Journal of Integrative Neuroscience

J. Integr. Neurosci. **2022**; 21(6): 161
https://doi.org/10.31083/j.jin2106161

Original Research

Characterization of the Sphingolipidome of the Peri-Infarct Tissue during Hemorrhagic Transformation in a Mouse Model of Cerebral Ischemia

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Academic Editor: Beata Sarecka-Hujar
Submitted: 4 May 2022 Revised: 3 June 2022 Accepted: 15 June 2022 Published: 26 September 2022

Abstract

Background: Cardiovascular diseases like stroke cause changes to sphingolipid mediators like sphingosine 1-phosphate (S1P) or its ceramide analogs, which bear the potential to either alleviate or exacerbate the neurological damage. Therefore, the precise identification of alterations within the sphingolipidome during ischemic stroke (IS) and hemorrhagic transformation (HT) harbors a putative therapeutic potential to orchestrate local and systemic immunomodulatory processes. Due to the scarcity of research in this field, we aimed to characterize the sphingolipidome in IS and HT. **Methods**: C57BL/6 mice underwent middle cerebral artery occlusion (MCAO) and specimens of the peri-infarct tissue were taken for sphingolipid profiling. **Results**: Ischemic stroke resulted in reduced S1P whilst ceramides were elevated six hours post ischemia onset. However, these differences were nearly revoked at 24 hours post ischemia onset. Moreover, the topmost S1P and ceramide levels were linked to the presence of HT after MCAO. In this study we show the characterization of the sphingolipidomic landscape of the peri-infarct tissue after ischemic stroke and HT. Especially, highest values of S1P, C_{18} lactosylceramide, C_{18} glucosylceramide, and $C_{24:1}$ ceramide were nearly entirely expressed by mice with HT. **Conclusions**: Our results warrant further investigations into the immunomodulatory consequences of altered sphingolipid species for the development of HT after IS.

Keywords: sphingosine 1-phosphate; ceramide; $S1P_1$; ischemic stroke; hemorrhagic transformation; sphingolipid profiling; MCAO; C57BL/6

1. Introduction

Globally, stroke is the second leading cause of death and is a major cause of disability and long-term care. This bears serious socioeconomic consequences for the affected person, their relatives, and society [1]. In 2016, the worldwide prevalence of stroke was 80.1 million, with cerebral ischemia accounting for 84.4% of the cases recorded [2]. Acute ischemic stroke is a medical emergency. The primary goal of ischemic stroke therapy is to achieve a safe, rapid, and effective reperfusion [3,4]. Current therapeutic approaches are systemic intravenous thrombolysis with recombinant tissue plasminogen activator (rtPA) within a narrow time or endovascular thrombectomy. Although vividly studied more recently, immunomodulatory therapeutic approaches in the acute phase of stroke have yet to demonstrate a benefit regarding clinical outcomes in large scale clinical trials.

Hemorrhagic transformation (HT) represents a complication of ischemic stroke occurring mainly after reperfusion [5,6]. Thrombolytic therapy (rtPA) increases the risk for HT by approximately 10-fold [7,8].

However, endovascular mechanical thrombectomy has also been shown to increase the risk of hemorrhagic complications [9]. More than three retrieval attempts during endovascular therapy were associated with an increased rate of symptomatic intracerebral hemorrhage and an Alberta Stroke Program Early CT Score (ASPECTS) $\geq$8 showed a significant negative correlation with sICH [10]. Moreover, shorter time to endovascular reperfusion therapy was associated with a lower risk of sICH [11].

Activation of matrix metalloproteases (MMPs) [12,13] and severe endothelial damage after ischemia/reperfusion compromise endothelial integrity and foster the development of HT [14,15]. After transient focal cerebral ischemia, the blood-brain barrier exhibits increased permeability as early as 25 minutes after reperfusion possibly persisting for several weeks [16,17].

Sphingolipids represent ubiquitous components of cellular membranes involved in cell-cell contacts but also serve as signaling molecules. There is a growing body of evidence regarding their regulatory function following stroke [18–20]. Ceramides, precursors of the signaling molecule sphingosine 1-phosphate (S1P), play a prominent role as central hubs of the sphingolipid metabolism [21]. As such, they confer and regulate apoptosis [22]. Several studies have provided evidence of ceramide accumulation during cerebral ischemia [23,24]. The induction of reperfusion is considered to trigger increased ceramide synthesis [25,26]. Ceramides cause apoptosis through mitochondrial dysfunction [27]. Furthermore, an increase in ceramide synthesis via increased acid sphingomyelinase (ASMase) activity has been demonstrated in animal models of stroke [24,28–30]. ASMase-deficient mice exhibited a reduced infarct size and improved neurological deficits after transient focal cerebral ischemia [31]. In contrast, S1P regulates the blood-brain-barrier (BBB) function by conferring its signaling on the vascular endothelium via $S1P_1$. This fosters a resistance to inflammation-induced vascular leakage whilst tight junctions and BBB selectivity is maintained [32].

In the present study, we aimed at analyzing the sphingolipid metabolome within the peri-infarct cortex following HT. Due to the chemotactic ability of sphingolipids that can regulate local and systemic immunomodulatory processes by recruiting immune cells, the aim of this study was to identify sphingolipid subspecies as putative risk factors for HT after stroke.

2. Materials and Methods

For all experiments, male C57BL/6 mice (strain J, 11–12 weeks, Charles River Laboratories, Sulzfeld, Germany) were used and kept on a 12:12 h light-dark cycle with food and water ad libitum. All animal experiments in this study conformed to the German Protection of Animals Act and the guidelines for care and use of laboratory animals as determined by the local institutional review board (Regierungspräsidium Darmstadt, Germany, code FU/1049, approved on 2nd of April 2015). Where experiments required sampling of whole blood isolated from human volunteers, informed consent was obtained.

2.1 Experimental Model of Transient Middle Cerebral Artery Occlusion

Transient middle cerebral occlusion (MCAO) was performed as described previously [33]. In brief, mice were anesthetized with 1.5% isoflurane (Forene, Abbott, Wiesbaden, Germany) and received 0.1 mg kg^{-1} buprenorphine (Temgesic, Essex Pharma, Munich, Germany) under spontaneous respiration. The focal ischemia was induced by inserting a standardized silicon-coated monofilament with a tip diameter of 0.23 mm (Doccol, Redlands, CA, USA). A midline cervical incision was followed by the introduction of the monofilament along the internal carotid artery until it occluded the ostium of the right middle cerebral artery. The reperfusion was initiated by withdrawing the monofilament after 3 hours of focal cerebral ischemia. After the surgery, mice were monitored and received food and regular drinking water ad libitum. Animals were assessed either 6 hours or 24 hours after ischemia onset, in order to describe their global neurological functions before being euthanized.

