Induced Systemic Inflammation

At the time of stroke induction, the presence of periodontitis was determined by assessing alveolar bone resorption. High-resolution micro-computerized tomography was used to measure the distance between the alveolar bone crest and cement-enamel junction at baseline and at the day before stroke induction. Although no significant difference was observed between baseline and at the day before stroke induction in sham mice, a significant increase in bone resorption was observed between baseline and at the day before stroke induction in mice subjected to tooth-ligature with *P gingivalis* inoculation (LIP-Pg group) (Figure S1A

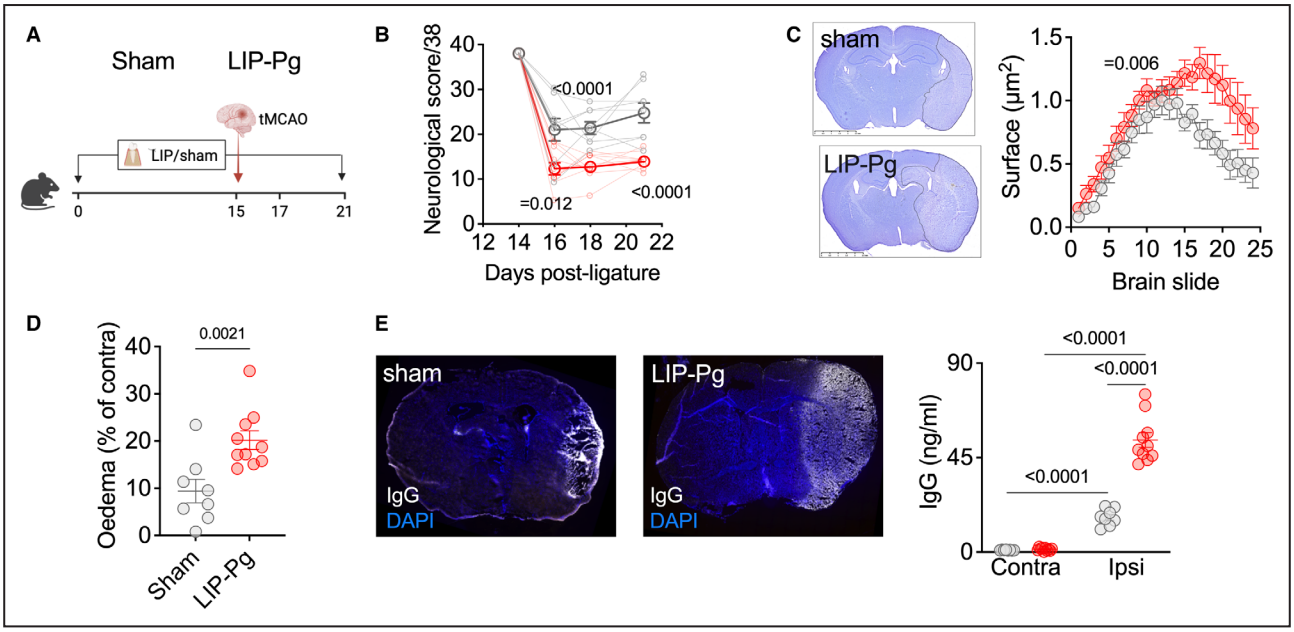


**Figure 1. *Porphyromonas gingivalis*-associated periodontitis worsens stroke outcome.**
**A**, Study overview: Male C57BL6/J mice (10 weeks old) were divided into ligature-induced periodontitis with ligature soaked in *P gingivalis* (LIP-Pg, n=10) and control (sham, n=8) groups. Fifteen days after inducing periodontitis, mice underwent tMCAO and were euthanized either 2 or 7 days poststroke. **B**, Sensorimotor deficit: There were 11 tests conducted the day before tMCAO induction as well as 1, 3, and 7 days later in LIP-Pg and sham mice. Mice without any neurological deficit were assigned a score of 38. Statistical analysis was performed using the Mann-Whitney test to compare LIP-Pg vs sham at each time point. **C**, Stroke volume: Brain sections (24 slices) stained with cresyl violet assessed stroke surface. Left: representative. Right: mean ischemic surface of LIP-Pg (n=7) and sham (n=5) mice (numbers differ from initial group sizes due to technical issues during tissue processing). **D**, Brain edema: Expressed as the percentage increase in volume of the ischemic hemisphere relative to contralateral hemisphere in LIP-Pg mice compared with sham mice. Statistical significance was assessed using the Mann-Whitney test. **E**, Blood–brain barrier disruption: Representative image of IgG immunostaining (white signal) and concentration of IgG measured in homogenates obtained from ipsilateral and contralateral hemispheres of LIP-Pg and control mice. Statistical analysis was performed using the Kruskal-Wallis test, followed by an uncorrected Dunn test to determine significance. Contra indicates contralateral; DAPI 4′,6′-iaminido-2-phenylindole; IgG, immunoglobulin G; Ipsi, ipsilateral; LIP-Pg, ligature-induced periodontitis-*Porphyromonas gingivalis*; and tMCAO, transient middle cerebral artery occlusion.

