CHANGES IN MICROBIAL HOMEOSTASIS

FOLLOWIGN ACQUIRED BRAIN INJURY IN A PORCINE MODEL

by

JULIE H. JEON

(Under the Direction of HEA JIN PARK)

ABSTRACT

Acquired brain injury, such as stroke and traumatic brain injury, are devastating injuries that result in long-term disability and death. Recently, inter-organ communication has gained attention due to its pivotal role in homeostasis and disease pathophysiology. Particularly, the microbiome-gut-brain axis is the major inter-organ communication for maintaining homeostasis in brain injuries. The microbiome is the genetic material of all the microorganisms that live on and inside the body and is essential for nutrition, human development, and immunity. In particular, the gut microbiome is a key regulator of the gut-brain axis and is closely related to neurological function and mental health of the host. The microbiome is also present in other parts of the human body, including oral cavity, skin, and hair, etc. This dissertation aims to evaluate the effect of acquired brain injury on microbiome and homeostasis in gut and oral cavity. In manuscript #1, gut microbiome changes were characterized during the acute stage of stroke in a pig model. Ischemic stroke significantly induced shifts in microbial diversity, increased levels of opportunistic pathogens, and reduced beneficial microbes, which were associated with the stroke severity. In manuscript #2, changes in gut inflammation and gut membrane integrity was investigated following the intracisternal

administration of Tanshinone IIA-loaded nanoparticles (Tan IIA-NPs) and induced pluripotent stem cell-derived neural stem cell (iNSCs) transplantation therapy during the long-term ischemic stroke in a pig model. Tan IIA-NP+iNSC treatment increased fecal SCFAs levels, reduced protein levels of TNF-α and TNF-α receptor 1 and increased expression of gut tight junction proteins occludin, claudin1, and ZO-1. In manuscript #3, we investigated whether the traumatic brain injury (TBI) alters oral microbiota composition, which is the second largest microbial community in body. TBI reduced Faith's Phylogenetic Diversity and altered beta-diversity of the oral microbiome compared to the sham surgery without altering the taxonomical composition. Findings from these studies suggest that acquired brain injury such as stroke and TBI modulate microbial composition in gut and oral cavity and the crosstalk between the microbiome and the host may be a potential therapeutic target for conditions involving neurological disorders.

INDEX WORDS: ISCHEMIC STROKE, TRAUMATIC BRAIN INJURY, SECONDARY BRAIN INJURY, GUT-BRAIN AXIS, MICROBIOME-HOST INTERACTION, GUT MICROBIOME, ORAL MICROBIOME, MICROBIAL DIVERSITY, GUT HOMEOSTASIS, SHORT-CHAIN FATTY ACIDS, TIGHT JUNCTION PROTEINS, PORCINE MODEL, TRANSLATIONAL LARGE ANIMAL MODEL.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Acquired brain injuries such as stroke and traumatic brain injury (TBI) are fatal diseases, as they result in high mortality rates and permanent disability. The number of stroke and TBI patients increases every year, and until recently, more than 795,000 people suffer from a stroke or recurrent stroke each year [1], and approximately 288,000 people are hospitalized for TBI [2]. Ischemic stroke is the most abundant type of stroke, caused by a blockage of a blood vessel [3], while TBI is occurred by a sudden shock or pressure on the brain [4], both of which induce serious brain damage and death of neuronal cell. Following the primary injury, secondary injury causes significant deleterious effects including excitotoxicity, apoptosis, oxidative stress, and neuroinflammation, breaking down the blood-brain barrier [5]. Increased brain permeability allows inflammatory molecules to move between the brain and the circulatory system [6], implying that alterations in brain homeostasis can influence peripheral organs, particularly the gut, and vice versa [7]. Previous studies have shown that ischemic stroke commonly induces bowel discomfort in patients [8] with intestinal microbial disturbance [9-11], and impairments in microbial composition, gut barrier function, structure, and integrity were also observed in rodent model of stroke [12, 13] and TBI [14, 15]. Interestingly, systemic administration of prebiotic or beneficial microbes improved neurological dysfunctions by lowering gut inflammation and enhancing gut microbial homeostasis in brain injury animal models [16, 17], suggesting the close relationship between the gut and the brain. Collectively, those findings from the previous studies suggested the significant involvement of inter-organ communication in acquired brain injuries.

Inter-organ communication has been proposed to have a crucial role in homeostasis and disease pathophysiology [18]. In particular, the microbiota-gut-brain axis is important for maintaining homeostasis in brain diseases, and the gut microbiome is one of the key regulators of gut-brain function. The gut microbiome is the most diverse and largest bacterial community in the human body [19], and it plays a role in defense against pathogen, vitamin synthesis, immune system development, central nervous system modulation, as well as the short-chain fatty acids production, which are important for energy metabolism [20]. Drastic changes in microbiota can cause imbalances in the bacterial composition, which is called dysbiosis, and it includes the loss of beneficial bacteria, overgrowth of opportunistic pathogens, and loss of overall microbial diversity [21]. Microbial dysbiosis has been observed in various disease such as inflammatory bowel disease, cancer, cardiovascular disease, and obesity in humans [22-24], and has also been found in neurological disease such as stroke. This suggests that the close relationship between the gut and the brain may play an important role in neurological diseases through a bidirectional communication called the gut-brain axis [7]. The gut-brain axis consists of several connecting pathways through the neural system, the immune system, and the endocrine system [7, 25]. Briefly described, in brain, hypothalamus-pituitary-adrenal axis (HPA axis) is well known system for stress respond and pathway of neuroendocrine transmission [26]. Those stimulation results in release of stress hormone, the glucocorticoids from the adrenal glands, and influence gastrointestinal organs including bowel motility, permeability, and inflammation [26]. The gastrointestinal system also has enteric nervous system, also known as second brain, since it has about 200-600 million neurons of network similar to that of the brain. This system is independently controlling the gut functions but rely on the autonomic nervous system, especially the vagus nerve [27]. Vagus nerve is the major nerve of the parasympathetic system and mostly transmit the signals

from the gut to the brain with the vagal afferent nerve terminals located in the gut tissue, sensing the hormonal or mechanical signals [27]. Therefore, the neural system mediates the gut-brain axis, either directly or indirectly. In the gastrointestinal system, bacteria and their metabolites mediate the gut-brain interaction. The microbes in the gut can directly interact with immune cell and stimulate the release of cytokines that can affect neurophysiology [28]. Additionally, gut microbes produce several neurotransmitters, such as serotonin, tryptophan, GABA (Gamma-Aminobutyric Acid), and gut metabolites (bile acids and short-chain fatty acids), which can transmit signals to the brain through the nerve and circulatory system [25, 29]. Through this inter-organ communication, changes in brain or gastrointestinal homeostasis have been shown to influence each other in brain diseases. Therefore, it is critical to understand changes in the gastrointestinal system following brain injuries in order to comprehend the communication between the brain and the gut.

Following the gut microbiome, oral microbiome is the second largest microbial community after gut microbiome in human body. Unlike the bacteria in the gut that lives in the intestinal cavity with mucus, the bacteria in oral cavity make a bacterial biofilm that coats the oral cavity surfaces [30]. Each area of the oral cavity, including tongue, teeth, cheeks, throat, saliva, subgingival plaque, etc. has a slightly different microbial composition but the most abundantly existing genus is *Streptococcus* [31]. Although most studies to investigate the role of microbiome in host health were conducted on the gut microbiome, it has been suggested that oral microbiome may also be relevant to host's health [32]. Interestingly, dysbiosis in oral microbial composition has been recently found in neurological disorders such as Alzheimer's disease and Parkinson's disease. In patients with Alzheimer's disease, reduced microbial diversity and increased levels of endotoxin-releasing gram-negative bacteria such as *Moraxella*, *Leptotrichia*, and *Sphaerochaeta* were found

[33]. In patients with Parkinson's disease, potential opportunistic oral pathogens including *Prevotealla, Veillonella, Lactobacillaceae,* and *Coriobacteriaceae* were increased [34]. This result suggests a potential connection between oral microbiome and brain diseases. As studies investigating the role of oral microbiome in brain injuries are lacking at present, more research is needed on this subject.

Diet is not only an important energy source of humans, but also a fuel source for the gut microbiota; thus, dietary changes may govern alterations in microbiota composition. A healthy diet is a major source of various nutritional components that have antioxidant and anti-inflammatory properties [35, 36]. Poor diet and nutritional status has been linked to several comorbidities and undesirable health-related outcomes in brain injury patients. Acquired brain injuries such as stroke [37] and TBI [38], have distinct disease processes such as increased oxidative stress, inflammation, and poor energy metabolism in brain. Therefore, plant-based, low-fat, high-fiber diets rich in antioxidants, may minimize the risk of disease [39, 40] and influence the harmful pathophysiological processes, improving clinical outcomes [41]. A previous study indicated that a intake of western dietary pattern was related to an elevated risk of stroke [39, 40], whereas an intake of the Mediterranean diet or Dietary Approaches to Stop Hypertension (DASH) diet were associated with decreased risk of stroke [40]. In animal models of brain injury, dietary changes affected gut microbiota composition, intestinal inflammation, and membrane integrity, modifying the gut-brain axis and impacting neuronal function [42, 43]. Therefore, a variety of diet or nutrients consumed by the host will help to reduce risk factors of brain injuries and modulate gut microbiota composition and homeostasis, both of which affect the brain via gut-brain interaction.

Animal models have been used to investigate a wide range of scientific issues, including mechanisms and novel therapies, before applying the findings to humans. Particularly, pigs are strong translational animal models for biomedical research, such as the brain and gastrointestinal (GI) system, due to their structural and physiological similarities to the humans over rodents' model [44-46]. Pigs have anatomical brain size and structure similar to humans, with pigs having more than 60% of white matter (i.e., white matter-predominant gyrencephalic brains) [47, 48] with gyrencephalic brain structure [49], whereas rodents only have about 10% of white matter with a smooth cerebral cortex [49]. Furthermore, pig brain development is similar to that of humans, for much of their brain growth, composition, and myelination occurs around birth [50]. In addition to brain, pigs have similar physiological and anatomical traits of the GI tract to humans. Mice are originally granivore animals, whereas pigs are omnivorous mammal [51], thus pigs have comparable transit times, digestive and absorption processes, and nutritional requirements compare to human [52, 53]. Additionally, pigs use the cecum as the major site for fermentation of plant and dietary fiber, unlike mice that use the large colon [52]. Surprisingly, 96% of the functional genes in the human gut metagenome were also found in the pig gut metagenome [54], while 85% of gut microbiota in mice was absent in the human flora [55], suggesting that the complexity of the pig microbiome is comparable to that of the human microbiome. Therefore, the use of pig model as a clinically translational model will be an essential research tools for investigating the association between the brain and gut in human neurological diseases.

The literature review (Chapter 2) provides an overview of the preventive and therapeutic effects of nutrients on acquired brain injuries, such as stoke and TBI, and how nutrients affect disease outcomes by modulating gut-brain axis. Chapter 3 details a study that investigated how the gut microbiome including microbial composition and diversity, is altered following an ischemic

stroke for up to 5 days post-stroke and the correlation of this alterations with the stroke severity in a pig model. Chapter 4 presents the results of an investigation of the changes in gut homeostasis following the intracisternal administration of Tanshinone IIA-loaded nanoparticles (Tan IIA-NPs) and transplantation of induced pluripotent stem cell-derived neural stem cells (iNSCs) in the brain, and how those intestinal changes are correlated with stroke recovery during the long-term ischemic stroke. Although fecal samples are widely used to investigate the microbiome in the gut, bowel inconsistencies are commonly found in patients with brain injury, making it difficult to collect fecal samples. Therefore, examining the oral microbiome, the second most diverse microbial community, will be an additional or alternative area for studying microbial-host interaction in brain injuries. Chapter 5 examines the changes in the oral microbial composition and diversity during the acute stage of traumatic brain injury using a piglet model.

