

Peripheral and central galactose/ β -galactoside-related alterations in ischemic stroke: Clinical associations of circulating LPH and experimental evidence for Glb1 and galectin-3

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Abstract

Aims

Circulating metabolic biomarkers for acute ischemic stroke (AIS) remain incompletely characterized. We examined galactose-related alterations at both peripheral and central levels, focusing on serum lactase-phlorizin hydrolase (LPH) as a candidate circulating biomarker and on the roles of Glb1 and galectin-3 (Gal-3) in ischemic neuronal injury.

Methods

Untargeted serum metabolomics was first performed in an exploratory AIS cohort to identify altered carbohydrate-related metabolic patterns. Serum LPH was then assessed in an independent hospital-based cohort and a community-based cross-sectional cohort of 1,810 participants. Transient middle cerebral artery occlusion/reperfusion (tMCAO/R) in rats and oxygen-glucose deprivation/reoxygenation (OGD/R) in primary neurons were used to examine the effects of *Glb1* knockdown and Gal-3 inhibition with GB1107, alone and in combination, on neuronal injury and oxidative stress.

Results

Serum LPH was significantly higher in patients with AIS and discriminated AIS from healthy controls with an AUC of 0.908. In the community cohort, higher LPH remained associated with stroke status after adjustment for major vascular risk factors. Metabolomic analysis also showed alterations in galactose-related metabolism. Glb1 and Gal-3 expression increased in ischemic rat brain tissue and OGD/R-exposed neurons. Targeting either Glb1 or Gal-3 reduced neuronal injury and oxidative stress. Combined intervention produced greater protection than either treatment alone.

Conclusion

Circulating LPH is associated with ischemic stroke and may have value as a peripheral metabolic biomarker. Glb1 and Gal-3 contribute to ischemic neuronal injury and may represent neuroprotective targets. Together, these findings reveal complementary peripheral metabolic alterations and central injury responses involving Glb1 and Gal-3 in ischemic stroke.

1. Introduction

Ischemic stroke (IS) remains a leading cause of mortality and long-term disability worldwide, imposing a substantial clinical and socioeconomic burden[1–3]. Although reperfusion therapies, including intravenous thrombolysis and mechanical thrombectomy, have markedly improved outcomes in eligible patients, their benefits remain constrained by narrow therapeutic windows, hemorrhagic complications,

and incomplete neurological recovery in a considerable proportion of patients[4–6]. These limitations highlight the need for accessible biomarkers associated with IS and a better understanding of the molecular events underlying ischemic brain injury.

Metabolic reprogramming is a prominent feature of cerebral ischemia[7]. Beyond impaired energy production, ischemia alters redox homeostasis, glycosylation, lysosomal function, and inflammatory responses[7, 8]. Most previous studies have focused on glucose utilization and energy failure, but ischemia-related metabolic disturbances extend to other carbohydrate pathways, including galactose-related processes[9, 10]. Galactose- and β -galactoside-related biology is involved in glycan metabolism, lysosomal homeostasis, and cellular stress responses, all of which may influence neuronal vulnerability after ischemia[11–13]. Importantly, the enzymes and substrates involved in these processes have distinct tissue distributions. Galactose-related alterations may therefore manifest differently in the peripheral circulation and the brain, yet their peripheral and central features in IS remain incompletely characterized.

Lactase-phlorizin hydrolase (LPH), encoded by *LCT*, is predominantly expressed at the brush border of the small intestine, where it catalyzes lactose hydrolysis into D-glucose and D-galactose[14, 15]. Previous work has largely focused on lactose digestion, lactase deficiency, and lactose intolerance[14, 15]. More broadly, galactose-related metabolic disturbances have been associated with oxidative stress, insulin resistance, vascular dysfunction, and inflammatory responses[16, 17]. Whether circulating LPH levels is altered in IS and whether it has potential value as a peripheral metabolic biomarker remain unclear.

In the brain, β -galactosidase 1 (Glb1) and galectin-3 (Gal-3) participate in distinct aspects of galactose- and β -galactoside-related biology. Glb1, encoded by *Glb1* **gene**, is a lysosomal hydrolase involved in the degradation of β -galactoside-containing glycoconjugates and contributes to intracellular glycan and lysosomal homeostasis[18]. Lysosomal dysfunction and impaired glycan degradation may increase neuronal susceptibility to ischemic injury[18], and altered *Glb1* expression has been reported in ischemic brain tissue[19]. Gal-3, encoded by *Lgals3*, is a β -galactoside-binding lectin involved in inflammatory signaling, oxidative stress, and tissue remodeling[20–22]. Increased Gal-3 expression has been observed after cerebral ischemia and has been associated with stroke severity and neurological outcomes[23, 24]. These separate lines of evidence implicate Glb1 and Gal-3 in post-ischemic brain injury, yet their functional contributions have rarely been examined within the same experimental framework, particularly through direct comparison of single and combined targeting in complementary in vivo and in vitro models. Their functional relationship therefore remains poorly defined.