and S1B). Additionally, we detected the presence of lipopolysaccharide in the plasma of LIP-Pg mice (Figure S1C), indicating bacteria entry into the bloodstream. Furthermore, LIP-Pg mice displayed systemic inflammation, as evidenced by a significant increase in circulating neutrophils and monocytes (Figure S1D) as well as elevated concentration of proinflammatory cytokines IL (interleukin)-1β, IL-6, and TNF (tumor necrosis factor)-α after 14 days of periodontitis (Figure S1E). In addition, these mice exhibited significantly higher plasma levels of chemokine (C-X-C motif) ligand 1 (CXCL1), intercellular molecule adhesion-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), further supporting the notion that periodontitis enhances the systemic inflammation by promoting inflammatory cell recruitment and vascular adhesion (Figure S1E).

## Periodontitis Provoked Worse Stroke Outcome

The impact of *P gingivalis*-induced periodontitis on stroke outcome was evaluated by assessing the severity of the sensorimotor deficit before stroke induction, and at 1, 2, and 7 days post-tMCAO. LIP-Pg mice exhibited exacerbated sensorimotor deficits (Figure 1B) compared with mice with tMCAO but without periodontitis. The neurological score markedly declined at 1 day poststroke in both groups of mice, with a more sustained reduction observed in LIP-Pg mice. Although sham-operated mice showed a trend ($P$=0.05) toward sensorimotor functions recovery between day 16 (score: 19/38) and day 21 (score: 25/38), LIP-Pg mice maintained significantly lower and stable neurological scores (day 16 score: 12/38; day 21 score: 14/38) during the 7 days following stroke induction. In tMCAO sham-operated mice, the neurological score remained within the normal range, independent of the presence or absence of periodontitis (Figure S2A).

## LIP-Pg Increased Stroke Size and Potentiated BBB Disruption

Stroke size was measured 7 days after tMCAO, revealing a significantly increased stroke volume in LIP-Pg mice compared with that of sham-operated mice (area under the curve [AUC] LIP-Pg: 20.8±1.1 versus AUC sham: 1.7±0.7; Figure 1C). Additionally, LIP-Pg mice exhibited increased brain edema (Figure 1D). BBB integrity following tMCAO was assessed through the measurement of Ig (immunoglobulin)G extravasation (Figure 1E). Both groups of mice showed significant influx of plasma IgG into the ipsilateral hemispheres (ipsilateral versus contralateral in sham: $P$<0.01 and in LIP-Pg: $P$<0.001) with a more pronounced difference in LIP-Pg mice (ipsilateral sham: 15.3±1.0 and ipsilateral LIP-Pg: 53.3±3.5; $P$<0.05). Importantly, IgG concentration within the contralateral hemisphere was close to the limit of detection, with no difference between both groups of mice.

Furthermore, LIP-Pg and control mice that underwent sham surgery for tMCAO did not display any infarct areas as shown by cresyl violet staining and showed no IgG extravasation, indicating the absence of BBB disruption (Figure S2B and S2C, respectively).

## LIP-Pg Exacerbated Inflammation and Brain Leukocyte Infiltration in Stroke Mice

As previously mentioned, LIP-Pg mice exhibited a systemic inflammatory response (Figure S1). Following tMCAO, elevated circulating neutrophil (LIP-Pg: 1.68±0.21 versus sham: 0.73±0.07) and monocyte (LIP-Pg: 0.27±0.01 versus sham: 0.11±0.01) count (Figure 2A) and increased concentrations of IL-1β, IL-6, and TNF-α were observed 48 hours poststroke (Figure 2B).

We have also assessed the impact of sham surgery for tMCAO on blood cell profiles in both LIP-Pg and control mice. We found that sham surgery for tMCAO did not significantly alter the blood formula, particularly neutrophil and monocyte counts (Figure S2D).

Neutrophils and monocytes are among the first inflammatory cells to be recruited to the site of brain injury, peaking in infiltration at 2 to 3 days post-tMCAO.[22] Using flow cytometry on cerebral hemisphere cell suspension, we monitored neutrophil ($CD11b^+$, $Ly6G^+$, $Ly6C^{low}$) and monocyte ($CD11b^+$, $Ly6G^-$, $Ly6C^{high}$) recruitment.

Forty-eight hours after stroke, LIP-Pg mice exhibited a higher infiltration of neutrophils and monocytes into the ipsilateral hemisphere compared with sham mice (Figure 3A). Conversely, few neutrophils and monocytes were detected in the contralateral hemisphere in both groups. Additionally, we confirmed neutrophil accumulation and their activation in the ischemic brain by quantifying myeloperoxidase concentration. We observed a significant increase of myeloperoxidase within the ischemic brain of mice with *P gingivalis*-associated periodontitis as compared with those of mice without periodontitis (Figure 3B).