References

1. Tsao, C.W., et al., *Heart Disease and Stroke Statistics 2022 Update: A Report From the American Heart Association.* Circulation, 2022. **145**(8): p. e153-e639.
2. Peterson, A.B., et al., *Surveillance report of traumatic brain injury-related emergency department visits, hospitalizations, and deaths, United States, 2014.* 2019.
3. Amarenco, P., et al., *Atherosclerotic Disease of the Aortic Arch and the Risk of Ischemic Stroke.* 1994. **331**(22): p. 1474-1479.
4. Faul, M., et al., *Traumatic Brain Injury in the United States: Emergency Department Visits, Hospitalizations and Deaths*. 2010.
5. Borgens, R.B. and P. Liu-Snyder, *Understanding Secondary Injury.* The Quarterly Review of Biology, 2012. **87**(2): p. 89-127.
6. Galea, I., *The blood–brain barrier in systemic infection and inflammation.* Cellular & Molecular Immunology, 2021. **18**(11): p. 2489-2501.
7. Suganya, K. and B.-S. Koo, *Gut-Brain Axis: Role of Gut Microbiota on Neurological Disorders and How Probiotics/Prebiotics Beneficially Modulate Microbial and Immune Pathways to Improve Brain Functions.* International journal of molecular sciences, 2020. **21**(20): p. 7551.
8. Camara-Lemarroy, C.R., B.E. Ibarra-Yruegas, and F. Gongora-Rivera, *Gastrointestinal complications after ischemic stroke.* J Neurol Sci, 2014. **346**(1-2): p. 20-5.
9. Tremlett, H., et al., *The gut microbiome in human neurological disease: A review.* Ann Neurol, 2017. **81**(3): p. 369-382.

10. Yin, J., et al., *Dysbiosis of Gut Microbiota With Reduced Trimethylamine-N-Oxide Level in Patients With Large-Artery Atherosclerotic Stroke or Transient Ischemic Attack.* J Am Heart Assoc, 2015. **4**(11).

11. Xia, G.-H., et al., *Stroke Dysbiosis Index (SDI) in Gut Microbiome Are Associated With Brain Injury and Prognosis of Stroke.* Frontiers in neurology, 2019. **10**: p. 397-397.

12. Singh, V., et al., *Microbiota Dysbiosis Controls the Neuroinflammatory Response after Stroke.* J Neurosci, 2016. **36**(28): p. 7428-40.

13. Houlden, A., et al., *Brain injury induces specific changes in the caecal microbiota of mice via altered autonomic activity and mucoprotein production.* Brain Behav Immun, 2016. **57**: p. 10-20.

14. Hang, C.H., et al., *Alterations of intestinal mucosa structure and barrier function following traumatic brain injury in rats.* World J Gastroenterol, 2003. **9**(12): p. 2776-81.

15. Chen, G., et al., *Effects of progesterone on intestinal inflammatory response, mucosa structure alterations, and apoptosis following traumatic brain injury in male rats.* J Surg Res, 2008. **147**(1): p. 92-8.

16. Yuan, Q., et al., *Lactulose Improves Neurological Outcomes by Repressing Harmful Bacteria and Regulating Inflammatory Reactions in Mice After Stroke.* Frontiers in Cellular and Infection Microbiology, 2021. **11**.

17. Li, H., et al., *Clostridium butyricum exerts a neuroprotective effect in a mouse model of traumatic brain injury via the gut-brain axis.* Neurogastroenterology & Motility, 2018. **30**(5): p. e13260.

18. Bodine, S.C., et al., *An American Physiological Society cross-journal Call for Papers on "Inter-Organ Communication in Homeostasis and Disease".* American journal of physiology. Lung cellular and molecular physiology, 2021. **321**(1): p. L42-L49.

19. Group, N.H.W., et al., *The NIH Human Microbiome Project.* Genome research, 2009. **19**(12): p. 2317-2323.

20. Amon, P. and I. Sanderson, *What is the microbiome?* Archives of disease in childhood - Education & practice edition, 2017. **102**(5): p. 257.

21. DeGruttola, A.K., et al., *Current Understanding of Dysbiosis in Disease in Human and Animal Models.* Inflammatory bowel diseases, 2016. **22**(5): p. 1137-1150.

22. Sarkar, S., B. Das, and S. Banerjee, *Insights into the human gut microbiome and cardiovascular diseases.* 2018. **4**(1): p. 10-14.

23. Davis, C.D., *The Gut Microbiome and Its Role in Obesity.* Nutrition today, 2016. **51**(4): p. 167-174.

24. Franzosa, E.A., et al., *Gut microbiome structure and metabolic activity in inflammatory bowel disease.* Nature Microbiology, 2019. **4**(2): p. 293-305.

25. Carabotti, M., et al., *The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems.* Annals of gastroenterology, 2015. **28**(2): p. 203-209.

26. de Punder, K. and L. Pruimboom, *Stress induces endotoxemia and low-grade inflammation by increasing barrier permeability.* Front Immunol, 2015. **6**: p. 223.

27. Waise, T.M.Z., H.J. Dranse, and T.K.T. Lam, *The metabolic role of vagal afferent innervation.* Nature Reviews Gastroenterology & Hepatology, 2018. **15**(10): p. 625-636.

28. El Aidy, S., T.G. Dinan, and J.F. Cryan, *Immune modulation of the brain-gut-microbe axis.* Frontiers in microbiology, 2014. **5**: p. 146-146.

29. Martin, C.R., et al., *The Brain-Gut-Microbiome Axis.* Cellular and molecular gastroenterology and hepatology, 2018. **6**(2): p. 133-148.

30. Deo, P.N. and R. Deshmukh, *Oral microbiome: Unveiling the fundamentals.* Journal of oral and maxillofacial pathology : JOMFP, 2019. **23**(1): p. 122-128.

31. Sampaio-Maia, B., et al., *Chapter Four - The Oral Microbiome in Health and Its Implication in Oral and Systemic Diseases,* in *Advances in Applied Microbiology*, S. Sariaslani and G. Michael Gadd, Editors. 2016, Academic Press. p. 171-210.

32. Willis, J.R. and T. Gabaldón, *The Human Oral Microbiome in Health and Disease: From Sequences to Ecosystems.* Microorganisms, 2020. **8**(2): p. 308.

33. Liu, X.X., et al., *Analysis of Salivary Microbiome in Patients with Alzheimer's Disease.* J Alzheimers Dis, 2019. **72**(2): p. 633-640.

34. Pereira, P.A.B., et al., *Oral and nasal microbiota in Parkinson's disease.* Parkinsonism Relat Disord, 2017. **38**: p. 61-67.

35. Stromsnes, K., et al., *Anti-Inflammatory Properties of Diet: Role in Healthy Aging.* Biomedicines, 2021. **9**(8): p. 922.

36. Kris-Etherton, P.M., et al., *Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer.* The American Journal of Medicine, 2002. **113**(9, Supplement 2): p. 71-88.

37. Robbins, N.M. and R.A. Swanson, *Opposing effects of glucose on stroke and reperfusion injury: acidosis, oxidative stress, and energy metabolism.* Stroke, 2014. **45**(6): p. 1881-1886.

38. McKee, A.C. and D.H. Daneshvar, *The neuropathology of traumatic brain injury.* Handbook of clinical neurology, 2015. **127**: p. 45-66.

39. Fung, T.T., et al., *Prospective Study of Major Dietary Patterns and Stroke Risk in Women.* Stroke, 2004. **35**(9): p. 2014-2019.

40. Lopez-Romero, L., F. Silva-Sieger, and E. Gamboa-Delgado, *[Dietary factors associated with stroke: a literature review].* Rev Neurol, 2016. **63**(5): p. 211-8.

41. Ramírez-Salazar, S.A., et al., *Dietary Insights in Neurological Diseases.* Curr Neurol Neurosci Rep, 2021. **21**(10): p. 55.

42. Trachsel, J., et al., *Dietary Resistant Potato Starch Alters Intestinal Microbial Communities and Their Metabolites, and Markers of Immune Regulation and Barrier Function in Swine.* Frontiers in Immunology, 2019. **10**(1381).

43. Kim, K.-A., et al., *High Fat Diet-Induced Gut Microbiota Exacerbates Inflammation and Obesity in Mice via the TLR4 Signaling Pathway.* PLOS ONE, 2012. **7**(10): p. e47713.

44. Kaiser, E.E. and F.D. West, *Large animal ischemic stroke models: replicating human stroke pathophysiology.* Neural Regen Res, 2020. **15**(8): p. 1377-1387.

45. Lind, N.M., et al., *The use of pigs in neuroscience: modeling brain disorders.* Neuroscience & Biobehavioral Reviews, 2007. **31**(5): p. 728-751.

46. Swindle, M.M., et al., *Swine as models in biomedical research and toxicology testing.* Veterinary pathology, 2012. **49**(2): p. 344-356.

47. Gieling, E.T., et al., *The Pig as a Model Animal for Studying Cognition and Neurobehavioral Disorders*, in *Molecular and Functional Models in Neuropsychiatry*, J.J. Hagan, Editor. 2011, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 359-383.

48. Cullen, D.K., et al., *A Porcine Model of Traumatic Brain Injury via Head Rotational Acceleration.* Methods Mol Biol, 2016. **1462**: p. 289-324.

49. Baker, E.W., et al., *Induced Pluripotent Stem Cell-Derived Neural Stem Cell Therapy Enhances Recovery in an Ischemic Stroke Pig Model.* Sci Rep, 2017. **7**(1): p. 10075.

50. Dickerson, J.W.T., J. Dobbing, and R.A. McCance, *Prenatal and postnatal growth and development of the central nervous system of the pig.* Proceedings of the Royal Society of London. Series B. Biological Sciences, 1967. **166**(1005): p. 384-395.

51. Heinritz, S.N., R. Mosenthin, and E. Weiss, *Use of pigs as a potential model for research into dietary modulation of the human gut microbiota.* Nutrition Research Reviews, 2013. **26**(2): p. 191-209.

52. Sciascia, Q., G. Daş, and C.C. Metges, *REVIEW: The pig as a model for humans: Effects of nutritional factors on intestinal function and health1*. Journal of Animal Science, 2016. **94**(suppl_3): p. 441-452.

53. Miller, E.R. and D.E. Ullrey, *The Pig as a Model for Human Nutrition.* Annual Review of Nutrition, 1987. **7**(1): p. 361-382.

54. Xiao, L., et al., *A reference gene catalogue of the pig gut microbiome.* Nat Microbiol, 2016. **1**: p. 16161.

55. Ley, R.E., et al., *Obesity alters gut microbial ecology.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 11070-5.

CHAPTER 2

REVIEW OF THE LITERATURE

Stroke

Stroke is the fifth major cause of mortality and a leading cause of disability in the United States, contributing to about 795,000 new cases or recurrences each year [1]. Stroke occurs when blood flow to the brain area is blocked or leaked from vessels, classifying two types of strokes: 1) ischemic stroke and 2) hemorrhagic stroke [2, 3]. Ischemic stroke accounts for 87% of all strokes and is caused by a lack of blood flow and is further classified into cerebral infarction and transient ischemic attack (TIA) [3]. Cerebral infarction is necrotic tissue damage in the brain due to the lack of oxygen, while TIAs are defined as neurological dysfunction caused by cerebral ischemia and recovers within 24 hours of stroke. Hemorrhagic stroke is induced by bleeding and is divided into subarachnoid hemorrhage and intracerebral hemorrhage. Subarachnoid hemorrhage is caused by compromised blood vessels on the brain surface while intracerebral hemorrhage is induced by bleeding into the brain commonly due to high blood pressure [3]. As a result, stroke causes a limited blood supply to brain cells regardless of the type of stroke. The cerebral blood flow (CBF) in a normal brain is about 50 ±10 ml/100 g/min [4, 5], and CBF in acute stage of stroke patients was decreased to 14.1 to 35ml/100 g/min in the penumbra and 4.8 to 8.4ml/100 g/min in infarct core, which are beyond the critical range for brain cell blood supply [6], considering that neuronal cells start to be damaged at 8 - 10ml/100 g/min or lower of CBF [5, 7]. The neuronal cell death causes short- or long-term physical and cognitive impairment resulting in permanent disability. There is only one Food and Drug Administration (FDA)-approved drug for ischemic stroke called

tissue plasminogen activator (tPA), which dissolves the blood clot. However, this treatment has a narrow therapeutic window (needs to be treated within 4.5hr after stroke incidence) and has associated adverse effects such as increased risk of intracranial hemorrhage [8]. To treat hemorrhagic stroke, generic medications are used to control the bleeding and reduce brain pressure. Therefore, it is important to understand the physiological aspects of stroke to develop an alternative treatment. The injuries following stroke are progressively spread and exacerbated by secondary injuries including excitotoxicity, inflammation, oxidative stress, and apoptosis that destroying cells survive from the primary injury or are undamaged cells [9]. Targeting those secondary injuries is suggested as a therapeutic strategy to improve stroke severity and outcomes.