Accordingly, the present study investigated peripheral and central galactose-related alterations associated with IS. We first characterized serum metabolic changes and evaluated circulating LPH as a candidate peripheral metabolic biomarker in independent clinical cohorts. We then examined Glb1 and Gal-3 in cerebral ischemia and assessed their functional contributions using complementary tMCAO/R and OGD/R models, including single and combined interventions. The study had two primary aims: to

determine whether circulating LPH represents a clinically relevant peripheral metabolic feature of IS and to characterize the functional roles and potential relationship of Glb1 and Gal-3 in ischemic neuronal injury.

2. Materials and Methods

Experimental procedures, cohort definitions, metabolomics methods, reagents, and key materials are summarized here; detailed protocols, materials, and references are provided in the *Supplementary Materials*.

2.1 Clinical cohorts

Three clinical cohorts were included for exploratory metabolomic profiling, hospital-based validation, and community-based association analyses. The exploratory cohort consisted of 10 patients with acute ischemic stroke (AIS) and 10 age-matched healthy controls recruited from the Affiliated Hospital of Guizhou Medical University between January 2020 and June 2022. Serum samples from this cohort were used for untargeted metabolomic profiling.

A second hospital-based cohort was recruited from the same hospital between January and December 2022 and included 20 patients with AIS and 20 healthy individuals undergoing routine health examinations. This cohort was used to measure serum LPH and evaluate its ability to distinguish patients with AIS from healthy controls.

The third cohort was derived from community stroke screening programs conducted in Guiyang and Zunyi, Guizhou Province, between February 2017 and December 2022. A total of 1,810 permanent residents aged ≥ 40 years were included. Participants were classified into five categories: low risk, intermediate risk, high risk, transient ischemic attack (TIA), or previous ischemic stroke, according to predefined stroke-screening criteria. This cohort was used to examine the association of serum LPH with cerebrovascular risk and stroke status.

2.2 Clinical data and serum collection

Demographic characteristics, lifestyle factors, vascular risk factors, and laboratory data were collected according to the design of each cohort. For the hospital-based cohorts, fasting venous blood was collected in the morning, and serum was separated and stored at -80°C until analysis.

2.3 Untargeted metabolomics analysis

Untargeted serum metabolomic profiling was performed using ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) in both positive- and negative-ion modes. Raw data were processed using MS-DIAL, and metabolic differences between the AIS and control groups were examined by orthogonal partial least squares discriminant analysis (OPLS-DA). Differential metabolites were defined by a variable importance in projection (VIP) value > 1 and $P <$

0.05. Pathway enrichment was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Small Molecule Pathway Database (SMPDB), with the Human Metabolome Database (HMDB) used for subsequent galactose metabolism-related analysis.

2.4 Enzyme-linked immunosorbent assay (ELISA)

Serum LPH levels were quantified using a commercial sandwich ELISA kit (Human Lactase ELISA Kit, Catalog No. DL-LCT-Hu; Wuxi Donglin Sci & Tech Development Co., Ltd., China) according to the manufacturer's instructions.

2.5 Rat tMCAO/R model and interventions

Adult male Sprague–Dawley (SD) rats were subjected to transient middle cerebral artery occlusion/reperfusion (tMCAO/R; 2 h ischemia followed by 24 h reperfusion). Animals were randomly assigned to experimental groups (n = 6 per group). Two experimental sets were used: (1) Sham and tMCAO/R groups for model characterization; and (2) tMCAO/R + DMSO + sh-NC, tMCAO/R + GB1107 + sh-NC, tMCAO/R + DMSO + sh-Glb1, and tMCAO/R + GB1107 + sh-Glb1 groups for intervention experiments. GB1107 was administered intraperitoneally at 0.1 mg/kg/day, and sh-Glb1 lentivirus was delivered by intracerebroventricular injection; both interventions were initiated one week before tMCAO/R induction.

2.6 Neurological deficit scoring

Neurological deficits were assessed using the Longa scoring system, with higher scores indicating more severe impairment.