Furthermore, DNA encoding for 16S rRNA of *P gingivalis* was detected in the ipsilateral hemisphere of LIP-Pg mice with stroke (Figure 3C), whereas none was found in the contralateral hemispheres of LIP-Pg mice, suggesting that *P gingivalis* could only infiltrate the injured hemisphere in our experimental conditions.

Therefore, these results suggest that *P gingivalis*-induced periodontitis primes the organism to a stronger inflammatory response following an injury.

## Role of *P gingivalis* Bacteremia on Stroke

Independently of the impact of the ligature on systemic inflammation, periodontal pathogens or their

by-products, such as lipopolysaccharide, can disseminate from periodontal tissues into the bloodstream, contributing to inflammatory response and potentially affecting distant tissues. Therefore, we postulated that *P gingivalis* could directly contribute to the pathophysiology of stroke. To test this hypothesis, we injected $1.10^8$ colony forming units of *P gingivalis* as previously described[23] or saline solution before inducing stroke with tMCAO (Figure 4A). Mice injected with *P gingivalis* exhibited significantly elevated circulating levels of lipopolysaccharide (Figure 4B). Remarkably, we observed a direct effect of systemic administration of *P gingivalis* on stroke severity. The volume of the necrotic area as detected by tetrazolium chloride staining was significantly increased (Figure 4C) in mice injected with *P gingivalis* (AUC *P gingivalis*: 13.9±0.8 versus sham: 7.6±0.7, $P<0.05$). Additionally, BBB disruption assessed by IgG staining (Figure 4D) and neutrophil accumulation evaluated by Ly6G staining (Figure 4E) were markedly heightened in the ischemic brain hemispheres following *P gingivalis* administration.

## Role of *P gingivalis* on BBB Disruption

As demonstrated above, the LIP-Pg experimental model and systemic administration of *P gingivalis* provoked BBB disruption, evidenced by IgG infiltration into the ischemic brain parenchyma. Consequently, we aimed to investigate the interaction between *P gingivalis* and the BBB using hCMEC/D3 cells as an in vitro model.[21]

In the first set of experiments conducted under normal oxygen conditions, we examined whether *P gingivalis* could destabilize the BBB, by using the xCELLigence system (Figure S3A). Upon reaching confluence (after 72 hours), hCMEC/D3 cells were exposed to increasing multiplicity of infection (MOI) of *P gingivalis*, and impedance was monitored every 10 minutes over a 6-hour period. Representative normalized cell index curves demonstrated a dose-dependent disruption effect of *P gingivalis* on the BBB function. Specifically, *P gingivalis* induced a dose-dependent reduction of the BBB integrity with a significant effect observed after 1 hour of culture with a MOI of 1:10 and 1:100. BBB integrity was further compromised at 4 hours and even more at 6 hours. It is noteworthy that the significant reduction of normalized cell index observed at a MOI of 1:10 and MOI 1:100 after 6 hours of Pg stimulation is attributed to cell death, which was assessed by alamarBlue assay (data not shown).

Given the critical role of platelet endothelial cell adhesion molecule-1 (PECAM-1) in maintaining the integrity of BBB[24] and its sensitivity to protease activity, we investigated whether *P gingivalis* proteases could disrupt the BBB by inducing proteolytic shedding of

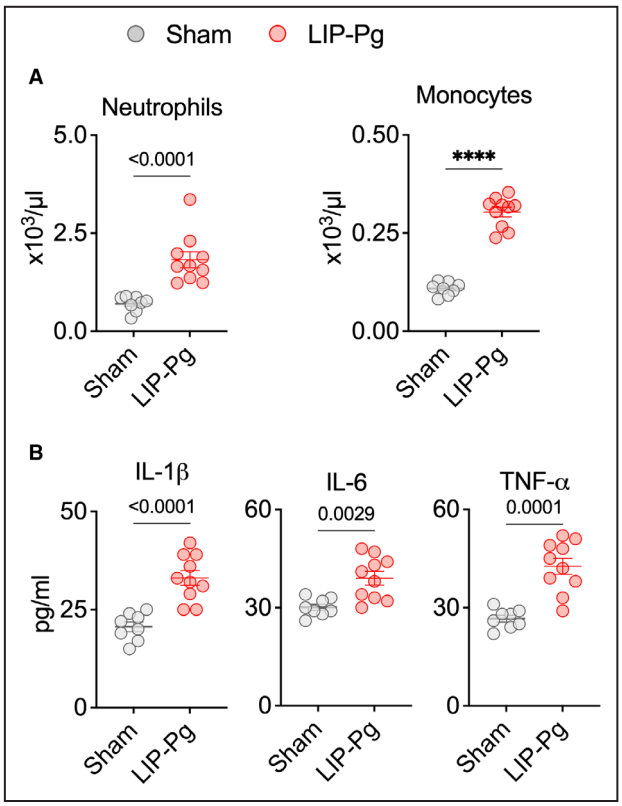


**Figure 2. *Porphyromonas gingivalis*-associated periodontitis induced systemic inflammation after tMCAO.**
**A**, Analysis of circulating neutrophil and monocyte blood counts by flow cytometry. **B**, Plasma cytokines by ELISA 2 days after tMCAO in sham (n=8) and LIP-Pg (n=10) mice. Statistical analysis was conducted using a nonparametric Mann-Whitney test. IL-1β indicates interleukin 1β; IL-6, interleukin 6; LIP-Pg, ligature-induced periodontitis-*Porphyromonas gingivalis*; tMCAO, transient middle cerebral artery occlusion; and TNF-α, tumor necrosis factor α.