Traumatic brain injury

Traumatic brain injury (TBI) is another major cause of death and disability in the United States [10]. Pathophysiology of the TBI begins with the initial injury, which is caused by mechanical damage to the blood-brain barrier (BBB), brain tissue, and vasculature [11]. There are the following two types of primary injuries in TBI: 1) focal brain injury which is usually seen in patients who had a closed head TBI or penetrating TBI that have localized contusion at the core of the injury site [11], and 2) diffuse brain injuries caused by rapid deceleration and acceleration involving high-speed motor vehicle accidents [12]. As a result, TBI induces bleeding and white matter shearing, leading to neuronal cell damage [11, 12]. Both kinds of damage often coexist in individuals who have had a moderate to severe TBI although diffuse axonal injury (DAI) accounts for about 70% of TBI cases [13]. The cellular and physiological events that arise during primary injury often progress to delayed and long-term secondary injuries that can last from hours to years [14]. These secondary injuries trigger significant deleterious effects on the brain through some

molecular and cellular responses including excitotoxicity, mitochondrial dysfunction, oxidative stress, and inflammation [9, 15]. Therefore, targeting those secondary injuries can be a major target for treating TBI by limiting neuronal cell death and facilitating recovery and outcomes.

Secondary injuries (Appendix Figure 1)

Secondary injuries occur through the biochemical, molecular and physiological processes during the primary brain injury developed from hours to days and contribute to further destruction of neuronal cells [16]. Stroke and TBI experimental models have been shown to induce an increased expression of proinflammatory cytokines [17-20], apoptotic molecules [20, 21], and oxidative stress [17-20, 22], while disrupting the antioxidant defense system [17-20] in the brain lesion site. Following primary injury, ion homeostasis in the brain is disrupted due to the lack of oxygen and glucose with a restricted blood supply [23]. Then, the ion pumps lose their normal function, resulting in a potassium (K+) efflux and a sodium (Na+) influx with an adenosine triphosphate (ATP) depletion. It causes neuronal membrane depolarization of the nerve membrane and excessive release of glutamate [23]. The extracellular accumulation of glutamate activates glutamate receptors of neurons (NMDA or AMPA receptors) and allow an intake of high levels of calcium [24]. The glutamate-activated NMDA receptor increases the generation of reactive oxygen species (ROS) and nitric oxide (NO), which exacerbate the cell injuries [25, 26]. The excessive influx of calcium in neurons also activates enzymes such as phospholipases, endonucleases, and proteases [27], as well as caspase-processed apoptosis [28], mitochondria impairment [29], and deregulation of ROS homeostasis [30], leading to neuronal cell death. The increased oxidative stress damages the BBB that generates a bidirectional flow of the inflammatory molecules between the systemic circulation and the brain [31]. Concurrently, in the injured brain tissue, the resting

microglia (ramified phenotype) turned into the activated form [32] and was recruited to the ischemic area [33]. The released proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin 1β (IL-1β) from the resident cells stimulate and aggravate an inflammatory response and also damage the BBB, resulting in infiltration of circulatory inflammatory cells that causes neuronal cell death or axonal damage [34], leading to loss of function of the brain in stroke and TBI cases.

Preventive effects of nutrients on stroke

Carbohydrate

Carbohydrates are the main source of body energy and provide different health consequences depending on their types of carbohydrates. Dietary glycemic index and dietary glycemic load both reflect the ability to raise blood sugar in the body. Glycemic index measures the quality of carbohydrates [35], while the dietary glycemic load evaluates both the quality and quantity of carbohydrates [36]. Excessive carbohydrate intake with a high glycemic index or glycemic load has been shown to negatively affect lipid and glucose metabolism, increasing the risk of cardiovascular disease [37-39]. Interestingly, Hajushafiee et al found that stroke patients consumed more energy and refined carbohydrates (such as sugar, jelly, sweet beverage, candy, etc…) in the preceding year before stroke than healthy people [40]. That carbohydrate intake with a high glycemic index and the glycemic load were associated with an increased risk of stroke [41, 42]. Contrary, a high intake of low glycemic index food was correlated with reduced risk of stroke [42-44]. For example, adding 10 grams of total dietary fiber per day lowered the incidence of stroke by 12% [45], and increased fiber consumption was linked with reduced stroke risk in a dose-dependent manner [46]. Various types of fiber, including that of fruit [44, 47], vegetable [44, 47],

cereal [42], and whole-grain [48], were also significantly correlated with decreased risk of stroke, indicating the beneficial role of fibers against stroke risk. Dietary fiber consumption may reduce stroke risk by lowering the incidence of hypertension [49] and improving blood lipid profiles [50, 51] and insulin resistance [52], which are risk factors for stroke. It also helps to reduce bowel dysfunction, including constipation and impaired bowel movement which are commonly found in stroke patients [53]. Overall, consumption of carbohydrates with a high glycemic index and load was associated with an increased risk of stroke, and it is important to consider both the quantity and quality of carbohydrate intake to prevent incidence of stroke.

Protein

Proteins are essential nutrients for body development and repair. Dietary protein intake was associated with stroke risk depending on the source of protein. High intake of red meat was related to an increased risk of stroke [54-56], whereas poultry intake [54] was linked with reduced risk of stroke. Red meat is processed with high sodium concentration and has a high amount of heme iron which may contribute to the development of hypertension [57] or atherosclerosis [58]. In contrast, poultry has a lower amount of heme iron and a higher level of polyunsaturated fat that benefits lipid profiles or blood pressure [59]. Similarly, the population that consumed fish as a major source of animal protein had a reduced risk of stroke [60-62], possibly due to a high percentage of the n-3 polyunsaturated fatty acids found in fish. Compared to animal proteins, vegetable proteins have more nonessential amino acids, including arginine, glycine, alanine, and serine, which can promote gluconeogenesis and lower insulin levels [63], both of which were linked to reduced risk of stroke [64]. In addition, high intake of arginine may reduce blood pressure by increasing nitric oxide, a

vasodilator [65]. Collectively, dietary protein intake from poultry, fish, or vegetables is recommended rather than processed red meat to prevent risk of stroke.

Fat

Reducing excessive high fat diets is generally recommended to prevent cardiovascular disease due to the high saturated fatty acids (SFAs) levels [66]. Stroke patients had consumed more hydrogenated fats, butter, cream, and mayonnaise than the healthy controls in the year before their stroke [67], suggesting a potential correlation between fat intake and stroke incidence. However, whether SFAs consumption is positively correlated with risk of stroke is controversial. Meta-analysis studies reported that increased SFAs consumption is not linked with an elevated risk of stroke [68-70], and the association with stroke risk differs by ethnicity [71-73], sex [71-73], BMI [73], or SFAs dose [73]. Interestingly, some studies have reported that low SFAs intake was associated with high risk of hemorrhagic stroke [74-76], and ischemic stroke patients with a history of hyperlipidemia had reduced white matter hyperintensity, which is a predictor of infarct progression [77]. A possible explanation for the association between low fat intake and high risk of stroke is that low cholesterol levels caused by reduced SFAs intake may cause cell membrane abnormalities and arterial necrosis in the brain [78]. Collectively, the impact of SFAs consumption on stroke risk remains debatable. The discrepancy between studies may depend on genetic and environmental differences among populations, food source, or specific subtypes of SFAs, suggesting the necessity for further research.

Polyunsaturated fatty acids (PUFAs), which comprise docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA), are one of the major components of fat. PUFAs consumption demonstrated beneficial effects on human health by reducing platelet aggregation, and

inflammation, and improving lipid metabolism [79]. Stroke patients presented a low level of PUFAs in the blood compared to healthy individuals [80], indicating that PUFAs potentially contribute to stroke risk. Studies showed that women who consumed fish more than 5 times per week had lower risk of ischemic stroke than women who consumed it 1-3 times per month [81], suggesting PUFA intake may be beneficial for preventing incidence of ischemic stroke. Interestingly, the effect of fish or fish oil intake on stroke risk disappeared in hemorrhagic stroke [81, 82]. Park et al stated that w-3 PUFA may raise the risk of hemorrhagic stroke by increasing the tendency to bleed with high pharmacological doses, such as the amount consumed by Greenland Eskimos (≥ 10 g/d) [83]. In conclusion, it is still controversial whether SFAs intake is associated with risk of stroke; meanwhile, PUFAs intake may be beneficial for reducing risk of ischemic stroke, but not hemorrhagic stroke, due to their different pathophysiological traits.

Vitamins

As a secondary injury, oxidative stress damages cellular compounds, and that stress is considered the major target in stroke. Therefore, antioxidant vitamins are suggested as beneficial supplements for stroke prevention. Vitamin C (ascorbic acid) is a well-known water-soluble antioxidant, and the plasma level of vitamin C was inversely associated with stroke mortality [84, 85] and risk of stroke [86-88]. A meta-analysis study reported that stroke risk was inversely related to dietary vitamin C intake and circulating vitamin C level, but not related to supplemental vitamin C intake [89]. Interestingly, circulating vitamin C level was saturated after taking 100mg of vitamin C per day, suggesting that people with high levels of vitamin C in their circulation may not benefit from vitamin C supplementation [89]. In a dose-dependent manner, the stroke risk remained low up to an intake of 550mg/day, but relative risk increased after an intake of

200mg/day, indicating that higher amount of vitamin C intake should be treated with caution [89]. Interestingly, a recent study showed that dietary vitamin C intake was inversely linked with a risk of total and ischemic stroke in non-smokers but not in smokers, assuming that smokers would consume more vitamin C to counteract the oxidative stress induced by smoking and may need a high dose of vitamin C [90]. Therefore, establishing a proper dosage of vitamin C is critical for its effectiveness in stroke prevention. Vitamin E (alpha-tocopherol), also known to have anti-oxidative and neuroprotective properties [91], showed inconsistent results for stroke risk. Vitamin E supplementation had no effect on incidence [92-94] and mortality of stroke [85, 95], nor was it related to decreased risk of ischemic stroke [96, 97]. It was, however, linked to an increased risk of hemorrhagic stroke [92, 96, 98], indicating that the effect of vitamin E on stroke risk is still controversial and may vary depending on the type of stroke. Another antioxidant vitamin, beta-carotene is a precursor for vitamin A and can reduce oxidative stress and neuronal damage [99]. In healthy male humans, the baseline level of plasma beta-carotene tended to be inversely related to ischemic stroke risk [100]. However, beta-carotene supplementation was linked to an increased risk of hemorrhagic stroke in male smokers [96], but it was not related to stroke incidence in women [93]. Moreover, supplementation with the three antioxidant vitamins (250mg vitamin C, 600mg vitamin E, and 20mg β-carotene daily) did not have a significant protective effect on stroke risk compared to the placebo group [101]. Therefore, it is premature to conclude that antioxidant vitamins are protective against risk of stroke, and further studies are needed to define a proper dose of vitamins (especially vitamin C) and their effects depending on the type of stroke.

Other vitamins have also been studied for their association with stroke risk, but data remains insufficient. Hyperhomocysteine is suggested to be linked to arterial dissection, thrombosis, or early atherosclerosis [102], as well as high risk of stroke [7, 103]. B vitamins or

folic acid intake can lower the blood level of total homocysteine [104, 105]. Previous studies showed that B vitamin supplementation reduced homocysteine levels and risk of stroke [106, 107]. Interestingly, the protective effect of B vitamin on stroke risk was absent in patients with kidney disorders, who have reduced renal excretion of homocysteine [106], supporting the fact that homocysteine blood level may be a critical risk factor for stroke incidence. Therefore, intake of B vitamins may be beneficial against risk of stroke, and dosage should be adjusted depending on the patient's condition.

Poor vitamin D blood level has been linked with hypertension [108] and an increased risk of stroke [109-114], showing that severe vitamin D deficiency causes the highest risk of stroke [112, 114]. However, conflicting findings reported that low levels of vitamin D in blood were not associated with stroke risk [115], or were only associated with an increased risk of ischemic stroke but not hemorrhagic stroke [116], which may be due to lower statistical power with a smaller number of hemorrhagic stroke patients than for ischemic stroke in the study [116].

In conclusion, vitamins C and B may protect against stroke risk, but other vitamins showed some conflicting findings, and there is insufficient evidence that they reduce stroke risk. More studies are warranted to determine the role of vitamins depending on the different time points of vitamin administration, different routes, improper dosage, and different types of strokes.

Minerals

Minerals such as magnesium, potassium, sodium, and zinc are essential micronutrients for body metabolism and composition. Magnesium intake protects against cardiovascular disease, with an inverse association with the risk of hypertension [117, 118] or type 2 diabetes [119, 120]. Magnesium can compete with sodium as a binding site in vascular smooth muscle cells, induces

endothelial vasodilation, and improves endothelial function [121], which appears to be effective in preventing stroke. Previous studies showed that a high intake of magnesium was negatively correlated with the risk of stroke [118, 122-125] in a dose dependent manner (150 – 550mg/day) [126]. Interestingly, after adjusting for hypertension and diabetes, the degree of inverse correlation between magnesium consumption and stroke risk was reduced, indicating that hypertension and diabetes due to low magnesium intake may impact risk of stroke [127]. Similarly, intake of potassium was negatively correlated with the risk of stroke [125, 128-131], possibly due to the improvement of vascular endothelial function and vascular flow by the release of nitric oxide [132].