114 mice were subjected to 3 hours of MCAO followed by immediate imaging and reperfusion. Reperfusion was performed either 6 hours (n = 60) or 24 hours (n = 54) after ischemia onset. Within the 6-hour cohort, 22 mice were treated with warfarin and 38 without, whilst 15 mice received warfarin within the 24-hour cohort (**Supplementary Fig. 1**). 10 mice were used as sham-operated control. The operations were performed in an unblinded fashion since the operator did not apply any modifications such as drug treatment, however, sample provision for the mass spectrometry or 2,3,5-triphenyltetrazolium chloride (TTC) imaging were done in a blinded fashion.

2.2 Warfarin Administration

To increase the chance of spontaneous HT following ischemic stroke (IS) induced by MCAO, we evaluated mice under warfarin treatment at the onset of ischemia versus non-anticoagulated mice. Accordingly, we administered warfarin through drinking water dissolving a 5 mg Coumadin tablet (warfarin sodium crystalline; Bristol Myers Squibb, Munich, Germany) in 375 mL tap water in accordance with previous reports [34]. Assuming a water consumption in rodents of 15 mL/100 g and a body weight of 20 g per 24 hours, a warfarin uptake of 0.033 mg (0.83 mg kg^{-1}) per mouse would be achieved within a 20-hour feeding period. Warfarin administration through bottled water was started at the same time. Following 20-hours of feeding ($\pm$ Warfarin), MCAO was induced.

2.3 Assessment of Neurological Deficits

The neurological examination was performed 6 hours and 24 hours after ischemia onset, respectively. The modified Neurological Severity Score (mNSS) was applied to assess neurological deficits [35]. The mNSS is one of the most frequently referred to neurological scores for the functional assessment of mice after an induced stroke. A scoring system is used to quantify neurological deficits. Hemiparesis, walking behavior, coordination, and sensory function are the main areas assessed. The maximum score for mice is 18 points. The Bederson score was originally developed as an evaluation tool for the success of MCAO in the rat [36]. The neurological deficit is assessed using a 5-point scale and captures behavioral changes in the mouse based on its spontaneous movements. The grip test is used to evaluate motor function as well as coordination. The mice were placed on a wooden bar 30 cm above the ground and the time period to fall off was assessed.

2.4 Determination of Sphingolipid Concentrations by High-Performance Liquid Chromatography-Tandem Mass Spectrometry

The quantification of sphingoid bases and ceramides in tissue from the peri-infarct cortex collected 6 or 24 hours after ischemia onset as described in detail elsewhere [33,37]. Briefly, samples of the peri-infarct cortex were snap-frozen with liquid nitrogen and stored at –80 °C until required for further analyses. The tissue samples were first mixed with water:ethanol (75:25, v/v) and homogenized to a suspension of 0.05 mg/μL tissue, using zirconium oxide grinding balls and a Mixer Mill MM400 (Retsch, Haan, Germany). For lipid extraction a volume of 20 μL of the homogenate was used and mixed with 200 μL extraction buffer (citric acid 30 mM, disodium hydrogen phosphate 40 mM, pH 4.2) and 20 μL of the internal standard solution. The mixture was extracted once using 600 μL methanol:chloroform:hydrochloric acid (15:83:2, v/v/v). Afterwards, the organic phase was divided into two aliquots (one for the determination of sphingoid bases and one for the determination of ceramides), evaporated and reconstituted using 50 μL of tetrahydrofuran:water (9:1, v/v) containing 0.2% formic acid and 10 mM ammonium formate (ceramides) and 50 μL methanol containing 5% formic acid (sphingolipids). The LC-MS/MS system consisted of a triple quadrupole mass spectrometer QTRAP 5500 (Sciex, Darmstadt, Germany) equipped with a Turbo Ion Spray source operated in positive electrospray ionization mode and an Agilent 1290 Infinity LC-system with binary HPLC pump, column oven and autosampler (Agilent, Waldbronn, Germany). Chromatographic separation of ceramides was achieved using a Zorbax RRHD Eclipse Plus C18 column (1.8 μm 50 $\times$ 2.1 mm ID, Agilent, Waldbronn, Germany) and for the sphingoid bases using a Zorbax Eclipse Plus C8 UHPLC column (1.8 μm 30 $\times$ 2.1 mm ID, Agilent, Waldbronn, Germany). For all analytes, the concentrations of the calibration standards, quality controls and samples were evaluated by Analyst software 1.7.1 and MultiQuant Software 3.0.3 (both Sciex, Darmstadt, Germany) using the internal standard method (isotope-dilution mass spectrometry).

2.5 Flow Cytometric Analysis of Immune Cells

For flow cytometric analysis of peripheral blood immune cells, 1 mL of venous blood after peripheral venipuncture was sampled from a Heparin-lithium monovette. Flow cytometry was performed as described elsewhere [38]. In brief, $S1P_1$expression was fixed for 30 minutes at 4 °C in a total volume of 10 mL in calcium- and magnesium-depleted PBS/2mM EDTA/2% fatty-acid free BSA/0.1% paraformaldehyde (PFA). Subsequently, the sample was centrifuged (10 minutes, 4 °C, 400 g), the supernatant removed, and the red cells lysed at room temperature in the dark for 15 minutes according to the manufacturer's instructions (420301, Biolegend). The solution was centrifuged (5 minutes 4 °C, 400 g) and immune cells resuspended in calcium- and magnesium-depleted PBS/2mM EDTA/2% fatty-acid free BSA (staining buffer). 5×10^5 cells were dispensed into polystyrene tubes, stained for 60 minutes at 4 °C in the dark, washed with 1 mL of staining buffer and assessed on a FACS Canto II (BD Biosciences, Heidelberg, Germany). The following antibodies were used: anti-human CD45-FITC (clone 30F11, Miltenyi Biotec, Bergisch Gladbach, Germany), anti-human CD3-PerCP (clone UCHT1, Biolegend, San Diego, CA, USA), anti-human S1P1-eFluor660 (clone SW4GYPP, ThermoFisher, Waltham, MA, USA), Mouse IgG_1 kappa isotype control (clone P3.6.2.8.1, ThermoFisher).