2.7 2,3,5-Triphenyltetrazolium chloride (TTC) staining

Cerebral infarct area was assessed via TTC staining at 24 h post-reperfusion. Fresh brain slices were incubated with 2% TTC solution, and infarct sizes were quantified with ImageJ software. Red staining represented viable tissue, while infarcted regions appeared pale white.

2.8 Hematoxylin and eosin (H&E) staining

Brain tissues were subjected to hematoxylin and eosin (H&E) staining for histopathological evaluation. Tissue sections were prepared using standard procedures, and morphological changes were examined under a light microscope.

2.9 Oxidative stress and biochemical assays

Levels of reactive oxygen species (ROS), malondialdehyde (MDA), glutathione (GSH), D-glucose, and D-galactose were determined using commercially available assay kits according to the manufacturers' instructions.

2.10 Immunofluorescence staining and western blotting

Glb1 and Gal-3 protein expression was assessed by western blotting. Immunofluorescence staining was performed to examine the expression and cellular localization of Glb1 and Gal-3 in neurons.

2.11 Primary culture of rat cortical neurons and oxygen–glucose deprivation/reoxygenation (OGD/R) model

Primary cortical neurons were isolated from E16-18 SD rat embryos and cultured in Neurobasal complete medium. Primary rat cortical neurons were subjected to oxygen–glucose deprivation/reoxygenation (OGD/R; 2 h deprivation followed by 24 h reoxygenation). Two independent experimental sets were performed: (1) Control and OGD/R groups; and (2) OGD/R + DMSO + sh-NC, OGD/R + GB1107 + sh-NC, OGD/R + sh-Glb1 + DMSO, and OGD/R + GB1107 + sh-Glb1 groups. GB1107 was applied at a final concentration of 1 µg/mL. OGD/R treatment was initiated 24 h after lentiviral transduction.

2.12 Cell viability and cytotoxicity assays

Cell viability was assessed using a Cell Counting Kit-8 (CCK-8) assay, and cytotoxicity was evaluated by measuring lactate dehydrogenase (LDH) release.

2.13 TUNEL staining

Apoptosis of OGD/R-injured primary cortical neurons was detected using the One-Step TUNEL Apoptosis Assay Kit (green fluorescence, Cat. No. C1088, Beyotime Biotechnology, Shanghai, China).

2.14 Statistical analysis

Normality of continuous variables was assessed using the Shapiro–Wilk test. Normally distributed data are presented as mean ± standard deviation, non-normally distributed data as median (interquartile range), and categorical variables as n (%). For in vivo rat and in vitro neuronal experimental data, comparisons between two groups were performed using the independent-samples t-test or Mann–Whitney U test, as appropriate. Multiple-group comparisons were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, or the Kruskal–Wallis H test followed by Dunn's post-hoc test for datasets that violated normality assumptions.

For clinical cohort data, receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic discriminatory capacity of serum LPH for AIS, with the area under the curve (AUC) and 95% confidence intervals calculated. Univariable and multivariable binary logistic regression analyses were performed to explore associations between serum LPH levels and stroke status. Multivariable models were adjusted for age, hypertension, diabetes mellitus, and dyslipidemia, and results were reported as odds ratios (ORs) with 95% confidence intervals (CIs).

All statistical analyses were performed using SPSS version 29.0 and GraphPad Prism version 9.0. All statistical tests were two-tailed, and a *P* value < 0.05 was considered statistically significant.

3. Results

3.1 Serum metabolomic profiling reveals candidate metabolic alterations in AIS

Untargeted serum metabolomic profiling was performed in an exploratory cohort comprising 10 patients with AIS and 10 healthy controls. Baseline demographic, clinical, and lipid characteristics did not differ significantly between groups (all $P > 0.05$; **Supplementary Table S1**).

In the negative-ion mode, orthogonal partial least-squares discriminant analysis (OPLS-DA) demonstrated a separation tendency between the AIS and control groups between the AIS and control groups, with an R^2Y of 0.993 and a Q^2 of 0.755 (Fig. 1A). None of the 200 permuted models yielded a Q^2 value exceeding that of the original model ($P < 0.005$; Fig. 1B). The corresponding S-plot highlighted variables contributing most strongly to the modeled group distinction (Fig. 1C).

Across the positive- and negative-ion modes, 95 metabolites met the prespecified exploratory screening criteria, including 38 metabolites detected in the negative-ion mode (Fig. 1D). The heatmap further demonstrated distinct abundance patterns of these candidate metabolites between the two groups (Fig. 1E).