The American Heart Association (AHA) has reduced the recommended daily sodium intake for all Americans to ≤1500mg due to the well-established link between excessive sodium consumption and hypertension [133]. Stroke patients had higher sodium intake than healthy people in the year before their stroke [134], and people who consumed more than 4000mg/day of sodium had a 2.6-fold increase in stroke risk than those who consumed less than 1500mg/day [135]. Similarly, people who had a high-salt diet had twice as high a risk of having an ischemic stroke as those on a low-salt diet [123], and positively correlated with stroke mortality [136, 137]. A meta-analysis study also described that a high intake of sodium and the sodium to potassium ratio were correlated with the increased risk of stroke [138], suggesting that high sodium intake is not recommended due to high risk of stroke.

Zinc is the second most abundant trace mineral in the body and has anti-inflammatory and antioxidant effects, protecting vascular cells from oxidative and inflammatory damage [139]. A meta-analysis study reported that plasma or serum zinc levels were higher in ischemic stroke patients compared to the control group, suggesting that zinc levels may be positively correlated with risk of ischemic stroke [140]. In contrast, plasma zinc levels were inversely correlated with

the risk of first hemorrhagic stroke [141]. Zinc deficiency or overload can induce blood-brain barrier disruption under oxygen and glucose deprivation conditions, which is the main cause of complications following stroke, such as inflammation and edema [142]. Therefore, it may be critical to maintain zinc homeostasis in ischemic and hemorrhagic stroke patients. The association and underlying mechanisms between zinc and each type of stroke needs further investigation.

Other minerals, such as iron, phosphorus, and calcium have conflicting associations with stroke risk. A low serum level of iron in the elderly (age > 65) was linked to an increased risk of stroke [110], while the plasma level of iron or iron intake in people aged 50-69 years [122] or 25-64 years [143] was not associated with risk of stroke, indicating that age may be an important variable for risk of stroke due to iron deficiency. The association between stroke risk and phosphorus intake was different depending on the type of stroke. Higher serum phosphate levels were linked with an increased risk of hemorrhagic stroke and a reduced risk of brain infarction in hemodialysis patients [144]. Conversely, the phosphorus blood level did not correlate with either ischemic [145] or total stroke risk [146]. Lastly, calcium intake was inversely related to a low risk of stroke [122, 123, 146, 147] or there was no correlation [128, 134, 148].

In conclusion, the effect of mineral intake on stroke risk is still inconclusive. The mineral intake will be related to increased consumption of other potentially beneficial nutrients such as vitamins, dietary fiber, or bioactive compounds. Additionally, it may be related to other possible lifestyle variables such as smoking and alcohol consumption and other stroke risk factors.

Therapeutic effects of nutrients on acquired brain injuries

When glucose is depleted, lactate is a well-known alternative energy source for the brain. During the brain injury, lack of oxygen and increased glucose consumption result in glucose

depletion and cerebral energy dysfunction [149, 150]. Interestingly, lactate demands have been found to increase in brain-injured patients [151-153] and preclinical studies suggest that lactate administration has neuroprotective effects on acute brain injuries. Intravenous or intracerebroventricular injection of sodium L-lactate reduces lesion size and improves neurological outcomes in TBI and stroke rodent models [154-157]. It also promotes brain restoration processes such as angiogenesis and neurogenesis in intracerebral hemorrhage rat model [158]. Clinical studies have also shown that exogenous lactate supplementation improves neurological outcomes [159], reduces intracranial pressure [160], and enhances cerebral energy metabolism by increasing lactate concentration and glucose availability in TBI patients [160-162], suggesting that lactate supplementation may be potentially therapeutic for brain injuries, as it restores impaired energy metabolism.

Protein-energy malnutrition is common in brain injury patients, contributing to poor clinical outcomes with increased oxidative stress and inflammation [163]. Therefore, adequate protein intake is an important component of nutritional management for these patients [164]. Whey protein supplementation in stroke patients decreased the NIH stroke scale/score (NIHSS) [165, 166] modified Rankin scale (mRS) scores [166], improved mini-mental state exam (MMSE) [167], and reduced TNF-α [166], IL-6 [166, 168], and high-sensitive C-reactive protein (hs-CRP) [166] blood level compared to non-treated patients, showing beneficial effects on cognitive function and inflammation. Further, stroke survivors often experience muscle atrophy as a result of physical impairment and ongoing muscle loss due to aging [169]. Loss of muscle can reduce muscle strength and exercise capacity which affects daily activity and post-stroke recovery [169]. Providing patients with energy and protein-rich supplementation helps maintain body mass and body composition in the early stage of a stroke [170, 171] and prevents fat loss in female patients

[171]. The combination of protein supplementation with neuromuscular electrical stimulation or exercise reduced muscle atrophy [172] and improved aerobic capacity and functional activity performances in daily living [173] compared to the non-treated group. Collectively, protein-rich supplementation will improve body performance and functional recovery by reducing levels of proinflammatory substances in brain-injured patients.

Omega-3 polyunsaturated fatty acids (PUFAs) offer benefits against cardiovascular disease by improving blood profiles and endothelial function [174]. Docosahexaenoic acid (DHA) is an essential omega-3 PUFAs mostly found in fish and is necessary for the development of the nervous system and brain functioning [175]. Preclinical studies reported that administration of DHA in the MCAO rodent model reduced the production of proinflammatory cytokines [176-179], microglial activation [176, 177, 179], and total infarct volume [177-179] and improved behavior recovery [178, 179]. In the TBI rodents model, DHA supplementation also improved neurological function [180, 181] and attenuated neuronal apoptosis and oxidative stress [180, 181], whereas the depletion of DHA in the brain was associated with impaired functional recovery [182, 183]. In clinical studies, lower levels of eicosapentaenoic acid (EPA), DHA, and w-3 PUFAs were associated with a high NIHSS score on admission and poor functional outcomes in stroke patients [184]. The ratio of w-3/w-6 fatty acids including EPA/arachidonic acid (AA), DHA/AA, and EPA+DHA/AA was also negatively correlated with early neurological deterioration in the acute stage of stroke patients [185], indicating the important neuroprotective role of w-3 PUFAs in brain injury patients. The possible mechanism of fish oil intake against brain injuries may include an increase of cerebral blood flow [186], inhibition of platelet aggregation [187-189], reduced blood viscosity [189], and suppression of blood pressure [190].

Vitamins are important micronutrients that can limit secondary injuries such as oxidative stress and inflammation. Vitamin C and E are well-known free radical scavengers showing beneficial effects on experimental brain injury models by reducing oxidative stress [191-193], improving cognitive function [192, 194], and preventing severe infarct size in the striatum [195]. In clinical studies, low activity of blood antioxidants or low levels of plasma vitamin C were correlated with the severity of neurological impairments in stroke or head trauma patients [196, 197], suggesting the potential importance of vitamin C supplementation. However, studies have failed to show an improvement in the clinical or functional status of stroke patients who received 500mg/d [198] or 1000mg/d of vitamin C intake [199]. Similarly, low dose administration (500mg/day, intravenous) of vitamin C did not have any recognizable impact on perilesional edema progression in TBI patients, while high dose (10g, intravenous) of vitamin C limited its development compared to the placebo group [200]. At the same time, vitamin E supplementation (400IU/d, intramuscular) reduced TBI mortality rate and improved functional outcomes at discharge [200]. When stroke patients were orally given vitamin C (500mg/day) combined with vitamin E (800IU/d), they had reduced blood levels of malondialdehyde (MDA) and CRP, showing the synergistic effects of this vitamin combination [201]. Collectively, although the study results vary depending on different experimental conditions such as dosage, route, duration and timing of administration, synergistic effects of antioxidant vitamins may be beneficial in treating brain injuries.

Other vitamins such as B vitamins and vitamin D are also studied for their role in attenuating or treating brain injuries. Post-stroke patients had insufficient intake of dietary folate than in their healthy counterparts [202] with high plasma levels of homocysteine [203]. An increased level of homocysteine was associated with the risk of stroke such as vascular damage

[204, 205] and production of free radicals [206]. In stroke patients, B vitamins supplementation reduced plasma levels of MDA and CRP [201, 207] and was associated with a decreased risk of post-stroke depression [208], demonstrating the beneficial effects of B vitamins against inflammation and oxidative stress in stroke patients.

Vitamin D deficiency was linked to high levels of CPR [209], large infarct lesion volume [210, 211], poor outcomes [209], and an increased post-stroke hip fractures [212] in ischemic stroke patients. Moreover, it was associated with impaired cognitive function in TBI patients [213], indicating that vitamin D deficiency may serve as a potential marker of high risk of poor outcome and disease severity. Supplementing vitamin D improved functional outcomes in stroke patients [214], and a single oral dose of 120,000IU of vitamin D reduced proinflammatory cytokines and improved the level of consciousness in TBI patients [215]. A combination of vitamin D (50,000IU) with vitamin A (50,000IU) supplementation reduced the serum IL-1β level and improved clinical outcomes in stroke patients, indicating the synergistic effects of vitamins combination. In conclusion, given the beneficial properties shown from the results, B vitamins and vitamin D may be considered as potential therapeutic options for brain injury patients.

Minerals are essential micronutrients that help our bodies develop and function properly. Magnesium is one of the macrominerals that showed neuroprotective effects in experimental brain injury models. Treatment of magnesium resulted in reduced neuronal loss [216, 217], cognitive dysfunction [216-218], and motor deficit [219] in a TBI rodent model as well as reduced lesion volume [220] in an ischemic stroke rat model. This reduction may be due to its beneficial role in vessel vasodilation [221] or preventing cell death [222]. However, clinical studies reported that magnesium therapy did not improve disability in stroke patients [223], or have any neuroprotective effect in TBI patients [224]. Moreover, mortality rate was increased in those TBI patients who had

a high magnesium dose compared to the placebo group [224], revealing data conflicting with previous preclinical studies. Interestingly, when magnesium was combined with potassium-enriched salt, it increased the neurological performances (NIHSS, mRS, and Barthel index) in stroke patients compared to potassium or magnesium-only enriched salt patients groups [225]. Collectively, more clinical studies are warranted to understand the role of magnesium in brain injuries, and the synergistic effects of magnesium with other minerals may be considered a potential therapeutic option.

Zinc is an important cofactor regulating a variety of enzymes involved in cellular processes or signaling pathways, and it has antioxidant and anti-inflammatory effects [226]. Interestingly, in preclinical studies, zinc has been suggested to have both neuroprotective and neurotoxic properties in brain injury models. It was reported that zinc administration inhibited the glutamate-induced calcium influx and neuronal death in cultured neurons [227] as well as improved spatial learning and memory function in a TBI rat model [228]. On the other hand, it had been suggested that excessive neuronal zinc accumulation after brain injury may be toxic [227] and contribute to calcium influx, excitotoxicity, and cell death [229, 230]. However, removing zinc via chelation or chemicals did not improved neurobehavioral outcome [231], or it had detrimental effects on neuronal damage in a TBI mice model [232]. Since the serum level of zinc was significantly depleted in stroke patients [233] and the low level of zin ($\leq$ 65mcg/dL) was associated with higher stroke severity and poor functional outcomes at discharge [234], the focus of studies shifted towards investigating the protective role of zinc supplementation in patients with brain injury [235]. Zinc supplementation improved neurological recovery in a zinc deficient group compared to a placebo group in stroke patients [236], and reduced mortality and neurological dysfunction in TBI patients [237], suggesting the potential neuroprotective role of zinc intake in brain-injured patients.

Electrolyte disturbance was found in acute stroke or TBI patients with a decreased level of serum potassium [238] and sodium [239, 240], possibly due to the disrupted antidiuretic hormone secretion, medications, or excessive use of intravenous fluids [241]. However, there are not many studies investigating whether the restoration of such deficiencies is beneficial for better outcomes in brain-injured patients. A study showed that an increase in daily potassium consumption was linked to a 40% reduction in the incidence of stroke-related death [129], indicating the potential beneficial role of potassium against stroke.

Lower serum calcium level at hospital admission was also associated with the larger lesion volumes [242] and the presence of cerebral microbleeds in stroke patients [243]. Supplementing calcium with vitamin D improved stroke outcomes and reduced mortality [244], suggesting their beneficial effects against brain injuries.

Collectively, preclinical studies have shown promising effects of minerals on the severity or outcome of stroke and TBI, however clinical trials on the therapeutic effects of mineral supplementation are limited. Further studies are required to determine effective doses or routes of application in patients.