2.6 Visualization of the Ischemic Lesion

The ischemic lesions were visualized by staining murine coronal slices with a 2% solution of 2,3,5-triphenyltetrazoliumchloride (TTC) 6 hours or 24 hours after ischemia onset as described elsewhere [39]. In addition, MCAO mice were assessed by magnet resonance imaging (MRI) 3 hours after reperfusion. MRI measurements were acquired on a 3T Magnetom TRIO (Siemens Medical Solutions, Erlangen, Germany). During MRI, mice were spontaneously breathing after receiving intraperitoneal anesthesia comprising of Ketamine (Ketavet®, 100 mg kg^{-1} bw, Parke-Davis, Berlin, Germany), Xylazine (Rompun®, 20 mg kg^{-1} bw, Bayer, Leverkusen, Germany), and Acepromazine (Vetranquil®, 3 mg kg^{-1} bw, CEVA Tiergesundheit, Düsseldorf, Germany). 75–150 μL of each drug was injected intraperitoneally per mouse in accordance with its body weight. Due to the cardiodepressive effect of the anesthesia, a low volume of application was started and injected as needed. The intertoe reflex was used to assess the depth of anesthesia in mice. Mice were continuously protected from hypothermia with an infrared lamp during anesthesia and the subsequent MRI recording.

2.7 Statistical Analyses

GraphPad Prism 8 (GraphPad Software, LLC, La Jolla, CA, USA) was used for statistical analyses. Data are illustrated as median $\pm$ interquartile range (IQR) except where stated otherwise. Statistical significance was assessed using the Mann-Whitney test for unpaired samples unless stated otherwise. A p value of <0.05 was considered statistically significant.

3. Results

3.1 Peri-Infarct Cortex Tissue Sampling to Perform a Tailored Sphingolipid Profiling Approach to Delineate Differences between Ischemic Stroke and Hemorrhagic Transformation

We studied the sphingolipid profile in the acute phase after focal cerebral ischemia, in a model of middle cerebral artery occlusion for 3 hours, followed by reperfusion

after 6 hours and 24 hours after ischemia onset. To establish hemorrhagic transformation (HT) more frequently, mice were additionally selected to receive anticoagulation with warfarin (Fig. 1A), which exacerbates the risk of HT [34]. HT was evaluated either using TTC staining or using MRI (Fig. 1B). Mice with effective oral anticoagulation with the vitamin-K-antagonist (VKA) warfarin (0.83 mg kg^{-1}) at the onset of cerebral ischemia displayed a higher frequency of HT both at a follow up time of 6 hours and 24 hours alike (VKA *vs.* no VKA: 6 h: 71.4% *vs.* 34.4%; 24 h: 77.8% *vs.* 42.9%, Fig. 1C). However, HT did not further worsen the neurological deficit of the mice in this experimental paradigm with large ischemic lesions as there were no significant differences in all three neurological scores tested 6 hours and 2 hours after ischemia onset (Fig. 1C).

To determine subclinical changes, we next assessed changes on the molecular level in terms of the peri-infarct sphingolipidome by liquid chromatography mass spectrometry (LC-MS) (Fig. 1D). Tissue samples were isolated 6- or 24-hour after the MCAO intervention, homogenized, and analyzed for their sphingolipid metabolome. Results were correlated to the presence of HT that occurred in VKA-anticoagulated but also in non-anticoagulated mice of this study and were recorded for each individual animal.

3.2 Characterization of the Sphingolipidome in the Acute and Subacute Phase of Ischemic Stroke

We could show that in the acute phase after ischemic stroke S1P levels were significantly reduced and ceramides were almost unanimously more abundantly expressed. Indeed, S1P levels were significantly decreased in the peri-infarct cortex 6 h after the onset of focal cerebral ischemia in comparison to sham-operated animals (IS_{6h} *vs.* sham: 350.3 ± 151.1 pg mg^{-1} *vs.* 1298.0 ± 442.9 pg mg^{-1}, $p < 0.0001$), and restored after 24 hours of observation as compared to 6 hours of observation ($p = 0.06$) in accordance with our previous observations [33].

Ceramides, however, displayed a reversed kinetic. For example, C_{16} ceramide was nearly two-fold enhanced at 6 hours of follow up (IS_{6h} *vs.* sham: 5.8 ± 1.3 ng mg^{-1} *vs.* 2.7 ± 1.0 ng mg^{-1}, $p < 0.001$) and levels declined back to baseline at 24 hours), and significantly lower than at 6 hours ($p < 0.0001$). Next, C_{18} ceramides mirrored the previous kinetics (IS_{6h} *vs.* sham: 253.5 ± 101.8 ng mg^{-1} *vs.* 113.2 ± 24.8 ng mg^{-1}, $p = 0.0003$; IS_{24h} *vs.* sham: 98.9 ± 41.3 ng mg^{-1} *vs.* 113.2 ± 24.8 ng mg^{-1}, $p > 0.99$; IS_{24h} *vs.* IS_{6h}: $p < 0.0001$). Likewise, this behavior was also observed for $C_{18:1}$ ceramide, C_{20} ceramide, and $C_{24:1}$ ceramide. C_{24} ceramide displayed endogenously higher variability amongst sham-operated mice and such 24 hours after ischemia onset

Owing to the scarcity of analyte material, several special sphingolipidome species were only analyzed in sham-operated mice and such after 24 hours of observation. Here, no differences could be ascertained comparing mice receiving the MCAO intervention with sham ($p > 0.05$). Here, sphinganine (Spha), sphingosine (Spho), C_{18} Spha, $C_{24:1}$ Spha, C_{16} glucosylceramide (GlcCer), C_{18} GlcCer, $C_{24:1}$ GlcCer, C_{16} lactosylceramide (LacCer), C_{18} LacCer, and $C_{24:1}$ LacCer have all been tested (Fig. 2).

3.3 Gross Assessment of the Sphingolipidome in the Peri-Infarct Tissue does not Distinguish between HT and IS

Next, we aimed to characterize the sphingolipidomic changes arising from the establishment of HT after ischemic stroke (IS). Initially, we had observed that HT occurred in a higher frequency if mice had been anticoagulated with VKA prior to the MCAO intervention [34]. Despite some scientific indication that warfarin might affect ceramide expression [40] via inhibition of the production of pro-inflammatory ceramides, we initially treated mice with VKA treatment (rectangles) to enrich for this complication of IS. Indeed, HT (red coloring) did occur more frequently (Fig. 3), but mice also passed away more often (6 h: VKA *vs.* no VKA: 13.6% *vs.* 0%, **Supplementary Fig. 1**). C_{16} ceramide, a known pro-inflammatory ceramide for example, is a product of acid sphingomyelinase 1 [41], and was being produced less abundantly in mice comparing mice treated with VKA to such without in the absence of HT (VKA *vs.* no VKA: 4.9 ± 2.1 ng mg^{-1} *vs.* 5.8 ± 1.3 ng mg^{-1}), although statistical differences could not be ascertained owing to the small sample size. Since we did not know the pharmacological consequences of warfarin onto other sphingomyelinase, and as a result of these two observations, we then discontinued the VKA model to induce HT in order to establish an unbiased sphingolipidomic landscape arising from HT because of IS in the absence of VKA anticoagulation (circles).