extracellular domains of PECAM-1. To assess this, we used flow cytometry (Figure S3B) to measure the ratio of mean fluorescence intensity (MFI) of anti-CD31 (domain 1, conjugated to allophycocyanin [APC]) to MFI of anti-CD31 (C-terminal domain, conjugated to phycoerythrin [PE]). We observed a significant decrease in the MFI ratio due to APC fluorescence decrease after 4 hours of simulation, with an MOI of 1:10, suggesting that *P gingivalis* is able to induce the shedding of the extracellular domain of PECAM-1.

To further investigate the mechanism of *P gingivalis* on BBB disruption, we exposed D3 cells with *P gingivalis* in the presence of pharmacologic inhibitors KYT-41 targeting Arg- and Lys-gingipains or using mutant strains of *P gingivalis* strain (KDP136) devoid of both gingipains expression. Preincubation of *P gingivalis* with KYT inhibitor for 10 minutes or stimulation with the KDP *P gingivalis* strain did not result in a significant decrease of impedance compared with the wild-type *P gingivalis* strain (Figure S3C). Flow cytometry analysis

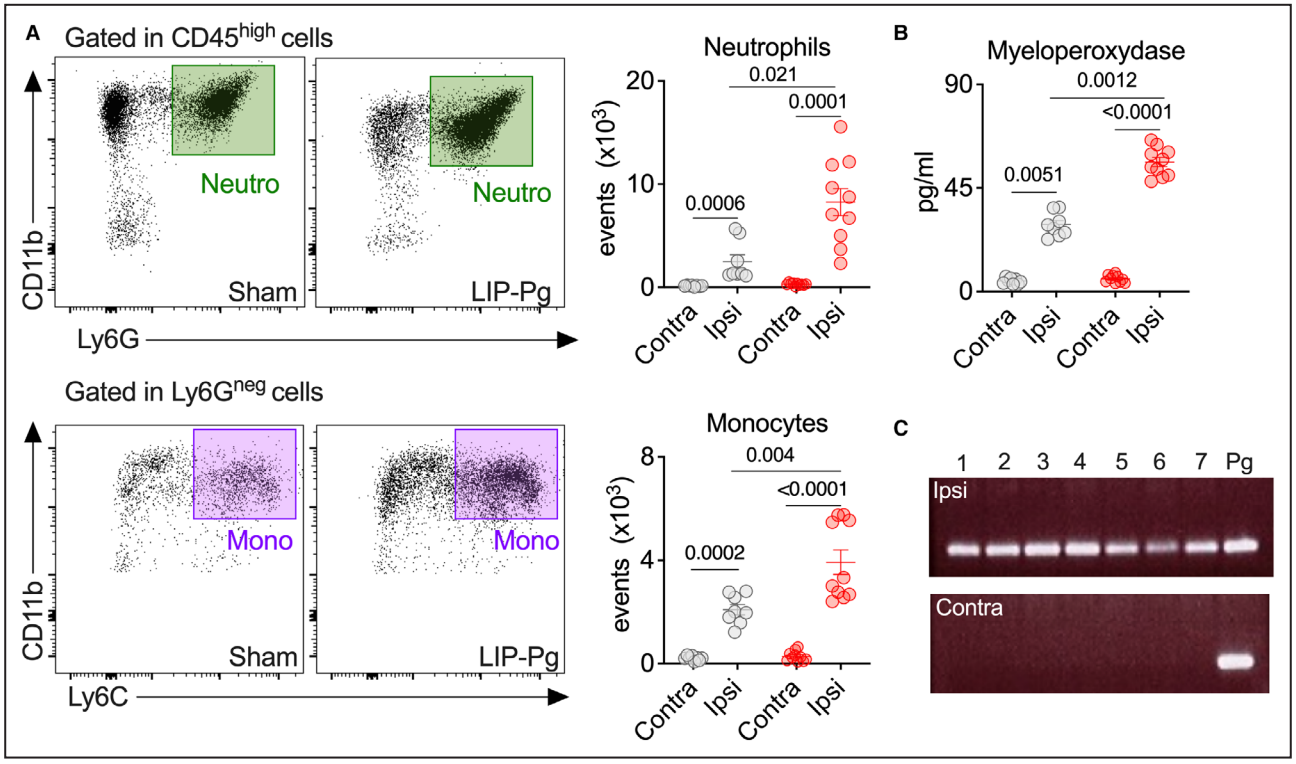


**Figure 3.** ***Porphyromonas gingivalis*-associated periodontitis induced brain inflammation and bacteria infiltration.**
**A**, Representative flow cytometry plots and quantification of neutrophils (CD11b⁺ Ly6G⁺) and monocytes (CD11b⁺ Ly6C⁺) in the ischemic hemispheres of LIP-Pg (n=10) and sham (n=8) mice. **B**, Quantification of myeloperoxidase by ELISA in homogenates of ipsilateral and contralateral hemisphere of sham (n=8) and LIP-Pg mice (n=10). **C**, Detection of *P gingivalis* in the ischemic brain of mice (n=7, no DNA available for 3 mice) in both the ipsilateral and contralateral hemispheres of LIP-Pg mice. DNA isolated from the culture of *P gingivalis* (ATCC33277) was used as a positive control. **A** and **B**, Kruskal-Wallis test followed by an uncorrected Dunn test. Contra indicates contralateral; Ipsi, ipsilateral; and LIP-Pg, ligature-induced periodontitis-*Porphyromonas gingivalis*.

of PECAM-1 revealed that the shedding of PECAM-1 from cells stimulated with KDP strain or KYT inhibitor (Figure S3D) was significantly reduced compared with the wild-type *P gingivalis* (MFI KYT: 2.7±0.2 and MFI KDP136: 2.8±0.3 versus *P gingivalis*: 1.7±0.1; $P<0.05$). These findings strongly indicate that gingipains plays a pivotal role in the disruption of BBB by *P gingivalis*.