Therapeutic effects of diet on acquired brain injuries via the gut-brain axis

The gut–brain axis, a bidirectional communication link between the gastrointestinal and the central nervous system, has gained attention in neurological injuries due to its important role in disease pathophysiology [245, 246]. Interestingly, stroke or TBI animal models had cognitive impairment [247, 248], increased neuroinflammation [247], and poor outcomes [249, 250], as well as gut dysfunction [247, 251] and gut dysbiosis [248-250], indicating that the gut and brain are somehow connected through this axis. Potential mechanisms of the gut-brain axis include immune pathways, neural pathways, and endocrine pathways involving microbial metabolites such as

neurotransmitters or short-chain fatty acids (SCFAs) [252]. Preclinical studies have suggested that dietary factors such as SCFAs, probiotics, prebiotics, Chinese medicine, or natural herbs that modulate the gut-brain axis may be effective therapeutic options for brain injuries, such as stroke and TBI.

SCFAs are byproducts produced by gut microbes and play an important role in regulating immune response, gut barrier function, and overall gut health [253]. In a stroke mice model, SCFAs supplementation reduced infarct volume and neurological impairment, both of which had a negative correlation with the levels of SCFAs including acetate, valerate, and, in particular, butyrate [254]. Butyrate is a primary energy source in the gut, and it helps to maintain the health of the intestinal mucosa [255]. Like SCFAs, butyrate supplementation reduced the neurological impairment, cerebral infarction, and cerebral edema and even improved blood lipid levels and risk of thrombosis in both an ischemic stroke and an ischemic stroke with type 2 diabetes rodent model [254, 256], by reducing opportunistic pathogens such as *Bacteroides*, *Klebsiella*, and *Haemophilus* [254] and increasing beneficial bacteria such as *Lactobacillus*, *Butyricicoccus*, *Megamonas* [254], and *Christensenellacea* (butyrate-producing bacteria) [256]. Butyrate supplementation also improved gut impairment following stroke by increasing the expression of gut tight junction proteins [254, 256], supporting the finding that SCFAs supplementation may modulate the gut microbial composition and restore gut integrity, thus affecting the severity and progression of brain injury.

Probiotics or SCFAs-producing bacteria are commensal or beneficial bacteria that have protective effects on human health. *Clostridium butrycum* (*C. butyricum*) is one of the butyrate-producing bacteria and its treatment has neuroprotective effects on stroke [257], TBI [258], and neurocognitive impairment rodent model [259]. In a stroke and TBI rodent model, *C. butyricum*

supplementation not only elevated beneficial microbiomes such as *Clostridium* cluster XIVab, *F. prausnitzii*, *Bifidobacterium*, and *Lactobacillus* [257], but also increased the intestinal secretion of glucagon-like peptide-1 (GLP-1) and expression of cerebral GLP-1 receptor (GLP-1R) [258]. The release of gut hormone GLP can be induced by butyrate through G-protein coupled receptor 41 (GPR41) or GPR43 [260]. Translocation of intestinal GLP into the bloodstream can bind to GLP-R in the brain, which is engaged in the PI3K/AKT pathway by improving neuronal growth, survival, and metabolism in the brain [257, 258]. *C. butyricum* supplementation also increased the levels of neuroprotective molecules, and brain-derived neurotrophic factor (BDNF) in the brain and reduced the neuroinflammation in the neurocognitive impairment mice model [259]. Therefore, SCFAs-producing bacteria may have beneficial effects on neurological disease by stimulating the release of gut metabolites, such as GLP and regulating its related signaling pathway in the brain.

As another probiotic, the *Lactobacillus* species supplementation also restored gut dysbiosis [261] by increasing the level of *Lactobacillus* (*Bacilli*, *Lactobacillales*, *Lactobacillaceae*) [262] and attenuating the impaired gut permeability in brain injury rodent models [261, 262]. In detail, *Lactobacillus* species supplementation increased the hippocampal neuroprotective protein level of BDNF and cAMP response element-binding response (CREB), a transcriptional regulator of BDNF, as well as reduced myelin damage and neural apoptosis [262]. Moreover, an increased level of *Lactobacillus* following supplementation had a significant positive correlation with behavioral test and protein expression of intestinal tight junction protein ZO-1, while it had a negative correlation with hippocampal levels of TNF-a, suggesting the regulatory effects of *Lactobacillus* on both cognitive and gut barrier functions [262]. Therefore, increased SCFAs levels or beneficial bacteria following probiotic or SCFA-producing bacteria supplementation may

attenuate the brain injury via the gut-brain axis by promoting the production of neuroprotective molecules or restoring gut function.

Prebiotics are food components that support gut microbiota and provide various health benefits [263]. Lactulose is one of the prebiotics and promotes the growth of beneficial microbes [264]. Lactulose supplementation in a stroke mice model restored functional outcomes and downregulated immune reaction by decreasing inflammatory cytokine expression (TNF-a and IL-1b) and increasing anti-inflammatory factors (TGF-b and Nrf2) in both the brain and gut [265]. In addition, it improved gut barrier function, and permeability, decreased levels of the harmful bacteria, *Desulfovibrionaceae*, and increased levels of beneficial metabolites such as eicosapentaenoic acid (EPA), allantoin, taurine, and D-mannose in blood [265], all of which have anti-inflammatory properties. Therefore, lactulose treatment may have neuroprotective effects by inhibiting harmful bacteria and regulating the immune response in the gut. When prebiotic (inulin) supplementation was combined with SCFAs-producing bacteria, the neuroprotective effects were better than the group with only SCFAs-producing bacteria, in terms of improving neurological deficits and reducing neuroinflammation in a stroke mice model [266]. Moreover, stroke-aged mice fed feces from the SCFA producers combined with inulin supplemented mice had increased protein expression of mucus producer, mucin 2 (MUC2) in goblet cells, and levels of SCFAs in the gut, blood, and brain compared to non-treated stroke-aged mice [266], suggesting the synergistic beneficial effects of gut microbiota and prebiotics against brain injury. Collectively, prebiotics may improve the severity or outcomes of brain injury by increasing gut-derived anti-inflammatory molecules and improving gut dysfunction.

Dietary components, such as Chinese traditional medicine or natural sources, have been studied to explore their beneficial effects on brain injuries via the gut-brain axis. Researchers

studied crocin [267], Radix Pueraiae with chuanxiong rhizome [268], Tong-Qiao-Huo-Xue decoction [269], rhubarb anthraquinone glycosides [270], Dioscorea polystachya from yam gruel [271], baicalin [272], β-asaron and paeonol [273], Panax notoginsenosides [274], and resveratrol [275] as therapeutic treatments in brain injury models. The treatments had neuroprotective effects showing reduced infarct volume [267-269, 274, 275], oxidative stress [267, 271], neuroinflammation [269, 272, 274, 275], and improved neurological function [267-269, 271, 272, 274, 275] by modulating gut microbiota, gut metabolites production, and systemic inflammation.

The role of the gut microbiota in brain injury was confirmed in a stroke mice model, as mice receiving fecal transplants from the treatment-supplemented mice group had better biological outcomes than those receiving fecal matter from the non-treated group [269, 275]. In addition, the therapeutic effect of the treatment disappeared when the gut microbiota was depleted [267], and poor health and functional outcomes following brain injury have been related to gut dysbiosis [276-278], indicating the importance of maintaining a normal gut microbiota in bettering brain injury status. Interestingly, Chinese medicine treatments restored the disrupted species richness and diversity (alpha-diversity) following ischemic stroke [268, 279] and had a similar microbial pattern (beta-diversity) with the sham surgery stroke group as compared to the non-treated stroke group [268, 269]. Treatment also restored the disrupted microbial composition following brain injuries [269, 272, 279] and modulated it by increasing the levels of beneficial or endogenous bacteria such as *Lactobacillus, Ruminococcus* [268, 271], *Clostridium* (SCFAs-producing bacteria) [271], *Bifidobacterium* [269], and *Bifidobacterium longum* (putative psychobiotics) [274], while reducing pathogenic bacteria including *Bacteroides, Escherichia_Shigella, Haemophilus, Eubacterium_nodatum_group, Collinsella, Enterococcus, Proteus, Alistipes, Klebsiella, Shuttleworthia and Faecalibacterium* [268]. This effect supports the claim that the Chinese

medicine treatments help to restore gut dysbiosis and ensure an abundance of beneficial bacteria following brain injuries.

Trimethylamine (TMA) is generated by the gut microbes that metabolize choline or L-carnitine, which is oxidized to trimethylamine N-oxide (TMAO) in the liver [280]. TMAO has been linked to adverse outcomes of cardiovascular diseases and is considered one of the risk factors of stroke [280]. A study found that baicalin treatment, an active flavonoid found in Chinese traditional medicine, reduced the plasma level of TMA, TMAO, and clusterin (a biomarker of neuroinflammation), improved cognitive function, increased functional connectivity between brain regions, elevated hippocampal plasticity and dendritic spine density, decreased neuroinflammation, and improved gut dysbiosis following stroke injury [272]. All these results suggest that baicalin may help to ameliorate brain injury by modulating gut microbes and reducing metabolites synthesis such as TMA and TMAO, which further influence neuroinflammation [272].

Cholecystokinin (CCK), a gut hormonal peptide, also acts as a neurotransmitter and binds to G protein-coupled receptors by stimulating vagal afferent fibers and transferring signals to the brain [281]. It has been shown that CCK may be involved in cognitive and memory function [282], showing that CCK knocked out mice had poor performance and spatial memory compared to the control [283]. He et al reported that β-asaron and paeonol treatments in an ischemic stroke rat model had increased protein expression of CCK, protein kinase A (PKA), and NF-kb inhibitor (Ikb) in both gut and brain tissue, as well as elevated protein expression of CCK type B receptor (CCKBR) in the brain [273]. The treatments also improved the behavioral performance and reduced brain tissue damage and systemic inflammation. Interestingly, the CCK level in the gut was positively correlated with the level of CCK in the brain, indicating that intestinal CCK levels may affect the levels in the brain via the gut-brain axis [273]. Although the exact mechanism of β-

asaron and paeonol has not been fully examined, a potential pathway is that released CCK from the intestine would bind to CCKBR in the brain through the vagus nerve and modulate neuroinflammation [284].

Gut microbiota produces neurotransmitters such as serotonin (5-HT) and γ-aminobutyric acid (GABA), which affect the central nervous system via the vagus nerve. 5-HT is an important signaling molecule involved in mood, appetite, sleep, and memory and learning function, and it is released from gut enterochromaffin cells to regulate visceral sensitivity and vasodilatory functions [285]. Disrupted levels of 5-HT in the brain have been linked to depression and the pathophysiology of a variety of neurological diseases [286], suggesting the importance of maintaining 5-HT levels for a healthy brain. A stroke rat model showed reduced levels of 5-HT and its metabolite 5-HIAA in both brain and gut as well as dysbiosis in the gut [270, 279]. However, those changes were restored by the treatment of Chinese medicine involving rhubarb anthraquinone glycoside (RGA) [270, 279], indicating that treatment may restore the 5-HT levels by modulating gut microbes. Similarly, Dioscorea polystachya treatment from a medicated diet of "yam gruel" also increased the protein level of cerebral 5-HT, GABA, and hippocampal BDNF in a stroke rat model, along with the increased amounts of beneficial bacteria such as SCFAs-producing bacteria (*Lactobacillus*, *Ruminococcus*, and *Clostridium*) and levels of intestinal SCFAs [271]. Intestinal 5-HT can stimulate BDNF production in the brain through vagus nerve stimulation [287], which is a neuroprotective molecule. Therefore, these treatments may restore the disrupted production of gut-derived neurotransmitters such as 5-HT or GABA following stroke and stimulate neuroprotective signaling by modulating gut microbiome composition.

Excitatory amino acids induce excitotoxicity, oxidative stress, and neuronal damage; therefore, the balance of excitatory and inhibitory response is important [288, 289]. GABA is an

inhibitory amino acid that suppresses the severity of brain injury and mitigates the toxicity of excitatory amino acids such as glutamate and aspartic acid [288, 289]. A recent study reported that gut microbiota, especially the *Bifidobacterium* species, are able to produce GABA [290]. Interestingly, Panax notoginsenosides (PNE) treatment increased the amount of beneficial bacteria *Bifidobacterium longum* (B.L) and upregulated the GABA receptor in the hippocampus, while administration of GABA antagonist significantly attenuated the neuroprotective effects of PNE and B.L [274]. In addition, rhubarb anthraquinone glycosides (RAG) treatment reduced the level of glutamate and aspartic acid and restored GABA in both brain and gut of the stroke rat model [270]. These results suggest that PNE and RAG treatments may help to increase the intestinal level of GABA and reduce the toxicity of excitatory amino acids via the gut-brain axis following brain injuries.