Our data demonstrates that HT is not associated with any alterations of the sphingolipidome encompassing the assessment of S1P and various ceramide species neither at 6 hours nor 24 hours after follow up (FU).

3.4 S1P Receptor Type 1 is Abundantly Expressed on Innate Immune Cells and a Risk Stratification Demasks Sphingolipidomic Profiles Associated with HT

S1P can enhance immune cell migration as a chemotactic agent [33,42] via $S1P_1$-mediated up-regulation of ICAM-1 and E-selectin on endothelial cells [43–46], which are important adhesion molecules for innate immune cells such as monocyte or granulocytes during diapedesis. Therefore, we sought to analyze the expression pattern of $S1P_1$ on peripheral immune cells to estimate their adhesiveness towards S1P-stimulated endothelium to obtain insights into putative pathophysiological consequences of this increase in peri-infarct cortex S1P levels. Indeed, $CD45^+$ immune cells of the adaptive lineage, i.e., T cells ($CD45^+CD3^+$ within the FSC/SSC properties for lymphocytes) or B cells ($CD45^+CD3^-$ within the FSC/SSC properties for lymphocytes) had an abundant expression of $S1P_1$ of 37.8% or 80.2%, respectively, compared to iso-

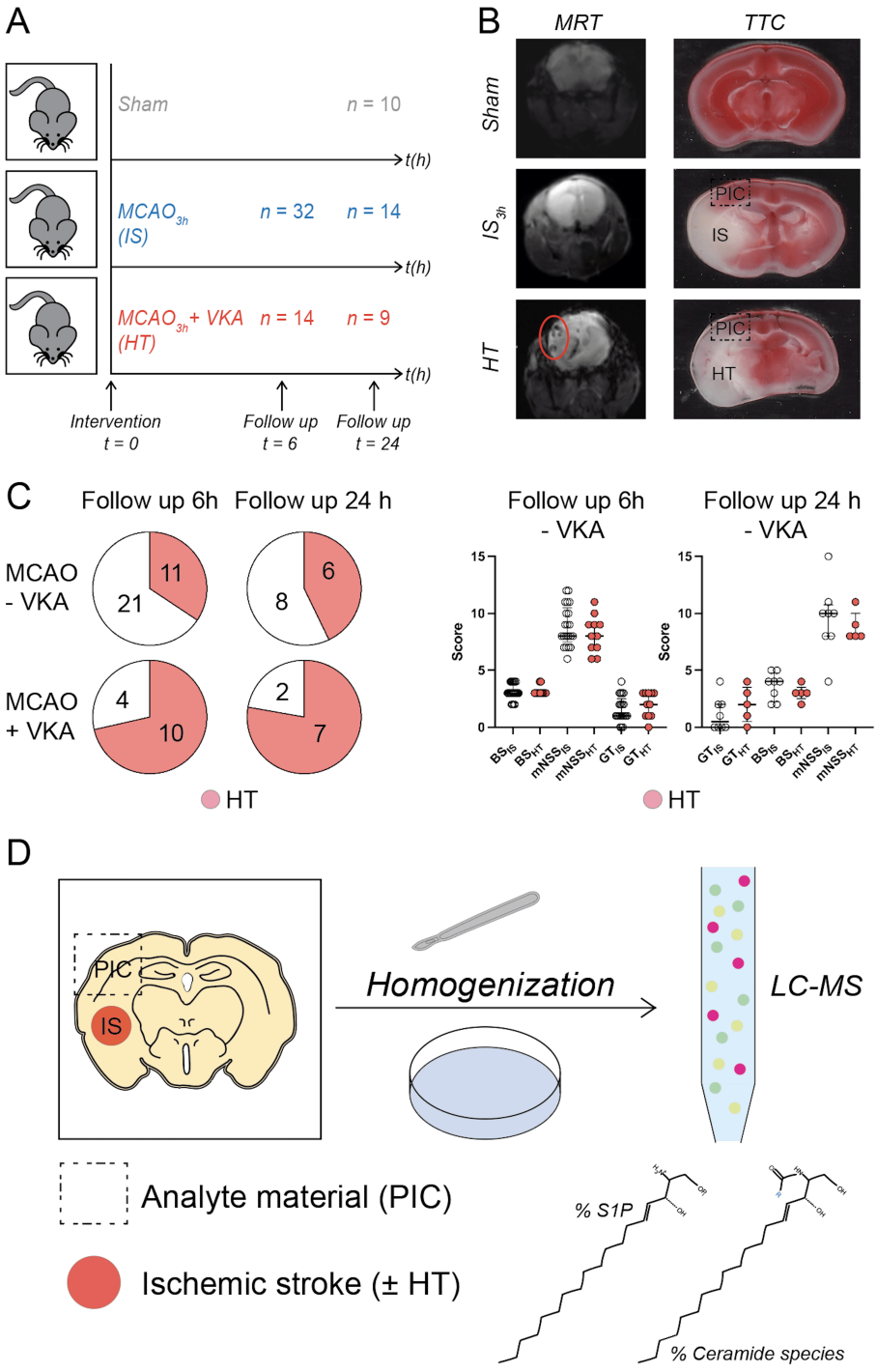

Fig. 1. Peri-infarct cortex tissue sampling to study and the impact of hemorrhagic transformation on sphingolipid mediator concentrations in an unbiased sphingolipid profiling approach. (A) Study design: C57/BL6 mice were either sham-operated (n = 10) or underwent ischemic occlusion of the middle cerebral artery for 3 hours (n = 69) either in the absence (n = 46) or presence (n = 23) of the vitamin-K-antagonist (VKA) warfarin. Mice were observed for 6 hours (n = 46) or 24 hours (n = 23) after ischemia onset. (B) Visualization of large hemispheric ischemic stroke (IS) and hemorrhagic transformation (HT) on TTC staining of native postmortem brain sections and MRI imaging. The peri-infarct cortex (PIC) from which analyte material was taken, is visualized. (C) C57/BL6 mice anticoagulated with VKA displayed hemorrhagic transformation after reperfusion with a higher frequency. In this model of large hemispheric infarctions, HT had no further significant impact on the functional status of the mice. The Mann-Whitney U-test was applied to calculate statistical differences. The data are presented as median ± IQR. (D) An unbiased temporal sphingolipid profiling approach by LC-MS/MS was utilized to assess the impact of HT occurring within the IS area on sphingolipid mediator tissue levels in the PIC (dashed rectangular box).