## Impact of *P gingivalis* on hCMEC/D3 Permeability and Cell Junctions Under Stroke Conditions

To simulate cerebral ischemia/reperfusion as seen in stroke, we induced oxygen and glucose deprivation (OGD) for 4 hours followed by a 4-hour reperfusion period (OGD/R).

Preconditioning hCMEC/D3 cells with *P gingivalis* intensified the effect of OGD/R on BBB disruption and paracellular barrier function. Specifically, that prestimulation with *P gingivalis* exacerbated the impact of ischemia/reperfusion on impedance (OGD/R+*P gingivalis* versus OGD/R, $P<0.05$) (Figure 5A) and paracellular transport of macromolecules evaluated by the measure of fluorescence of dextran-FITC (fluorescein isothiocyanate) in the basolateral compartment of wells (OGD/R+*P gingivalis* versus OGD/R, $P<0.05$) (Figure 5B).

For a deeper understanding of the molecular mechanisms underlying our observations, we examined the expression levels of PECAM-1, occludin, and zonula occludens-1 (ZO-1), which are both tight-junction proteins (Figure 5C and Figure S4). Stimulation of hCMEC/D3 cells with *P gingivalis* (MOI1:10) under normal oxygen and glucose conditions significantly decreased the expression of PECAM-1, ZO-1, and occludin. Furthermore, although OGD/R had only a slight effect on junction proteins (no significant difference), *P gingivalis* significantly enhanced the impact of OGD/R.

## DISCUSSION

Our comprehensive series of experiments delved into the intricate relationship between preexisting *P gingivalis*-associated periodontitis and stroke in mice. By using robust experimental models, we have revealed compelling evidence of the detrimental effect of periodontitis on stroke lesions and outcomes.

To our knowledge, our findings demonstrated for the first time that mice subjected to periodontitis