A stroke or TBI triggers local neuroinflammation while also disrupting the peripheral immune system [291, 292]. Studies have suggested the critical role of the gut microbiome in regulating immune response following brain injuries [293]. For instance, blood invasion of bacteria or proinflammatory substances from the gut can cross the BBB and alter its integrity by inducing neuroinflammation and affecting brain function [294]. Chinese medicine or natural source treatments inhibit the gut inflammation by decreasing the levels of IL-17α , IL-23, IFN-γ, TNF-α [275], and Th 17 cell [275], and inhibiting NF-kB activity [273], whereas increasing the amount of anti-inflammatory substances such as IL-10, IL-4 [275, 295], and T-reg cell [269, 275] in brain-injured models. Moreover, the treatments restored permeability in both the gut and brain by reducing the levels of blood gut leakage biomarker DAO, LPS, L-lactate [268, 269, 271] and fecal/plasma ratio of albumin [275], and restored the impaired tight junction protein in both brain [268] and gut [268, 269]. As a result, reduced blood levels of proinflammatory cytokines [271,

273, 275] were found with reduced infarct volume [267-269, 274, 275], improved neurological function [267-269, 271, 272, 274, 275], and reduced neuroinflammation [269, 272, 274, 275] or oxidative stress [267, 271] in the brain, indicating that treatments may reduce gut and systemic inflammation, which are further correlated to the severity and outcomes of brain injury.

Lastly, other studies investigated the role of drug or calorie restriction in brain injury models which also can modulate gut microbiota. Atorvastatin is an antithrombotic medicine that has been used to lower cholesterol and has been shown to decrease the risk of stroke and major coronary events [296]. Atorvastatin supplementation in the stroke mice model reduced the infarct volume, neurological deficits, and inhibited the microglia-mediated neuroinflammation by improving the impaired gut permeability and decreasing intestinal and systemic inflammation [295]. It also modulated the gut microbes by increasing beneficial bacteria *Lactobacillus* and decreasing *Bacteroideaceae* with a reduced level of circulating endotoxin [295]. Interestingly, Firmicutes was negatively correlated with brain proinflammatory cytokines (MCP1, IL-6, TNF-a) and positively related to intestinal anti-inflammatory cytokine IL-10, while *Parabacteroides goldsteinii*, and *Candidatus Alistipes marseilloanorexicus AP11* showed an opposite trend [295], suggesting that atorvastatin may improve the stroke outcome by modulating gut microbiome composition and reduce microglia-mediated neuroinflammation via the gut-brain axis.

Calorie restriction (CR) has been known to extend lifespan and inhibit age-related diseases [297]. Studies found that CR increased neurotrophic factors (BDNF and bFGF), anti-oxidant proteins (HO-1), and anti-inflammatory cytokine (IL-10), while decreasing proinflammatory cytokine (TNF-a and IL-6) [298] in a stroke animal model. CR also suppressed neuroapoptosis and microglia or astrocyte activation in TBI animal models [299, 300]. Another study also reported that pretreatment of CR (30% reduction of caloric intake) in a stroke mice model had improved

long-term outcomes including motor function, exploratory behavior, and reduced anxiety as well as reduced level of inflammatory cytokines (IL-1β and TNF-α) in the brain [301]. When these stroke mice received feces from CR-treated mice, long-term stroke outcomes also improved compared to stroke mice transplanted with feces from normal diet mice, indicating that changes in gut microbiota following CR are beneficial against stroke [301]. CR-treated mice were enriched with the probiotic, *Bifidobacterium,* and had increased levels of prostaglandin B1 in their feces, which is believed to be helpful for stroke recovery [301]. Prostaglandin B1 is an oligomer that has a potent inhibitor of phospholipases A2 activity, which is neurotoxic [302, 303]. As a follow-up experiment, when a cocktail of *Bifidobacterium* was administered to stroke mice, it also facilitated the long-term rehabilitation, suggesting that CR may improve the post-stroke recovery by improving beneficial bacteria and elevating beneficial metabolites in the gut. However, more studies are needed to investigate the role and mechanism of CR in stroke recovery.

Summary

Ischemic stroke and traumatic brain injury (TBI) are both major causes of death and disability in the United States. Both brain injuries result in significant neuronal cell death and secondary cascade injury including oxidative stress, inflammation, apoptosis, and blood-brain barrier disruption. A healthy diet contains a variety of beneficial nutrients with antioxidant and anti-inflammation properties. Therefore, many clinical studies have investigated the relationship between dietary nutrients on disease risk or outcomes. Fibers, protein (from poultry, fish, or vegetables), PUFAs, magnesium, potassium, and low sodium intake have been linked to reduced risk of stroke. Supplementation with lactate, high energy-dense protein, PUFAs, antioxidant vitamins, vitamin D, zinc, and calcium after brain injury has potentially beneficial effects on

recovery and outcomes of stroke and TBI patients. However, in many clinical and preclinical results, the effects of dietary nutrients on brain injury prevention and recovery varied between studies. The diet not only influences the body's metabolism, but it also modulates the gut microbiome, a key component that mediates gut-brain interaction. Bioactive compounds from the diet or traditional Chinese medicine have been shown to improve gut and brain homeostasis by regulating the gut-brain axis, suggesting dynamic inter-organ communication in brain injuries. Besides the gut microbiome, our body also contains microbiomes in various parts of the body, suggesting the feasibility of using other microbiomes in investigating their role in brain injuries. Therefore, this dissertation seeks to address three specific aims to investigate: 1) Gut microbiome changes in the acute stage of ischemic stroke and their correlation with stroke severity, 2) Changes in gut homeostasis following brain-targeted stroke therapy during long-term ischemic stroke, 3) Changes in the oral microbiome during the acute stage of traumatic brain injury.

References

1. Virani, S.S., et al., *Heart Disease and Stroke Statistics—2021 Update.* Circulation, 2021. **143**(8): p. e254-e743.
2. Amarenco, P., et al., *Atherosclerotic Disease of the Aortic Arch and the Risk of Ischemic Stroke.* 1994. **331**(22): p. 1474-1479.
3. Musuka, T.D., et al., *Diagnosis and management of acute ischemic stroke: speed is critical.* CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne, 2015. **187**(12): p. 887-893.
4. Lassen, N.A., *Normal average value of cerebral blood flow in younger adults is 50 ml/100 g/min.* J Cereb Blood Flow Metab, 1985. **5**(3): p. 347-9.
5. Zhou, D., et al., *Advances in chronic cerebral circulation insufficiency.* CNS neuroscience & therapeutics, 2018. **24**(1): p. 5-17.
6. Bandera, E., et al., *Cerebral Blood Flow Threshold of Ischemic Penumbra and Infarct Core in Acute Ischemic Stroke.* Stroke, 2006. **37**(5): p. 1334-1339.
7. Hassan, A., et al., *Homocysteine is a risk factor for cerebral small vessel disease, acting via endothelial dysfunction.* Brain, 2004. **127**(Pt 1): p. 212-9.
8. Katzan, I.L., et al., *Use of Tissue-Type Plasminogen Activator for Acute Ischemic StrokeThe Cleveland Area Experience.* JAMA, 2000. **283**(9): p. 1151-1158.
9. Borgens, R.B. and P. Liu-Snyder, *Understanding secondary injury.* Q Rev Biol, 2012. **87**(2): p. 89-127.
10. Peterson, A.B., et al., *Surveillance report of traumatic brain injury-related emergency department visits, hospitalizations, and deaths, United States, 2014.* 2019.

11. Ng, S.Y. and A.Y.W. Lee, *Traumatic Brain Injuries: Pathophysiology and Potential Therapeutic Targets.* Frontiers in cellular neuroscience, 2019. **13**: p. 528-528.

12. Smith, D.H., D.F. Meaney, and W.H. Shull, *Diffuse axonal injury in head trauma.* J Head Trauma Rehabil, 2003. **18**(4): p. 307-16.

13. Skandsen, T., et al., *Prevalence and impact of diffuse axonal injury in patients with moderate and severe head injury: a cohort study of early magnetic resonance imaging findings and 1-year outcome.* 2010. **113**(3): p. 556.

14. Pekna, M. and M. Pekny, *The neurobiology of brain injury.* Cerebrum : the Dana forum on brain science, 2012. **2012**: p. 9-9.

15. Greve, M.W. and B.J. Zink, *Pathophysiology of traumatic brain injury.* 2009. **76**(2): p. 97-104.

16. Carmichael, S.J.N., *The 3 Rs of Stroke Biology: Radial, Relayed, and Regenerative.* 2015. **13**: p. 348-359.

17. Zhang, R., et al., *Tenacissoside H promotes neurological recovery of cerebral ischaemia/reperfusion injury in mice by modulating inflammation and oxidative stress via TrkB pathway.* Clinical and Experimental Pharmacology and Physiology, 2021. **48**(5): p. 757-769.

18. Yao, Y., et al., *Chrysin Protects against Focal Cerebral Ischemia/Reperfusion Injury in Mice through Attenuation of Oxidative Stress and Inflammation.* International Journal of Molecular Sciences, 2014. **15**(11).

19. Zhuang, S., et al., *Germacrone alleviates neurological deficits following traumatic brain injury by modulating neuroinflammation and oxidative stress.* BMC Complementary Medicine and Therapies, 2021. **21**(1): p. 6.

20. Peng, Z., et al., *Gastrodin Alleviates Cerebral Ischemic Damage in Mice by Improving Anti-oxidant and Anti-inflammation Activities and Inhibiting Apoptosis Pathway.* Neurochemical Research, 2015. **40**(4): p. 661-673.

21. Raghupathi, R., et al., *Mild traumatic brain injury induces apoptotic cell death in the cortex that is preceded by decreases in cellular Bcl-2 immunoreactivity.* Neuroscience, 2002. **110**(4): p. 605-16.

22. Yu, G., et al., *Inhibition of Myeloperoxidase by N-Acetyl Lysyltyrosylcysteine Amide Reduces Oxidative Stress–Mediated Inflammation, Neuronal Damage, and Neural Stem Cell Injury in a Murine Model of Stroke.* Journal of Pharmacology and Experimental Therapeutics, 2018. **364**(2): p. 311.

23. Song, M. and S.P. Yu, *Ionic regulation of cell volume changes and cell death after ischemic stroke.* Translational stroke research, 2014. **5**(1): p. 17-27.

24. Puig, B., S. Brenna, and T. Magnus, *Molecular Communication of a Dying Neuron in Stroke.* International journal of molecular sciences, 2018. **19**(9): p. 2834.

25. Reynolds, I.J. and T.G. Hastings, *Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation.* The Journal of Neuroscience, 1995. **15**(5): p. 3318.

26. Gunasekar, P.G., et al., *NMDA Receptor Activation Produces Concurrent Generation of Nitric Oxide and Reactive Oxygen Species: Implications for Cell Death.* 1995. **65**(5): p. 2016-2021.

27. Weber, J., *Altered Calcium Signaling Following Traumatic Brain Injury.* 2012. **3**(60).

28. Abdul-Muneer, P.M., et al., *High Ca(2+) Influx During Traumatic Brain Injury Leads to Caspase-1-Dependent Neuroinflammation and Cell Death.* Mol Neurobiol, 2017. **54**(6): p. 3964-3975.

29. Duchen, M.R., *Mitochondria and calcium: from cell signalling to cell death.* The Journal of Physiology, 2000. **529**(1): p. 57-68.

30. Starkov, A.A., C. Chinopoulos, and G. Fiskum, *Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury.* Cell Calcium, 2004. **36**(3): p. 257-264.

31. Kahles, T., et al., *NADPH Oxidase Plays a Central Role in Blood-Brain Barrier Damage in Experimental Stroke.* Stroke, 2007. **38**(11): p. 3000-3006.

32. d'Avila, J.C., et al., *Microglial activation induced by brain trauma is suppressed by post-injury treatment with a PARP inhibitor.* Journal of Neuroinflammation, 2012. **9**(1): p. 31.

33. Li, T., et al., *Proliferation of parenchymal microglia is the main source of microgliosis after ischaemic stroke.* Brain : a journal of neurology, 2013. **136**.

34. Yang, C., et al., *Neuroinflammatory mechanisms of blood-brain barrier damage in ischemic stroke.* 2019. **316**(2): p. C135-C153.

35. Jenkins, D.J., et al., *Glycemic index of foods: a physiological basis for carbohydrate exchange.* Am J Clin Nutr, 1981. **34**(3): p. 362-6.

36. Vega-López, S., B.J. Venn, and J.L. Slavin, *Relevance of the Glycemic Index and Glycemic Load for Body Weight, Diabetes, and Cardiovascular Disease.* Nutrients, 2018. **10**(10): p. 1361.