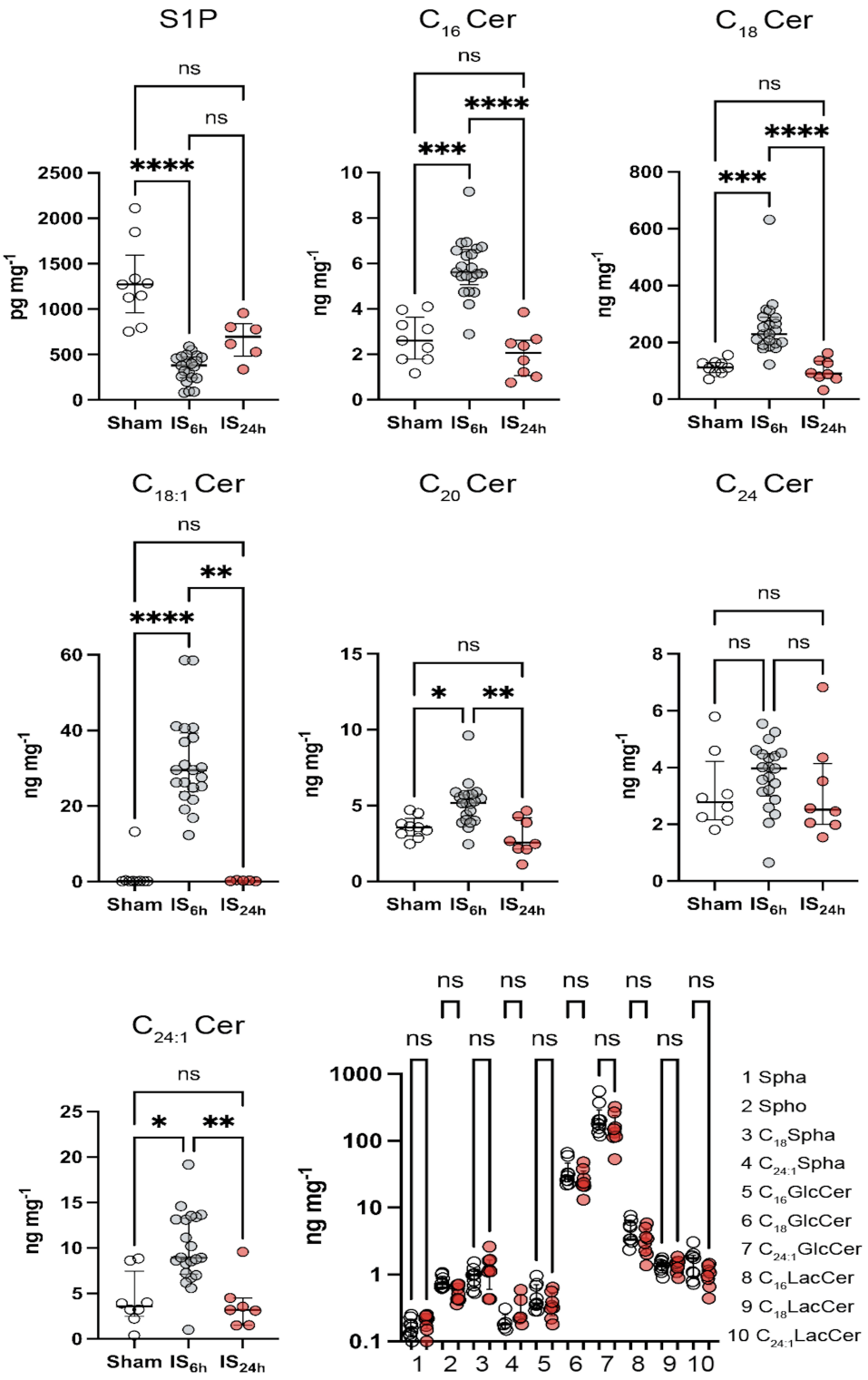

Fig. 2. Reversed kinetics of S1P and ceramide species in the peri-infarct tissue after ischemic stroke. Various sphingolipid species were tested by LC-MS from analyte material sampled from the peri-infarct tissue comparing mice receiving sham-intervention with such having undergone MCAO-intervention and followed up for 6 or 24 hours, respectively. S1P, C_{16} ceramide, C_{18} ceramide, $C_{18:1}$ ceramide, C_{20} ceramide, C_{24} ceramide, and $C_{24:1}$ ceramide were kinetically assessed. Due to scarcity of the analyte material, special sphingolipid species could only be characterized in sham mice and MCAO-operated mice with a follow up time of 24 hours (for abbreviations see text). A Kruskal-Wallis test with Dunn's multiple comparisons test was applied to calculate statistical differences. Data are presented as median ± IQR; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