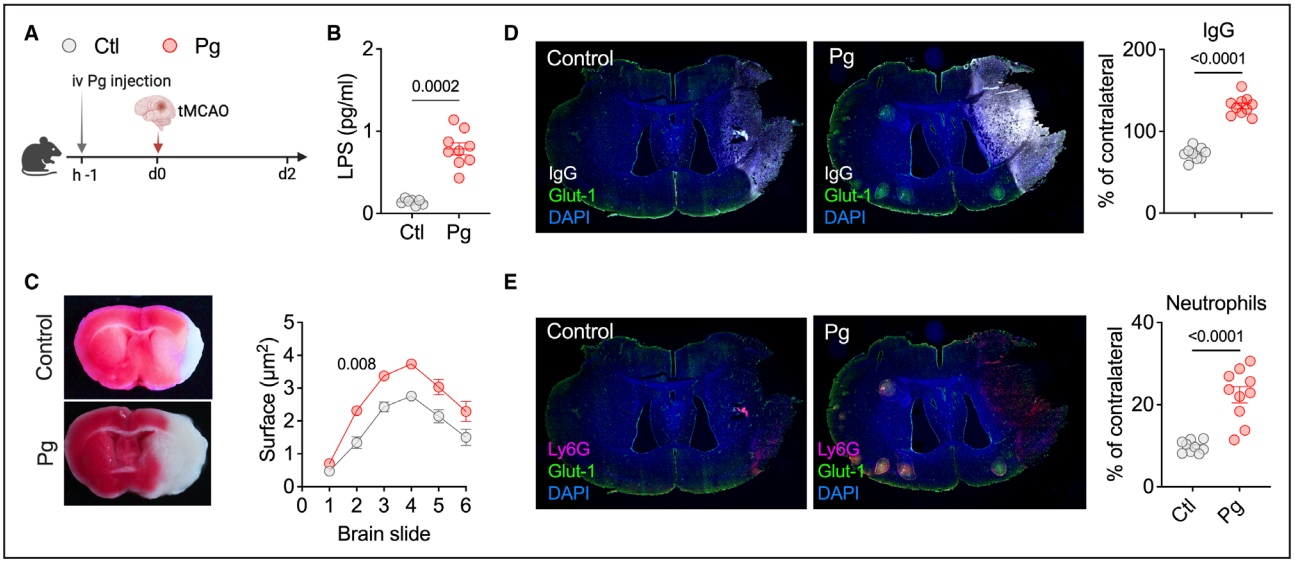


**Figure 4.** ***Porphyromonas gingivalis*** **bacteremia worsens stroke outcome.**
**A**, Study overview: *P gingivalis* or saline was injected into the mice Pg group (n=9) and control group (n=7), respectively, before inducing stroke by tMCAO. Euthanasia of mice occurred 2 days later. **B**, LPS concentration: Measured in plasma using a limulus amebocyte lysate kit for Pg and control mice. **C**, Stroke volume: representative sections of infarct areas labeled TTC in Pg and control mice and quantification. Each dot represents the mean obtained from all mice. **D** and **E**, BBB disruption and neutrophil infiltration: IgG staining (white) (**D**) and Ly6G+ cells (purple) (**E**) quantified in ischemic regions of Pg and control mice. Sections were costained with an anti-GLUT-1 (blood vessel marker) and DAPI. **A** through **E**, Mann-Whitney test. BBB indicates blood–brain barrier; Ctl, control; DAPI, (4′,6-diamidino-2phenylindole); GLUT-1, glucose transporter-1; IgG, immunoglobulin G; LPS, lipopolysaccharide; Pg, *Porphyromonas gingivalis*; tMCAO, transient middle cerebral artery occlusion; and TTC, tetrazolium chloride.

induced by *P gingivalis*-soaked tooth ligatures exhibited substantially larger stroke volume, increased brain edema, and more severe sensorimotor deficit following tMCAO compared with sham mice with stroke but without periodontitis. These results highlight the pathological role of periodontitis in exacerbating stroke severity. Although several studies[13–16] have demonstrated a link between periodontitis and ischemic stroke, our study introduces key advancements that significantly contribute to decrypt the mechanism underlying the relation between chronic periodontitis and stroke outcome. First, we used a clinically relevant ligature model of periodontitis with live *P gingivalis* and dynamic ligature changes ensuring robust and sustained periodontal inflammation. This approach addresses limitations of previous models that relied on *P gingivalis*-derived lipopolysaccharide, which fail to capture the full spectrum of host-pathogen interactions mediated by live bacteria, which can induce a broader range of host responses driven by the virulence factors such as gingipains and the ability to adhere and invade host cells. Our findings demonstrate for the first time that this model induces substantial systemic inflammation and exacerbates stroke outcomes, providing a more comprehensive understanding of the mechanisms underlying the periodontitis–stroke link.

Periodontitis, an inflammatory disease, is strongly associated with systemic inflammation and comorbidities including ischemic stroke. Here, we reported that LIP-Pg mice exhibited systemic inflammation as demonstrated by increased concentration of proinflammatory cytokines including IL-1β, IL-6, and TNF-α in plasma, which are important mediators of acute brain injury during stroke.[25,26] Additionally, we observed an elevated count of circulating neutrophils and monocytes in LIP-Pg mice. Neutrophils and monocytes are among the first line of defense after an ischemic stroke and infiltrate the brain 24 to 48 hours postischemia, participating in brain damage. The presence of a large number neutrophils is strongly associated with stroke severity, and neutrophil depletion has been shown to have a protective effect.[27] Here, we found that LIP-Pg-associated periodontitis, as compared with mice with tMCAO but without periodontitis, provoked a massive influx of neutrophils and monocytes at the border of the ischemic lesion. The increase of the chemokine CXCL1, which plays a pivotal role in attracting neutrophils to the site of inflammation, and of the adhesion molecules ICAM and VCAM, which facilitate the transmigration of immune cells across the BBB, highlight a potential mechanism through which periodontitis could exacerbate neutrophil infiltration following a stroke. After migration into