37. Ford, E.S. and S. Liu, *Glycemic Index and Serum High-Density Lipoprotein Cholesterol Concentration Among US Adults.* Archives of Internal Medicine, 2001. **161**(4): p. 572-576.

38. Mirrahimi, A., et al., *Associations of Glycemic Index and Load With Coronary Heart Disease Events: A Systematic Review and Meta‐Analysis of Prospective Cohorts.* Journal of the American Heart Association, 2012. **1**(5): p. e000752.

39. Burger, K.N.J., et al., *Dietary Glycemic Load and Glycemic Index and Risk of Coronary Heart Disease and Stroke in Dutch Men and Women: The EPIC-MORGEN Study.* PLOS ONE, 2011. **6**(10): p. e25955.

40. Hajishafiee, M., et al., *Dietary intake of different carbohydrates among incident stroke patients during previous year.* International journal of preventive medicine, 2013. **4**(Suppl 2): p. S290-S293.

41. Sieri, S., et al., *Dietary Glycemic Load and Glycemic Index and Risk of Cerebrovascular Disease in the EPICOR Cohort.* PLOS ONE, 2013. **8**(5): p. e62625.

42. Oh, K., et al., *Carbohydrate Intake, Glycemic Index, Glycemic Load, and Dietary Fiber in Relation to Risk of Stroke in Women.* American Journal of Epidemiology, 2005. **161**(2): p. 161-169.

43. Casiglia, E., et al., *High dietary fiber intake prevents stroke at a population level.* Clinical Nutrition, 2013. **32**(5): p. 811-818.

44. Larsson, S.C. and A. Wolk, *Dietary Fiber Intake Is Inversely Associated with Stroke Incidence in Healthy Swedish Adults.* The Journal of Nutrition, 2014. **144**(12): p. 1952-1955.

45. Chen, G.C., et al., *Dietary fiber intake and stroke risk: a meta-analysis of prospective cohort studies.* European Journal of Clinical Nutrition, 2013. **67**(1): p. 96-100.

46. Zhang, Z., et al., *Dietary fiber consumption and risk of stroke.* European Journal of Epidemiology, 2013. **28**(2): p. 119-130.

47. Larsson, S.C., J. Virtamo, and A. Wolk, *Total and specific fruit and vegetable consumption and risk of stroke: A prospective study.* Atherosclerosis, 2013. **227**(1): p. 147-152.

48. Liu, S., et al., *Whole Grain Consumption and Risk of Ischemic Stroke in WomenA Prospective Study.* JAMA, 2000. **284**(12): p. 1534-1540.

49. Sun, B., et al., *Exploration of the Association between Dietary Fiber Intake and Hypertension among U.S. Adults Using 2017 American College of Cardiology/American Heart Association Blood Pressure Guidelines: NHANES 2007–2014.* 2018. **10**(8): p. 1091.

50. Zhu, X., et al., *Quantitative assessment of the effects of beta-glucan consumption on serum lipid profile and glucose level in hypercholesterolemic subjects.* Nutrition, Metabolism and Cardiovascular Diseases, 2015. **25**(8): p. 714-723.

51. Jenkins, D.J., et al., *Soluble fiber intake at a dose approved by the US Food and Drug Administration for a claim of health benefits: serum lipid risk factors for cardiovascular disease assessed in a randomized controlled crossover trial.* The American Journal of Clinical Nutrition, 2002. **75**(5): p. 834-839.

52. Mao, T., et al., *Effects of dietary fiber on glycemic control and insulin sensitivity in patients with type 2 diabetes: A systematic review and meta-analysis.* Journal of Functional Foods, 2021. **82**: p. 104500.

53. Engler, T.M.N.d.M., et al., *Stroke: bowel dysfunction in patients admitted for rehabilitation.* The open nursing journal, 2014. **8**: p. 43-47.

54. Bernstein, A.M., et al., *Dietary Protein Sources and the Risk of Stroke in Men and Women.* Stroke, 2012. **43**(3): p. 637-644.

55. Larsson, S.C., J. Virtamo, and A. Wolk, *Red Meat Consumption and Risk of Stroke in Swedish Women.* Stroke, 2011. **42**(2): p. 324-329.

56. Larsson, S.C., J. Virtamo, and A. Wolk, *Red meat consumption and risk of stroke in Swedish men.* The American Journal of Clinical Nutrition, 2011. **94**(2): p. 417-421.

57. Grillo, A., et al., *Sodium Intake and Hypertension.* Nutrients, 2019. **11**(9): p. 1970.

58. Yang, W., et al., *Is heme iron intake associated with risk of coronary heart disease? A meta-analysis of prospective studies.* Eur J Nutr, 2014. **53**(2): p. 395-400.

59. Mozaffarian, D. and H.Y. Wu Jason, *Omega-3 Fatty Acids and Cardiovascular Disease.* Journal of the American College of Cardiology, 2011. **58**(20): p. 2047-2067.

60. Sauvaget, C., et al., *Animal protein, animal fat, and cholesterol intakes and risk of cerebral infarction mortality in the adult health study.* Stroke, 2004. **35**(7): p. 1531-7.

61. Ozawa, M., et al., *Dietary Protein Intake and Stroke Risk in a General Japanese Population.* 2017. **48**(6): p. 1478-1486.

62. Bouzan, C., et al., *A quantitative analysis of fish consumption and stroke risk.* 2005. **29**(4): p. 347-352.

63. Krajcovicova-Kudlackova, M., K. Babinska, and M. Valachovicova, *Health benefits and risks of plant proteins.* Bratisl Lek Listy, 2005. **106**(6-7): p. 231-4.

64. Folsom, A.R., et al., *Prospective associations of fasting insulin, body fat distribution, and diabetes with risk of ischemic stroke. The Atherosclerosis Risk in Communities (ARIC) Study Investigators.* Diabetes Care, 1999. **22**(7): p. 1077-83.

65. Dong, J.Y., et al., *Effect of oral L-arginine supplementation on blood pressure: a meta-analysis of randomized, double-blind, placebo-controlled trials.* Am Heart J, 2011. **162**(6): p. 959-65.

66. Kris-Etherton, P.M., K. Petersen, and L. Van Horn, *Convincing evidence supports reducing saturated fat to decrease cardiovascular disease risk.* BMJ Nutrition, Prevention & Health, 2018. **1**(1): p. 23.

67. Darvishi, L., et al., *Comparison of fat intake between patients with stroke and normal population.* Journal of research in medical sciences : the official journal of Isfahan University of Medical Sciences, 2013. **18**(Suppl 1): p. S59-S61.

68. de Souza, R.J., et al., *Intake of saturated and trans unsaturated fatty acids and risk of all cause mortality, cardiovascular disease, and type 2 diabetes: systematic review and meta-analysis of observational studies.* 2015. **351**: p. h3978.

69. Siri-Tarino, P.W., et al., *Meta-analysis of prospective cohort studies evaluating the association of saturated fat with cardiovascular disease.* Am J Clin Nutr, 2010. **91**(3): p. 535-46.

70. He, K., et al., *Dietary fat intake and risk of stroke in male US healthcare professionals: 14 year prospective cohort study.* 2003. **327**(7418): p. 777-782.

71. Muto, M. and O. Ezaki, *High Dietary Saturated Fat is Associated with a Low Risk of Intracerebral Hemorrhage and Ischemic Stroke in Japanese but not in Non-Japanese: A Review and Meta-Analysis of Prospective Cohort Studies.* Journal of atherosclerosis and thrombosis, 2018. **25**(5): p. 375-392.

72. Cheng, P., et al., *Can dietary saturated fat be beneficial in prevention of stroke risk? A meta-analysis.* Neurol Sci, 2016. **37**(7): p. 1089-98.

73. Kang, Z.-Q., Y. Yang, and B. Xiao, *Dietary saturated fat intake and risk of stroke: Systematic review and dose–response meta-analysis of prospective cohort studies.* Nutrition, Metabolism and Cardiovascular Diseases, 2020. **30**(2): p. 179-189.

74. Iso, H., et al., *Fat and Protein Intakes and Risk of Intraparenchymal Hemorrhage among Middle-aged Japanese.* American Journal of Epidemiology, 2003. **157**(1): p. 32-39.

75. Gillman, M.W., et al., *Inverse Association of Dietary Fat With Development of Ischemic Stroke in Men.* JAMA, 1997. **278**(24): p. 2145-2150.

76. Iso, H., et al., *Prospective Study of Fat and Protein Intake and Risk of Intraparenchymal Hemorrhage in Women.* 2001. **103**(6): p. 856-863.

77. Jimenez-Conde, J., et al., *Hyperlipidemia and reduced white matter hyperintensity volume in patients with ischemic stroke.* Stroke, 2010. **41**(3): p. 437-42.

78. D R Jacobs, J., *Why is Low Blood Cholesterol Associated with Risk of Nonatherosclerotic Disease Death?* 1993. **14**(1): p. 95-114.

79. Zárate, R., et al., *Significance of long chain polyunsaturated fatty acids in human health.* Clinical and translational medicine, 2017. **6**(1): p. 25-25.

80. Kim, Y.-J., et al., *Plasma phospholipid fatty acid composition in ischemic stroke: Importance of docosahexaenoic acid in the risk for intracranial atherosclerotic stenosis.* Atherosclerosis, 2012. **225**(2): p. 418-424.

81. Iso, H., et al., *Intake of Fish and Omega-3 Fatty Acids and Risk of Stroke in Women.* JAMA, 2001. **285**(3): p. 304-312.

82. Skerrett, P.J. and C.H. Hennekens, *Consumption of Fish and Fish Oils and Decreased Risk of Stroke.* 2003. **6**(1): p. 38-41.

83. Park, Y., et al., *Low level of n-3 polyunsaturated fatty acids in erythrocytes is a risk factor for both acute ischemic and hemorrhagic stroke in Koreans.* Nutrition Research, 2009. **29**(12): p. 825-830.

84. Gale, C.R., et al., *Vitamin C and risk of death from stroke and coronary heart disease in cohort of elderly people.* 1995. **310**(6994): p. 1563-1566.

85. Kubota, Y., et al., *Dietary Intakes of Antioxidant Vitamins and Mortality From Cardiovascular Disease.* 2011. **42**(6): p. 1665-1672.

86. Yokoyama, T., et al., *Serum Vitamin C Concentration Was Inversely Associated With Subsequent 20-Year Incidence of Stroke in a Japanese Rural Community.* 2000. **31**(10): p. 2287-2294.

87. Gey, K.F., H.B. Stähelin, and M. Eichholzer, *Poor plasma status of carotene and vitamin C is associated with higher mortality from ischemic heart disease and stroke Basel Prospective Study.* The clinical investigator, 1993. **71**(1): p. 3-6.

88. Vokó, Z., et al., *Dietary antioxidants and the risk of ischemic stroke.* The Rotterdam Study, 2003. **61**(9): p. 1273-1275.

89. Chen, G.-C., et al., *Vitamin C intake, circulating vitamin C and risk of stroke: a meta-analysis of prospective studies.* Journal of the American Heart Association, 2013. **2**(6): p. e000329-e000329.

90. Uesugi, S., et al., *Dietary intake of antioxidant vitamins and risk of stroke: the Japan Public Health Center–based Prospective Study.* European Journal of Clinical Nutrition, 2017. **71**(10): p. 1179-1185.

91. Khanna, S., et al., *Neuroprotective properties of the natural vitamin E alpha-tocotrienol.* Stroke, 2005. **36**(10): p. 2258-64.

92. Sesso, H.D., et al., *Vitamins E and C in the Prevention of Cardiovascular Disease in Men: The Physicians' Health Study II Randomized Controlled Trial.* JAMA, 2008. **300**(18): p. 2123-2133.

93. Cook, N.R., et al., *A Randomized Factorial Trial of Vitamins C and E and Beta Carotene in the Secondary Prevention of Cardiovascular Events in Women: Results From the Women's Antioxidant Cardiovascular Study.* Archives of Internal Medicine, 2007. **167**(15): p. 1610-1618.

94. Bin, Q., et al., *The role of vitamin E (tocopherol) supplementation in the prevention of stroke. A meta-analysis of 13 randomised controlled trials.* Thromb Haemost, 2011. **105**(4): p. 579-85.

95. Yochum, L.A., A.R. Folsom, and L.H. Kushi, *Intake of antioxidant vitamins and risk of death from stroke in postmenopausal women.* The American Journal of Clinical Nutrition, 2000. **72**(2): p. 476-483.

96. Leppälä, J.M., et al., *Controlled trial of alpha-tocopherol and beta-carotene supplements on stroke incidence and mortality in male smokers.* Arterioscler Thromb Vasc Biol, 2000. **20**(1): p. 230-5.