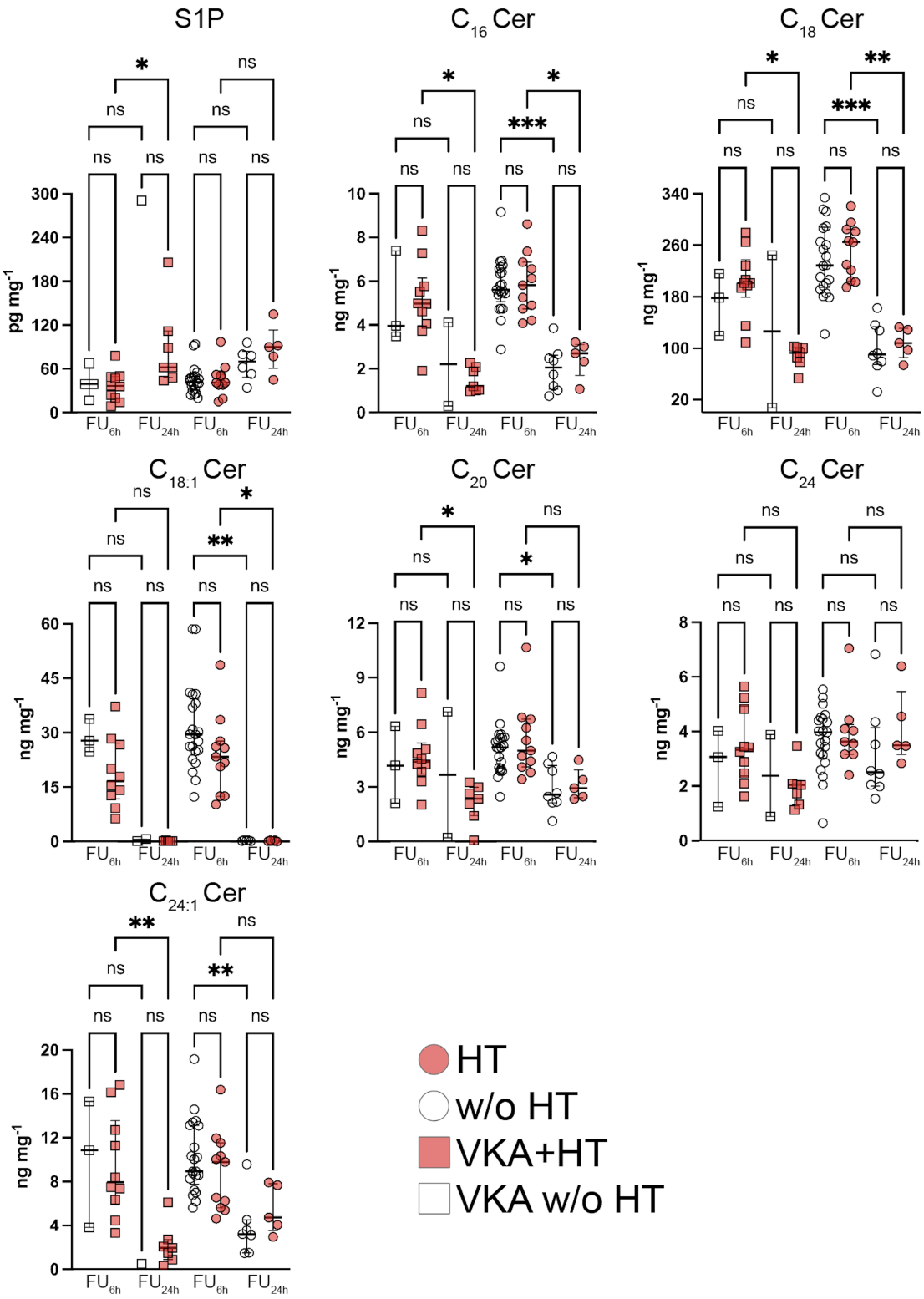

Fig. 3. Gross assessment of the sphingolipidome within the peri-infarct tissue does not distinguish between IS and HT. Cerebral specimens from the peri-infarct cortex of mice that have developed hemorrhagic transformation (HT) of the ischemic core after MCAO were analysed regarding their differential sphingolipid expression profile as compared to mice without HT. A Kruskal-Wallis test with Dunn's multiple comparisons test was applied to calculate statistical differences. Data are presented as median ± IQR; *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$. S1P, sphingosine 1-phosphate; Spho, sphingosine; Spha, sphinganine; Cer, ceramide.

type (<5%, Fig. 4A). Interestingly, innate immune cells captured in the granulocyte or monocyte gate by FSC/SSC-properties, typically first responders in the context of acute inflammation, had a way superior expression pattern of $S1P_1$ (Fig. 4A). Here, monocytes had an abundant expression of 80.7%, whilst granulocytes were almost unanimously positive for $S1P_1$. $S1P_1$ was shown to be a crucial factor regulating neutrophil recruitment [47].

Seeing that S1P is consumed (via receptor binding and subsequent internalization by effector cells) in the acute phase of IS, we intended to risk stratify our established sphingolipidomic profiles by the topmost and bottommost 35% of sphingolipid species measured and qualitative check whether HT had occurred or not (Fig. 4B). In doing so, we identified that apart from C_{18} sphinganine (Spha), the topmost expression levels of all other immediate S1P relatives and S1P itself were qualitatively linked to a more frequent conversion of HT. For example, the topmost 35% expression levels ($Hi_{35\%}$) for S1P showed HT in three out of four mice, whereas in the case of the bottommost 35% ($Lo_{35\%}$) only one mouse of four had converted to HT. Likewise, sphingosine, the closest relative of S1P had seen HT in four out of five mice in $Hi_{35\%}$ opposed to one out of five mice in $Lo_{35\%}$. Conversely, for C_{18} Spha, $Lo_{35\%}$ had seen HT in 60%, whilst in $Hi_{35\%}$ only one out of five mice had developed HT (20%). Apart from Spho, one ceramide subspecies also appeared to be closely linked to the establishement of HT. Here, C_{18} lactosylceramide had seen HT in 80% in $Hi_{35\%}$ as opposed to only 20% in $Lo_{35\%}$ (Fig. 4B). We report an association of multiple sphingolipid species as putative risk factors for the conversion of HT, however, would be keen to assess their relevance as causative agents to drive innate immune cell recruitment and subsequent loss of endothelial integrity predisposing for HT in the future. Yet, we are only reporting associations.

4. Discussion

This study provides comprehensive data on the metabolism of sphingolipid and ceramide species in the peri-infarct cortex after ischemic stroke in conjunction with prior oral anticoagulation therapy and HT.

We report that there is no association in terms of C57BL/6 mice's neurological performance in the acute to subacute phase (~24 hours) after reperfusion of cerebral ischemia in the context of non-space-occupying HT. Considering the large hemispheric infarctions induced in our experimental paradigm, this is not surprising. In humans, according to ECASS II (European Co-operative Acute Stroke Study II) hemorrhagic events can be distinguished into four subtypes: hemorrhagic infarction (HI) 1 and HI2, and parenchymal hemorrhage (PH) 1 and PH2, respectively [48]. However, further research has shown that predominantly PH2, characterized by >30% of space-occupying lesions within the infarcted area is associated with clinically detectable effects 24 hours after onset and an increased risk of death within three months post infarction [49]. Our model was not able to mimic PH2 as mice with more widely spread HT almost never survived. Yet, our model entailed all other facets of sICH as denoted by ECASS II and we therefore sought to scrutinize these facets of sICH for changes in the sphingolipid metabolome within the peri-infarct cortex. This appeared relevant to us since the peri-infarct cortex, commonly referred to as the penumbra, consists of neurological tissue that can be saved and benefits from neurological interventions, e.g., re-canalization [50].