3.2 Galactose-related metabolic alterations are identified in AIS serum metabolomics

In the negative-ion (ESI^-) mode, galactose metabolism was enriched in both the KEGG and SMPDB analyses (Fig. 2A). KEGG analysis also identified starch and sucrose metabolism, whereas SMPDB mapped the candidate metabolites to several related carbohydrate metabolic processes, including lactose degradation, lactose synthesis, glycolysis, and gluconeogenesis. Collectively, these cross-database results indicated that multiple carbohydrate-related pathways, including galactose metabolism, were represented among the candidate metabolites detected in the negative-ion mode.

In the ESI^+ mode, the candidate metabolites were primarily associated with amino acid metabolism, primary bile acid biosynthesis, purine metabolism, and steroid hormone biosynthesis in the KEGG and SMPDB analyses (Fig. 2B).

Given the concordant nominal enrichment of galactose metabolism in the ESI^- mode KEGG and SMPDB analyses, a focused cross-database intersection analysis was subsequently performed. Metabolites annotated to galactose metabolism were retrieved independently from KEGG and HMDB and intersected with the 95 candidate metabolites identified by untargeted metabolomics. Fourteen metabolites were shared between the KEGG and HMDB datasets, whereas D-glucose was the only metabolite common to KEGG, HMDB, and the metabolomics-derived candidate set (Fig. 2C).

The raw spectral intensity of D-glucose was higher in patients with AIS than in healthy controls ($P < 0.001$; Fig. 2D). As the sole metabolite shared by the KEGG, HMDB, and metabolomics-derived datasets, D-glucose was therefore carried forward for subsequent identification of metabolism-associated candidate genes.

3.3 Metabolite–enzyme network analysis reveals LPH as a circulating candidate associated with galactose metabolism

To explore the enzyme and protein associations underlying the observed metabolic alterations, the candidate metabolites were mapped to curated metabolite–reaction–enzyme/gene relationships using Metscape. The resulting network comprised five disconnected functional subnetworks related to steroid, carbohydrate, lipid, purine, and amino acid metabolism. Within the carbohydrate metabolism-associated subnetwork, the *LCT* gene node was linked to lactase and, through the corresponding lactose-hydrolysis reaction, to lactose, D-galactose, and D-glucose (Fig. 3A).

Thirty-one metabolite-associated proteins were subsequently analyzed using STRING to construct a protein–protein interaction network (Fig. 3B). The network contained multiple proteins involved in carbohydrate metabolism, including LPH, HK1, HK2, HK3, MGAM, and GANC. In the simplified carbohydrate metabolism-associated subnetwork, LPH, encoded by *LCT*, had a degree of 8 and was located within a connected protein group containing HK1, HK2, HK3, MGAM, and GANC (Fig. 3C).

Although LPH is not the node with the highest degree in the entire network, its participation in the lactose hydrolysis reaction, its position in the carbohydrate metabolism-related network, and its connectivity with other carbohydrate-processing proteins make it a candidate for network association in the subsequent exploratory clinical assessment.

3.4 Serum LPH is elevated in a hospital-based AIS cohort

Serum LPH concentrations were measured in a separate single-center cohort consisting of 20 patients with AIS and 20 healthy controls. No statistically significant between-group differences were observed in baseline demographic characteristics or vascular risk factors (all $P > 0.05$; **Supplementary Table S2**).

Serum LPH concentrations were significantly increased in patients with AIS compared with healthy controls (91.52 ± 3.85 vs. 86.00 ± 3.35 ng/mL, $P < 0.001$; **Supplementary Table S2**). ROC analysis yielded an AUC of 0.908 (95% CI, 0.820–0.995; $P < 0.001$) for distinguishing patients with AIS from healthy controls (95% CI, 0.820–0.995; $P < 0.001$; **Supplementary Fig. S1 and Supplementary Table S3**).

3.5 Serum LPH is associated with stroke status in a community-based cohort

To further evaluate the association between circulating LPH and cerebrovascular conditions, serum LPH concentrations were compared across the clinical groups in the community-based cohort. Serum LPH

concentrations differed across the five clinical categories ($P = 0.002$; **Supplementary Table S4**). The serum LPH levels were higher in stroke patients, while the LPH concentration was relatively lower in the TIA group. This suggests that LPH does not simply change linearly with the severity of cerebrovascular events.

We first examined the association between serum LPH levels and high-risk cerebrovascular status. Higher serum LPH levels were associated with greater odds of high-risk status in the community cohort (OR = 1.208, 95% CI: 1.132–1.289, $P < 0.001$; **Supplementary Table S5**). When participants were categorized according to the median serum LPH concentration, those in the higher LPH group also had greater odds of high-risk status than those in the lower LPH group (OR = 1.297, 95% CI: 1.154–1.458, $P < 0.001$; **Supplementary Table S6**).