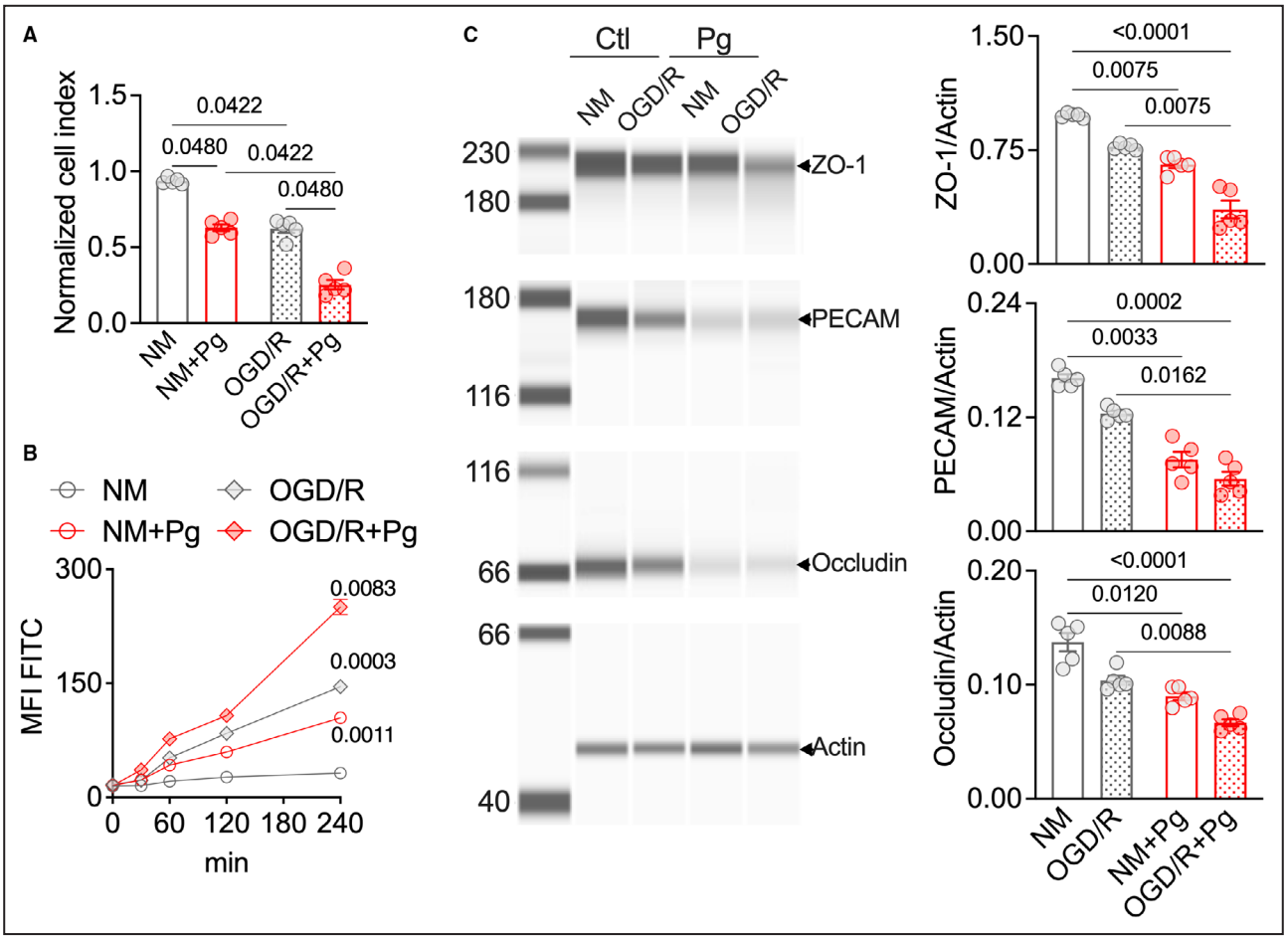


**Figure 5. Role of *Porphyromonas gingivalis* on in vitro blood–brain barrier disruption.**
Human cerebral microvascular endothelial (D3) cells were stimulated with Pg MOI 1:10 and subjected to OGD for 4 hours, followed by a 4-hour reperfusion period (O2+Glc) to simulate ischemia/reperfusion. **A**, Cell impedance: Blood–brain barrier function was assessed using xCELLigence technology (n=5). Impedance was monitored every 15 minutes during reperfusion (left). Normalized cell index was compared at 4 hours (right). **B**, Paracellular permeability FITC-labeled 70 kDa dextran was used to MFI at 30 minutes, 1 hour, 2 hours, and 4 hours (n=3). **C**, Junction protein expression: Representative images and quantification of PECAM-1, ZO-1, and occludin (n=5) via protein simple technology (the full-size blot can be viewed in Figure S4). **A** through **C**, Statistical analysis: Kruskal-Wallis/Dunn test; unstimulated normoxic cells served as controls. Ctl indicates control; FITC, fluorescein isothiocyanate; MFI, measure fluorescence intensity; MOI, multiplicity of infection; NM, normoxia; OGD, oxygen–glucose deprivation; OGD/R, oxygen–glucose deprivation/reperfusion; O2+Glc, xygen+golucose; PECAM, platelet endothelial cell adhesion molecule; Pg, *Porphyromonas gingivalis*; and ZO-1, zonula occludens-1.

the ischemic area, neutrophils and monocytes become activated and release several mediators, such as myeloperoxidase, that contribute to aggravated brain damage,[28] notably by generating reactive oxygen species that can promote apoptosis.