Sphingolipid signaling has emerged as important metabolic pathways in the context of stroke and HT [19,51–53]. According to previous studies, we report that ischemia enhances S1P signaling [53]. In an MCAO model based on the inbred mouse strain *ICR*, the exogenously derived intracerebral application of S1P resulted in an up-regulation of Sphk1 and $S1P_3$ with a down-regulation of $S1P_1$ [54], which was also recently confirmed by our group [33]. The intracerebral deposition augmented infarct size, adversely activated microglia and astrocytes, and induced the expression of the inflammatory cytokine TNFα 24 hours after reperfusion [54]. These adverse effects were in parts reversible by the inhibition of the sphingolipid pathway by the application of fingolimod. However, the endothelium in the penumbral region was recently shown to rely on a maintained S1P-$S1P_1$ engagement to reduce the augmentation of the ischemic core [19]. We report that the peri-infarct cortex in mice with HT is denoted by an enhanced S1P level. Indeed, it was shown that even in the plasma, S1P can be used to distinguish ischemic stroke from hemorrhagic stroke in the early acute phase [55]. It appears that S1P, subject to the respective $S1P_R$ engagement, exerts dichotomous functions. On the one hand, endothelial $S1P_1$ engagement preserves BBB integrity and oxygenation in the penumbra [19]. The mechanism by which the peri-infarct cortex in the context of HT answers to the significantly up-regulated S1P expression yet remains to be elucidated. The temporal relationship of S1P and the establishment of HT remains further characterization, too. Does the enhanced production of S1P precede HT or does it follow? On the other hand, however, microglial and astrocyte engagement via $S1P_2$ or $S1P_3$ can propagate a pro-inflammatory loop with subsequent neurological deterioration [54]. With regards to the latter, inhibition of S1P-mediated signaling improved neurological outcome in patients with HT after ischemic stroke [51].

We propose that S1P promotes its detrimental consequences by means of intracerebral but also extracerebral signaling. Regarding the latter, we show that $S1P_1$ is unanimously and abundantly expressed on innate immune cells, including monocytes and neutrophil granulocytes. It seems intuitive that these immune cells respond to this established S1P gradient by chemotaxis [33,42]. Engagement of $S1P_1$ in immune cells, which we show to be almost unanimously expressed on innate immune cells, is not merely

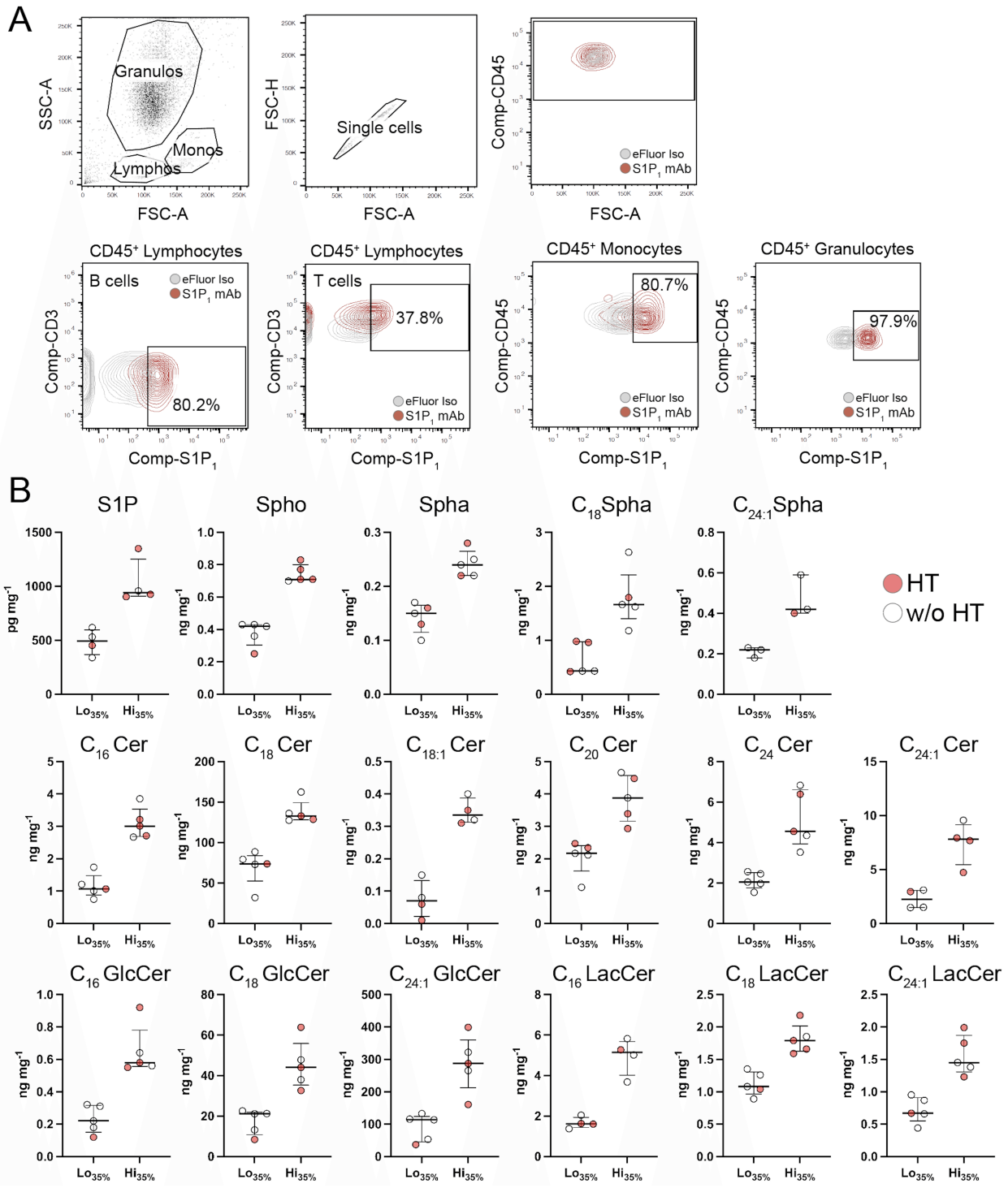

Fig. 4. Chemotactic recruitment of innate immune cells via sphingolipids and in-depth assessment of the sphingolipid subspecies as putative risk factors for HT. (A) As an example of chemotactic recruitment of innate immune cells, whole blood leukocytes were assessed for their expression pattern of the type 1 S1P receptor ($S1P_1$) by which cells are being recruited to effector organs. (B) Stratifying the assessed sphingolipid abundances by their topmost or bottommost 35% representative allowed the identification of putative species associated with HT in MCAO samples 24 hours after ischemia onset. Especially high levels of S1P and its immediate precursor sphingosine (Spho) or C_{18} lactosylceramide (LacCer) appeared to predispose for HT. In contrast, C_{18} sphinganine (Spha) or C_{16} LacCer were conversely regulated, i.e., lower levels were more frequently linked to HT. Data are presented as median ± IQR, and no statistical testing was performed.