We next evaluated the relationship between serum LPH levels and stroke status. In univariable logistic regression analysis, serum LPH levels, age, hypertension, diabetes mellitus, and dyslipidemia were significantly associated with stroke status (all $P < 0.05$; **Supplementary Table S7**). Serum LPH levels were positively associated with stroke status (OR = 1.264, 95% CI: 1.185–1.348, $P < 0.001$). This association remained significant after adjustment for age, hypertension, diabetes mellitus, and dyslipidemia (adjusted OR = 1.244, 95% CI: 1.139–1.359, $P < 0.001$; **Supplementary Table S8**).

3.6 Integrative bioinformatics prioritizes *Glb1* as a candidate associated with galactose-related metabolic alterations in ischemic stroke

To further investigate the molecular changes associated with galactose metabolic dysregulation identified by serum metabolomics, we performed an independent transcriptome-based integrative analysis to identify candidate genes involved in ischemic brain responses.

Genes associated with IS were collected from the GeneCards and CTD databases, while D-glucose metabolism-related genes were obtained from the HMDB database. Intersection analysis identified 14 genes shared between ischemic stroke-related genes and carbohydrate metabolism-associated genes (Fig. 4A). A protein–protein interaction (PPI) network was subsequently constructed using the STRING database. Several highly connected genes were identified within this network, including *HK1*, *GCK*, *MGAM*, *SI*, *AKR1B1*, and *Glb1* (Fig. 4B), suggesting their potential involvement in carbohydrate metabolism-related processes.

To further prioritize candidates with transcriptional alterations in ischemic brain tissue, differential expression analysis was performed using the rat MCAO transcriptomic dataset GSE61616. A total of 2,018 differentially expressed genes (DEGs) were identified between ischemic and sham groups (Fig. 4C–D). Integration of the 14 metabolism-associated candidate genes with the GSE61616 DEGs identified two overlapping genes, *Glb1* and *Si* (Fig. 4E). Expression analysis demonstrated that *Glb1* was significantly upregulated, whereas *Si* was downregulated in ischemic brain tissue (Fig. 4F).

Given the prominent upregulation of *Glb1* after ischemic injury, we further explored its associated molecular network using PPI analysis. The *Glb1*-centered network revealed several genes involved in lysosomal function and inflammatory responses. Among these interacting genes, *Lgals3* (encoding galectin-3, Gal-3) showed a close association with *Glb1* in the interaction network (Fig. 4G). Expression analysis further showed that *Glb1*-associated genes, including *Lgals3*, were consistently upregulated in ischemic samples (Fig. 4H).

Collectively, these integrative analyses identified *Glb1* as a candidate molecular mediator associated with galactose metabolic dysregulation in ischemic stroke and highlighted *Lgals3* as a potential interacting molecule within the *Glb1*-associated network, providing a rationale for subsequent functional validation.

3.7 Cerebral ischemia is accompanied by Glb1 and Gal-3 upregulation and galactose-related metabolic alterations

We characterized pathological, oxidative, molecular and metabolic phenotypes induced by ischemia-reperfusion injury using the tMCAO/R rat model. TTC staining and infarct quantification revealed extensive ipsilateral brain lesions in tMCAO/R rats, while sham rats displayed no infarct tissue (Fig. 5A, B). H&E staining of the peri-infarct cortex showed marked histopathological injury in the tMCAO/R group, including disorganized cell layout, cytoplasmic vacuolation and widespread neuronal loss (Fig. 5C).

Oxidative stress represents a hallmark of ischemic brain damage. Cortical assays showed significantly increased ROS and MDA levels and decreased GSH levels in ischemic tissue (Fig. 5D–F). We assessed Glb1 and Gal-3 expression via western blot and immunofluorescence. Immunoblotting verified significantly higher total Glb1 and Gal-3 protein abundance in the tMCAO/R group (Fig. 5G, H). Immunofluorescence co-staining with NeuN demonstrated neuronal expression of both Glb1 and Gal-3 in the peri-infarct cortex, with significantly increased Glb1- and Gal-3-positive neuronal staining in tMCAO/R rats (Fig. 5I, J).

Biochemical quantification showed significantly increased levels of D-glucose and D-galactose in the tMCAO/R group compared with the sham group (Fig. 5K), consistent with altered galactose-related carbohydrate metabolism following cerebral ischemia.