In addition to systemic inflammation, periodontitis may play a role in comorbidities through the dissemination of periodontal bacteria, including *P gingivalis*, and their by-products in the blood[29,30] and by reaching remote tissues, including atherosclerosis plaques[31,32] and thrombi from patients with stroke.[33] Although circulating live *P gingivalis* was not found in our experimental conditions, we detected lipopolysaccharide in the blood, and more importantly, we also detected DNA coding 16S RNA of *P gingivalis* within the ipsilateral (with stroke) hemisphere of LIP-Pg mice.

Importantly, we did not observe any brain lesions in mice subjected to periodontitis and sham surgery for tMCAO, as demonstrated by tetrazolium chloride staining and BBB disruption (assessed by IgG extravasation). These findings strongly indicate that the exacerbated brain damage observed in LIP-Pg+tMCAO mice is not due to a direct inflammatory effect or brain infection caused by *P gingivalis*, but rather results from a synergistic interaction of periodontitis and ischemia/reperfusion injury.

We also used a complementary approach in which *P gingivalis* is delivered via controlled intravenous injection at the time of stroke induction. The intravenous model

was designed to specifically address the relative contribution of localized periodontal disease from that of acute bacterial dissemination. This experiment mimics transient bacteremia episodes known to occur during routine oral activities or dental procedures. It also allows precise control of bacteremia dose and timing while isolating the systemic effects of *P gingivalis* from the confounding influence of local periodontal inflammation due to ligatures. Importantly, we demonstrated that both models achieved comparable systemic bacterial burdens, as indicated by plasma lipopolysaccharide levels, affirming the relevance IV injection in simulating bacteria dissemination associated with chronic periodontitis. Using this model, we confirmed that *P gingivalis* bacteremia alone is sufficient to exacerbate stroke injury, disrupt the BBB, and promote neutrophil infiltration.

We further elucidated the underlying mechanisms linking periodontitis and stroke revealing disrupted BBB integrity. LIP-Pg mice exhibited IgG extravasation in the ipsilateral hemisphere of mice with *P gingivalis*-associated periodontitis, suggesting that *P gingivalis* may exacerbate BBB disruption. To further investigate the cellular and molecular mechanisms of *P gingivalis* on the BBB, we used hCMEC/D3 cells[21] that were subjected to ischemia/reperfusion with oxygen glucose deprivation followed by normal oxygen and glucose conditions.[34] We found that OGD/R cells stimulated with *P gingivalis* exhibited highly impaired BBB functions and notably cell leakage, allowing the paracellular transport of macromolecules as compared with OGD/R cells, which might be due to the decrease of the expression of PECAM-1 and tight-junction proteins ZO-1 and occludin.

*P gingivalis* possesses numerous virulence factors that enable it to evade the immune system and provoke an inflammatory response and tissue damage. Among these factors, gingipain is a key mediator of *P gingivalis* toxicity and has been shown to degrade various host proteins, including collagen, fibrinogen, and immunoglobulins, and to modulate inflammatory and immune responses.[35] To identify the role of gingipains on BBB disruption, we used gingipain-deficient mutants of *P gingivalis* and pharmacological inhibitors. Our results showed that inhibition of gingipains protect the integrity of the BBB, notably by acting on junction proteins. Therefore, it will be interesting to evaluate the efficacy of gingipain inhibitors in vivo using our experimental model of stroke with preexisting *P gingivalis* periodontitis. In this sense, Dominy et al[36] previously observed that the lysine-gingipain inhibitor, COR, reduces brain *P gingivalis* infection and limits neurodegeneration in aged mice.

Even if *P gingivalis* is an important periodontal pathogen, a mono-infection experimental model does not closely reflect human periodontitis, which involves a broad range of periodontal pathogens and oral dysbiosis. Therefore, we propose developing a polymicrobial model of periodontitis in mice for future investigations, specifically incorporating periodontal bacteria composing the red complex[37] to elucidate the intricate interplay between the major periodontal bacteria and stroke. In addition, the restriction to male mice represents a limitation. Although supported by epidemiological patterns, inclusion of both sexes in future studies will be critical to enhance the translational relevance and generalizability of our findings.

In conclusion, this study marks the first direct demonstration of the impact of periodontitis on stroke in mice, underscoring the importance of considering periodontitis in stroke research and clinical management. Our study provides mechanistic insights into how periodontitis, particularly when associated with *P gingivalis*, may worsen stroke outcomes. The research presented here opens up several important perspectives for future investigations and potential clinical applications. Understanding the relationship between periodontitis and stroke may pave the way for the preventive measures that include improving the diagnostic of periodontitis in patients with ischemic stroke and encouraging the setting-up of interventional clinical trials to evaluate the benefit of periodontal treatment on ischemic stroke outcome and to propose personalized interventions for patients with stroke and finally to reduce the health and economic burden of stroke.

## ARTICLE INFORMATION

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### Disclosures

None.

### Supplemental Material

Data S1: Supplemental Methods
Tables S1–S4
Figures S1–S4

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