a crucial determinant for granulocyte recruitment [47], but also results in activation of the phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling pathway [33], which confers a metabolic on-switch denoted by cellular activation culminating in the adhesive β_2 integrin Mac-1 [56]. Likewise, endothelial cells in the peri-infarct cortex respond with S1P ligation by $S1P_1$ resulting in ICAM-1 and E-selectin up-regulation [43–46]. These adhesion molecules are of crucial importance for neutrophil-endothelial interactions during adhesion [57–59]. We therefore hypothesize that in the context of HT, monocytes and neutrophils are swiftly and more predominantly activated and recruited to the penumbra-associated endothelium by means of $S1P_1$ modulated endothelial ICAM-1 and E-selectin expression. This should be experimentally validated by intravascular imaging with CMFDA-labeled splenocytes and autologous re-infusion prior to the MCAO intervention or immunohistochemistry in the future. If this were true, inflammatory granulocytes could mechanistically answer for the extent of HT by means of the production of MMP9 or reactive oxygen species (ROS), respectively. A dramatic up-regulation of MMP9 was recently reported within 24 hours after MCAO treatment, which was paralleled with a breakdown of tight junction markers and maintained for 7 days post-MCAO [60]. Likewise, ROS have been shown to induce endothelial cell stress via NLRP3 [61,62], which facilitates the deterioration of endothelial integrity associated with HT [63]. The associated hyperglycemia further enhances loss in blood brain barrier permeability [63] and should be experimentally addressed in the future. Previous research at least suggests a crucial role for neutrophils and monocytes since they were shown to undergo the most imminent and profound transcriptomic changes after stroke [64], including an up-regulation of MMP9. In humans, the cerebral infiltration by MMP9-positive neutrophils is documented [65].

Platelets are another source of S1P release by means of thrombin and factor X activation [66], therefore we sought to investigate the influence of thrombin and factor X on the S1P level [67,68]. For this reason, vitamin K, the essential factor for prothrombin development, was inhibited by warfarin-supplementation 72 hours prior to assessment. We could not find any impact of VKA-pretreatment on the expression of any sphingolipid nor ceramide species assessed.

Besides S1P and its precursor Spho, our unbiased sphingolipid profiling approach has identified an association of the $Hi_{35\%}$ levels for various ceramide species measured in the peri-infarct cortex to be linked to a higher frequency of HT conversion. In this regard, previous research implicated C_{16} ceramide as a mediator of cerebral venule vasoconstriction, thus enhancing leukocyte-endothelial interactions required for leukocyte rolling [69]. The concentration-dependent spasms observed in this study enhanced the venular wall permeability, venule rupture, and micro-hemorrhaging [69]. In addition, *de novo* generation of C_{16} and C_{24} ceramide were identified as causative agents of caspase-3, -8, and -9 activity in neutrophils, inevitably inciting their commitment to apoptosis [70]. It was only recently, that evidence emerged tying the very-long-chain ceramide species like C_{24} to being a competitor of C_{16} ceramide-mediated channel formation [71], therefore, mediating the pathological processes observed during stroke [72]. Acute stroke can cause a disruption to the BBB selectivity for lipid species [73], which may therefore be detectable in the plasma serving as biomarkers [73–75]. Consequently, it was reported that particularly C_{16}, C_{24}, and $C_{24:1}$ ceramides were elevated in the plasma and associated with the degree of white matter hyperintensity [76]. Under the assumption of plasma trespassing of cerebral ceramides our data would corroborate this. However, ceramide levels were also experimentally characterized in a rat MCAO model in the peri-infarct cortex. In AAV-treated MCAO-operated rats, $C_{18:1}$ and $C_{18:2}$ ceramides were enriched in the peri-infarct cortex four days after reperfusion [77]. In accordance with our data, another study reported on the increase of long- and very-long-chain ceramide species in MCAO-treated mice 24 hours after reperfusion [78]. For this reason, the acid sphingomyelinase/ceramide system has drawn attention as a potential target for ischemic stroke therapies [79], and should find due consideration in future mechanistic studies to characterize their consequences for the peri-infarct cortex in the context of HT.

5. Conclusions

In this study, we report changes in the S1P and various ceramide species profile in the peri-infarct cortex associated with HT following IS. Future studies are needed to address the mechanisms within the sphingolipid metabolome by which conversion to these species occurs and which biological consequences are being conferred. Do these species confer a risk for HT or is the conversion of these species a consequence of HT? Do these species adversely affect long-term outcome of the penumbra and survival? What is the mechanistic link to innate immune cell recruitment in terms of chemotaxis, pro-inflammatory cellular activation patterns? Moreover, further experiments are needed to conclusively determine the contributing sources of sphingolipid synthesis and release (e.g., microvascular endothelial cells, neurons, astrocytes, microglia). And considering ongoing trials investigating sphingosine 1-phosphate antagonistic treatment (fingolimod) after stroke, how can long-term immunodepression post-stroke be averted in the context of sphingolipid-antagonistic treatment? Synergistic experiments utilizing intravascular imaging techniques in the context of Tet-On inducible systems could shed some light on these kinds of questions.

Abbreviations

AAV, adeno-associated virus; ApoM, apolipoprotein M; ASPECTS, Alberta Stroke Program Early CT Score;

BBB, blood-brain barrier; CBF, cerebral blood flow; CD, cluster of differentiation molecule; CNS, central nervous system; FC, fold change; GBD, global burden of disease; HT, hemorrhagic transformation; IQR, interquartile range; IS, ischemic stroke; LC-MS, liquid chromatography mass spectrometry; MCAO, middle cerebral artery occlusion; MMP, matrix metalloprotease; MRI, magnet resonance imaging; PIC, peri-infarct cortex; rtPA, recombinant tissue plasminogen activator; sICH, symptomatic intracerebral hemorrhage; S1P, sphingosine 1-phosphate; $S1P_1$, sphingosine 1-phosphate receptor 1; TTC, 2,3,5-triphenyltetrazoliumchloride; VKA, vitamin-K-antagonist.

Author Contributions

AL, RB, JS designed the research study. AL, ST, DT, KL performed the research. AL, ST, KL, JS analyzed the data. AL and JS wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final version of the manuscript.

Ethics Approval and Consent to Participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and all animal experiments conformed to the German Protection of Animals Act and the guidelines for care and use of laboratory animals by the local institutional review board. The study was approved by the Institutional Review Board (Regierungspräsidium Darmstadt, Germany, code FU/1049, approved on 2nd of April 2015). Informed consent was obtained from all subjects involved in the study.

Acknowledgment

Thanks to all the peer reviewers for their opinions and suggestions.

Funding

This research was funded by the German Research Foundation (SFB1039: Project ID 259130777-E02; SFB1039/Z1; SFB1039-TPB08 to R.B). AL is supported as a Clinician Scientist by the SFB1039. JS was funded by a scholarship from the German Academic Scholarship Foundation (Studienstiftung des deutschen Volkes) and by the German Cancer Aid as a fellow of the Mildred Scheel Early Career Center at the CRTD, Medical Faculty, Technische Universität Dresden.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.jin2106161.

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