Taken together, these baseline data demonstrate that tMCAO/R injury coincides with neuronal Glb1 and Gal-3 overexpression, aggravated oxidative stress and altered galactose metabolism. These coordinated phenotypes provided the rationale for subsequent investigation of the functional contributions of Glb1 and Gal-3 to ischemic brain injury.

3.8 Gal-3 inhibition and Glb1 knockdown attenuate ischemic brain injury and oxidative stress in vivo

To investigate the effects of Gal-3 inhibition and Glb1 knockdown on ischemic brain injury in vivo, rats subjected to tMCAO/R received GB1107, sh-Glb1 lentivirus, or their combined intervention.

Immunofluorescence staining and Western blotting were first performed to verify the effects of each intervention. Compared with the tMCAO/R + DMSO + sh-NC group, GB1107 treatment significantly reduced Gal-3 protein expression without significantly affecting Glb1 levels. Conversely, sh-Glb1 significantly decreased Glb1 expression but did not significantly alter Gal-3 expression. Combined treatment with GB1107 and sh-Glb1 significantly reduced both Gal-3 and Glb1 protein levels, confirming effective simultaneous targeting of the two proteins (Fig. 6A–E).

TTC staining revealed that either GB1107 treatment or Glb1 knockdown significantly reduced cerebral infarct area compared with the tMCAO/R + DMSO + sh-NC group. Moreover, the combined intervention group exhibited a significantly smaller infarct area than either single-intervention group, indicating greater protection against ischemic brain injury (Fig. 6F, G). Consistently, neurological deficit scores were significantly decreased following either GB1107 treatment or Glb1 knockdown, and rats receiving the combined intervention exhibited significantly lower neurological deficit scores than those receiving either intervention alone (Fig. 6H).

H&E staining revealed pronounced histopathological injury in the ischemic brain tissue of the tMCAO/R + DMSO + sh-NC group, characterized by disrupted tissue architecture, neuronal shrinkage, nuclear pyknosis, and widened intercellular spaces. These pathological alterations were alleviated by either GB1107 treatment or Glb1 knockdown. Furthermore, the combined intervention resulted in better preservation of tissue architecture and neuronal morphology than either single intervention (Fig. 6I).

We next assessed oxidative stress by measuring ROS accumulation, MDA content, and GSH levels. Compared with the tMCAO/R + DMSO + sh-NC group, either GB1107 treatment or Glb1 knockdown significantly decreased ROS and MDA levels while increasing GSH levels. Combined treatment further reduced ROS and MDA levels and increased GSH levels compared with either single intervention (Fig. 6J–L).

Collectively, these findings demonstrate that Gal-3 inhibition and Glb1 knockdown each alleviate cerebral infarction, neurological deficits, histopathological injury, and oxidative stress in tMCAO/R rats. Combined targeting of Gal-3 and Glb1 produced significantly greater neuroprotective and antioxidant effects than either intervention alone. Notably, GB1107 did not significantly alter Glb1 protein expression, whereas Glb1 knockdown did not significantly affect Gal-3 protein expression.

3.9 Gal-3 inhibition and Glb1 knockdown alleviate OGD/R-induced neuronal injury, apoptosis, and oxidative stress in vitro

To further investigate the effects of Gal-3 inhibition and Glb1 knockdown under ischemia/reperfusion-mimicking conditions in vitro, primary cortical neurons were subjected to OGD/R and treated with GB1107, sh-Glb1 lentivirus, or their combined intervention. Western blotting revealed that OGD/R significantly increased both Glb1 and Gal-3 protein expression compared with the control group (Fig. 7A). We then verified the effects of each intervention. Compared with the OGD/R + DMSO + sh-NC

group, GB1107 treatment significantly reduced Gal-3 protein expression without significantly affecting Glb1 levels. Conversely, sh-Glb1 significantly decreased Glb1 expression but did not significantly alter Gal-3 expression. Combined treatment with GB1107 and sh-Glb1 significantly reduced the expression of both proteins (Fig. 7B).

Cellular injury was subsequently evaluated by cell viability and LDH release assays. Compared with the OGD/R + DMSO + sh-NC group, either GB1107 treatment or Glb1 knockdown significantly increased cell viability, whereas the combined intervention resulted in significantly higher cell viability than either single intervention (Fig. 7C). Consistently, LDH release was significantly decreased following either GB1107 treatment or Glb1 knockdown and was further reduced by the combined intervention, indicating greater attenuation of OGD/R-induced cellular injury (Fig. 7D).

We next evaluated intracellular oxidative stress using DHE fluorescence staining. Pronounced ROS accumulation was observed in neurons in the OGD/R + DMSO + sh-NC group. Either GB1107 treatment or Glb1 knockdown significantly reduced DHE fluorescence intensity, while the combined intervention resulted in a further significant reduction compared with either single intervention (Fig. 7E, G). TUNEL staining showed a similar protective pattern. The apoptotic rate was significantly decreased following either Gal-3 inhibition or Glb1 knockdown and was further reduced by the combined intervention (Fig. 7F, H).

Consistent with the ROS findings, either GB1107 treatment or Glb1 knockdown significantly decreased MDA levels and increased GSH levels compared with the OGD/R + DMSO + sh-NC group. Combined targeting of Gal-3 and Glb1 resulted in significantly lower MDA levels and higher GSH levels than either single intervention (Fig. 7I, J).

Collectively, these findings demonstrate that OGD/R induces upregulation of Glb1 and Gal-3 in primary cortical neurons. Gal-3 inhibition or Glb1 knockdown each alleviates OGD/R-induced neuronal injury, apoptosis, and oxidative stress, whereas combined targeting of Gal-3 and Glb1 produces significantly greater cytoprotective and antioxidant effects than either intervention alone.

4. Discussion

This study characterized galactose-related alterations in ischemic stroke at both peripheral and central levels. Serum LPH concentrations were elevated in AIS and remained significantly associated with previous ischemic stroke status in the community cohort after adjustment for major vascular risk factors. Glb1 and Gal-3 were upregulated in ischemic brain tissue and OGD/R-exposed primary cortical neurons. Either Glb1 knockdown or pharmacological inhibition of Gal-3 alleviated neuronal injury and oxidative stress, whereas dual targeting produced greater protection than either intervention alone. These findings identify two complementary levels of IS-associated alterations: peripheral metabolic changes associated with circulating LPH and central neuronal injury responses involving Glb1 and Gal-3.

LPH has received little attention in cerebrovascular research. Encoded by *LCT*, it is predominantly expressed at the small intestinal brush border and catalyzes lactose hydrolysis into D-glucose and D-galactose[14, 15]. Existing studies mainly address lactose digestion, lactase deficiency, and related gastrointestinal disorders. In our metabolite–enzyme network, *LCT* emerged as a candidate node linking lactose hydrolysis with D-glucose and D-galactose. Subsequent clinical analyses further supported the potential relevance of circulating LPH. Serum LPH concentrations were significantly higher in patients with AIS, with an AUC of 0.908 for distinguishing patients with AIS from healthy controls. In the community cohort, higher serum LPH was also independently associated with previous ischemic stroke status after multivariable adjustment. These findings suggest that circulating LPH may provide complementary metabolic information in the assessment of ischemic stroke. Its interpretation, however, should remain cautious. LPH levels did not increase progressively across cerebrovascular risk groups, and the cross-sectional design precludes causal inference. At present, LPH is better regarded as an IS-associated biomarker candidate rather than a predictive biomarker or causal risk factor.

Metabolomic profiling also pointed to altered galactose-related metabolism in AIS. D-glucose was the only metabolite shared by KEGG, HMDB, and the differential-metabolite set. In tMCAO/R brain tissue, D-glucose and D-galactose levels were both increased, accompanied by elevated ROS and MDA and reduced GSH. Previous studies have shown that cerebral ischemia disrupts energy metabolism, redox homeostasis, glycan processing, and lysosomal function[7, 12]. Experimental evidence from D-galactose-challenged brain-ageing models indicates that galactose overload can trigger mitochondrial impairment and oxidative stress[25, 26]. Our data fit within this broader metabolic context. They do not, however, demonstrate that increased D-galactose directly induces Glb1 or Gal-3.

Glb1 emerged as a candidate from the integrative screening. As a lysosomal β -galactosidase, it participates in the degradation of β -galactoside-containing glycoconjugates and intracellular glycan turnover[27]. Impaired autophagy–lysosome function is known to increase neuronal vulnerability to ischemia[28], and previous multi-omics studies have reported altered Glb1 expression in cerebral ischemia[29]. Direct functional evidence, however, remains limited. Our loss-of-function experiments provide direct functional evidence in this context. Glb1 knockdown reduced infarct burden, neurological deficits, histopathological injury, and oxidative stress in tMCAO/R rats. Similar protection was observed in OGD/R-treated primary neurons, with improved cell viability and reduced LDH release and apoptosis. These findings support a functional contribution of Glb1 upregulation to ischemic neuronal injury.

Gal-3 has been studied more extensively in ischemic stroke. Encoded by *LGALS3*, Gal-3 is a β -galactoside-binding lectin involved in neuroinflammation, cellular stress, and tissue remodeling[30, 31]. Clinical studies have associated higher circulating Gal-3 with greater stroke severity and poorer neurological outcomes[32, 24], while experimental work has shown that suppression of Gal-3-related signaling can reduce neuroinflammation, apoptosis, and ischemic injury[33, 24]. Our findings are consistent with these observations. Gal-3 protein expression was increased in both ischemic rat brain tissue and OGD/R-treated primary cortical neurons. GB1107 reduced infarct burden, neurological deficits, histopathological injury, apoptosis, and cellular damage. The redox findings add another layer: Gal-3

inhibition also reduced ROS and MDA while restoring GSH, further linking Gal-3-associated injury to oxidative stress.

The combined-intervention experiments provide additional insight into the relationship between Glb1 and Gal-3. GB1107 reduced Gal-3 protein expression without significantly altering Glb1, whereas Glb1 knockdown decreased Glb1 expression without affecting Gal-3. This pattern does not support a simple upstream–downstream relationship between Glb1 and Gal-3 at the protein-expression level, despite their close association in the PPI network. Nevertheless, simultaneous targeting consistently produced greater protection than either intervention alone. This was evident across infarct size, neurological scores, cell viability, apoptosis, and oxidative stress markers. Formal interaction analysis was not performed, so the combined effect cannot currently be classified as additive or synergistic. One possibility is that Glb1- and Gal-3-associated processes are partly nonredundant but converge on shared injury phenotypes such as oxidative stress and neuronal death.

These findings may have distinct translational implications. Circulating LPH may serve as a candidate peripheral metabolic biomarker for ischemic stroke, although its incremental diagnostic value requires prospective validation. In contrast, Glb1 and Gal-3 represent potential therapeutic targets within the ischemic brain. Inhibition of either molecule reduced neuronal injury, while combined targeting produced greater protection, supporting further investigation of Glb1- and Gal-3-directed neuroprotective strategies.

Several limitations should be considered. First, the exploratory metabolomic and hospital-based validation cohorts were relatively small, and the community cohort was cross-sectional; therefore, the temporal and predictive relationships between circulating LPH and ischemic stroke cannot be established. Larger prospective multicenter studies are needed to validate these findings and assess the incremental clinical value of LPH. Second, the biological origin, release mechanism, and pathophysiological significance of circulating LPH remain unclear. Third, the metabolomic analysis was exploratory, and only static D-glucose and D-galactose levels were assessed experimentally, without metabolic flux analysis. Finally, the relationship between Glb1 and Gal-3 was evaluated primarily at the protein-expression and phenotypic levels, and formal interaction analysis was not performed.

In conclusion, this study identifies two complementary levels of galactose-related alterations associated with ischemic stroke. Circulating LPH is elevated in AIS and associated with stroke status, supporting its potential as a candidate peripheral metabolic biomarker. Glb1 and Gal-3 are both upregulated in ischemic neurons, and reducing either molecule attenuates ischemia/reperfusion-induced neuronal injury and oxidative stress in cell and animal models. Combined targeting provides greater protection than either intervention alone, although the present data do not support a simple linear regulatory relationship between Glb1 and Gal-3 at the protein-expression level. These findings provide a basis for further clinical evaluation of circulating LPH and mechanistic investigation of Glb1 and Gal-3 as potential therapeutic targets for neuroprotection.

Declarations

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Disclosure

The authors declare that they have no financial or other conflicts of interest to disclose.

Data Availability Statement

The metabolomics data supporting the conclusions of this article will be deposited in a public metabolomics repository upon acceptance of the manuscript. The public transcriptomic dataset analysed in this study is available in GEO repository under accession number GSE61616 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61616>).

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Ethical Statement

This study involving human participants was performed in accordance with the principles of the Declaration of Helsinki. The population-based stroke screening component was approved by the Medical Ethics Committee of the Affiliated Hospital of Guizhou Medical University (Approval No. 2024-LS-192), while the clinical sample analysis component was approved by the same committee (Approval No. GMUH-CE-2022019). Written informed consent was obtained from all participants prior to participation.

All animal experimental procedures complied with the institutional guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of the Affiliated Hospital of

Guizhou Medical University (Approval No. GMUH-AE-2022019). All efforts were made to minimize animal suffering and reduce the number of animals used.

Author Contribution

Jiayan Zhang: Conceptualization, Investigation, Data curation, Formal analysis, Writing—original draft
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Xin Zhou: Investigation, Data curation
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