97. Allahtavakoli, M., et al., *Ascorbic Acid Reduces the Adverse Effects of Delayed Administration of Tissue Plasminogen Activator in a Rat Stroke Model.* Basic Clin Pharmacol Toxicol, 2015. **117**(5): p. 335-9.

98. Del Rio, D., et al., *Total Antioxidant Capacity of the Diet Is Associated with Lower Risk of Ischemic Stroke in a Large Italian Cohort.* The Journal of Nutrition, 2010. **141**(1): p. 118-123.

99. Sies, H., W. Stahl, and A.R. Sundquist, *Antioxidant functions of vitamins: Vitamins E and C, Beta-Carotene, and other carotenoids a.* Annals of the New York Academy of Sciences, 1992. **669**(1): p. 7-20.

100. Hak, A.E., et al., *Prospective Study of Plasma Carotenoids and Tocopherols in Relation to Risk of Ischemic Stroke.* 2004. **35**(7): p. 1584-1588.

101. *MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20 536 high-risk individuals: a randomised placebo-controlled trial.* The Lancet, 2002. **360**(9326): p. 23-33.

102. Kelly, P.J. and K.L. Furie, *Management and prevention of stroke associated with elevated homocysteine.* Current Treatment Options in Cardiovascular Medicine, 2002. **4**(5): p. 363-371.

103. Eikelboom, J.W., et al., *Association between high homocyst(e)ine and ischemic stroke due to large- and small-artery disease but not other etiologic subtypes of ischemic stroke.* Stroke, 2000. **31**(5): p. 1069-75.

104. *Dose-dependent effects of folic acid on blood concentrations of homocysteine: a meta-analysis of the randomized trials.* Am J Clin Nutr, 2005. **82**(4): p. 806-12.

105. Flicker, L., et al., *Efficacy of B vitamins in lowering homocysteine in older men: maximal effects for those with B12 deficiency and hyperhomocysteinemia.* Stroke, 2006. **37**(2): p. 547-9.

106. Saposnik, G., *The Role of Vitamin B in Stroke Prevention.* Stroke, 2011. **42**(3): p. 838-842.

107. Ji, Y., et al., *Vitamin B supplementation, homocysteine levels, and the risk of cerebrovascular disease: a meta-analysis.* Neurology, 2013. **81**(15): p. 1298-307.

108. Tamez, H. and R.I. Thadhani, *Vitamin D and hypertension: an update and review.* Current Opinion in Nephrology and Hypertension, 2012. **21**(5).

109. Carrelli, A.L., et al., *Vitamin D deficiency is associated with subclinical carotid atherosclerosis: the Northern Manhattan study.* Stroke, 2011. **42**(8): p. 2240-5.

110. Marniemi, J., et al., *Dietary and serum vitamins and minerals as predictors of myocardial infarction and stroke in elderly subjects.* Nutr Metab Cardiovasc Dis, 2005. **15**(3): p. 188-97.

111. Anderson, J.L., et al., *Relation of vitamin D deficiency to cardiovascular risk factors, disease status, and incident events in a general healthcare population.* Am J Cardiol, 2010. **106**(7): p. 963-8.

112. Leung, R.Y., et al., *Serum 25-hydroxyvitamin D and the risk of stroke in Hong Kong Chinese.* Thromb Haemost, 2017. **117**(1): p. 158-163.

113. Judd, S.E., et al., *Vitamin D deficiency and incident stroke risk in community-living black and white adults.* International journal of stroke : official journal of the International Stroke Society, 2016. **11**(1): p. 93-102.

114. Berghout, B.P., et al., *Vitamin D Status and Risk of Stroke.* Stroke, 2019. **50**(9): p. 2293-2298.

115. Skaaby, T., et al., *Vitamin D status and incident cardiovascular disease and all-cause mortality: a general population study.* Endocrine, 2013. **43**(3): p. 618-25.

116. Brøndum-Jacobsen, P., et al., *25-Hydroxyvitamin D and symptomatic ischemic stroke: An Original Study and Meta-Analysis.* Annals of Neurology, 2013. **73**(1): p. 38-47.

117. Ma, J., et al., *Associations of serum and dietary magnesium with cardiovascular disease, hypertension, diabetes, insulin, and carotid arterial wall thickness: The aric study.* Journal of Clinical Epidemiology, 1995. **48**(7): p. 927-940.

118. Bain, L.K.M., et al., *The relationship between dietary magnesium intake, stroke and its major risk factors, blood pressure and cholesterol, in the EPIC-Norfolk cohort.* International Journal of Cardiology, 2015. **196**: p. 108-114.

119. Rodríguez-Morán, M. and F. Guerrero-Romero, *Oral Magnesium Supplementation Improves Insulin Sensitivity and Metabolic Control in Type 2 Diabetic Subjects.* Diabetes Care, 2003. **26**(4): p. 1147.

120. Lopez-Ridaura, R., et al., *Magnesium Intake and Risk of Type 2 Diabetes in Men and Women.* Diabetes Care, 2004. **27**(1): p. 134.

121. Houston, M., *The Role of Magnesium in Hypertension and Cardiovascular Disease.* The Journal of Clinical Hypertension, 2011. **13**(11): p. 843-847.

122. Larsson, S.C., et al., *Magnesium, calcium, potassium, and sodium intakes and risk of stroke in male smokers.* Arch Intern Med, 2008. **168**(5): p. 459-65.

123. Liang, W., A.H. Lee, and C.W. Binns, *Dietary intake of minerals and the risk of ischemic stroke in Guangdong Province, China, 2007-2008.* Preventing chronic disease, 2011. **8**(2): p. A38-A38.

124. Larsson, S.C., N. Orsini, and A. Wolk, *Dietary magnesium intake and risk of stroke: a meta-analysis of prospective studies.* The American Journal of Clinical Nutrition, 2011. **95**(2): p. 362-366.

125. Adebamowo, S.N., et al., *Association between intakes of magnesium, potassium, and calcium and risk of stroke: 2 cohorts of US women and updated meta-analyses.* The American Journal of Clinical Nutrition, 2015. **101**(6): p. 1269-1277.

126. Zhao, B., et al., *The Effect of Magnesium Intake on Stroke Incidence: A Systematic Review and Meta-Analysis With Trial Sequential Analysis.* Frontiers in Neurology, 2019. **10**(852).

127. Ohira, T., et al., *Serum and dietary magnesium and risk of ischemic stroke: the Atherosclerosis Risk in Communities Study.* American journal of epidemiology, 2009. **169**(12): p. 1437-1444.

128. Iso, H., et al., *Prospective Study of Calcium, Potassium, and Magnesium Intake and Risk of Stroke in Women.* Stroke, 1999. **30**(9): p. 1772-1779.

129. Khaw, K.-T. and E. Barrett-Connor, *Dietary Potassium and Stroke-Associated Mortality.* New England Journal of Medicine, 1987. **316**(5): p. 235-240.

130. Bazzano, L.A., et al., *Dietary Potassium Intake and Risk of Stroke in US Men and Women.* Stroke, 2001. **32**(7): p. 1473-1480.

131. Seth, A., et al., *Potassium intake and risk of stroke in women with hypertension and nonhypertension in the Women's Health Initiative.* Stroke, 2014. **45**(10): p. 2874-2880.

132. Blanch, N., et al., *Effect of sodium and potassium supplementation on vascular and endothelial function: a randomized controlled trial.* The American Journal of Clinical Nutrition, 2015. **101**(5): p. 939-946.

133. Appel, L.J., et al., *The Importance of Population-Wide Sodium Reduction as a Means to Prevent Cardiovascular Disease and Stroke.* Circulation, 2011. **123**(10): p. 1138-1143.

134. Ghasemi, S., et al., *Dietary intake of minerals in the patients with stroke.* Journal of research in medical sciences : the official journal of Isfahan University of Medical Sciences, 2013. **18**(Suppl 1): p. S55-S58.

135. Gardener, H., et al., *Dietary Sodium and Risk of Stroke in the Northern Manhattan Study.* Stroke, 2012. **43**(5): p. 1200-1205.

136. Umesawa, M., et al., *Relations between dietary sodium and potassium intakes and mortality from cardiovascular disease: the Japan Collaborative Cohort Study for Evaluation of Cancer Risks.* The American Journal of Clinical Nutrition, 2008. **88**(1): p. 195-202.

137. Nagata, C., et al., *Sodium intake and risk of death from stroke in Japanese men and women.* Stroke, 2004. **35**(7): p. 1543-7.

138. Jayedi, A., et al., *Dietary sodium, sodium-to-potassium ratio, and risk of stroke: A systematic review and nonlinear dose-response meta-analysis.* Clinical Nutrition, 2019. **38**(3): p. 1092-1100.

139. Olechnowicz, J., et al., *Zinc status is associated with inflammation, oxidative stress, lipid, and glucose metabolism.* The Journal of Physiological Sciences, 2018. **68**(1): p. 19-31.

140. Huang, M., et al., *Serum/Plasma Zinc Is Apparently Increased in Ischemic Stroke: a Meta-analysis.* Biological Trace Element Research, 2021.

141. Zhang, J., et al., *Baseline Plasma Zinc and Risk of First Stroke in Hypertensive Patients.* 2019. **50**(11): p. 3255-3258.

142. Qi, Z. and K.J. Liu, *The interaction of zinc and the blood-brain barrier under physiological and ischemic conditions.* Toxicology and Applied Pharmacology, 2019. **364**: p. 114-119.

143. Ekblom, K., et al., *Iron Stores and HFE Genotypes Are Not Related to Increased Risk of Ischemic Stroke.* Cerebrovascular Diseases, 2007. **24**(5): p. 405-411.

144. Yamada, S., et al., *Association Between Serum Phosphate Levels and Stroke Risk in Patients Undergoing Hemodialysis.* 2016. **47**(9): p. 2189-2196.

145. Talebi, A., et al., *Cerebrovascular disease: how serum phosphorus, vitamin D, and uric acid levels contribute to the ischemic stroke.* BMC Neurology, 2020. **20**(1): p. 116.

146. Zhu, H.-L., et al., *Dietary calcium, magnesium, and phosphorus intakes and risk of stroke in Chinese adults.* Scientific Reports, 2021. **11**(1): p. 11270.

147. Umesawa, M., et al., *Dietary Intake of Calcium in Relation to Mortality From Cardiovascular Disease.* Stroke, 2006. **37**(1): p. 20-26.

148. Hsia, J., et al., *Calcium/Vitamin D Supplementation and Cardiovascular Events.* Circulation, 2007. **115**(7): p. 846-854.

149. DeVience, S.J., et al., *Metabolic imaging of energy metabolism in traumatic brain injury using hyperpolarized [1-13C]pyruvate.* Scientific Reports, 2017. **7**(1): p. 1907.

150. Vagnozzi, R., et al., *Changes of Cerebral Energy Metabolism and Lipid Peroxidation in Rats Leading to Mitochondrial Dysfunction After Diffuse Brain Injury.* Journal of Neurotrauma, 1999. **16**(10): p. 903-913.

151. Chen, T., et al., *Brain lactate uptake increases at the site of impact after traumatic brain injury.* Brain Res, 2000. **861**(2): p. 281-7.

152. Jalloh, I., et al., *Lactate uptake by the injured human brain: evidence from an arteriovenous gradient and cerebral microdialysis study.* J Neurotrauma, 2013. **30**(24): p. 2031-7.

153. Patet, C., et al., *Neuroenergetic Response to Prolonged Cerebral Glucose Depletion after Severe Brain Injury and the Role of Lactate.* Journal of Neurotrauma, 2015. **32**(20): p. 1560-1566.

154. Rice, A.C., et al., *Lactate administration attenuates cognitive deficits following traumatic brain injury.* Brain Research, 2002. **928**(1): p. 156-159.

155. Buscemi, L., et al., *Extended preclinical investigation of lactate for neuroprotection after ischemic stroke.* Clinical and Translational Neuroscience, 2020. **4**(1): p. 2514183X20904571.

156. Berthet, C., et al., *Neuroprotective Role of Lactate after Cerebral Ischemia.* Journal of Cerebral Blood Flow & Metabolism, 2009. **29**(11): p. 1780-1789.

157. Berthet, C., et al., *New evidence of neuroprotection by lactate after transient focal cerebral ischaemia: extended benefit after intracerebroventricular injection and efficacy of intravenous administration.* Cerebrovasc Dis, 2012. **34**(5-6): p. 329-35.

158. Zhou, J., et al., *Lactate potentiates angiogenesis and neurogenesis in experimental intracerebral hemorrhage.* Experimental & Molecular Medicine, 2018. **50**(7): p. 1-12.

159. Ichai, C., et al., *Sodium lactate versus mannitol in the treatment of intracranial hypertensive episodes in severe traumatic brain-injured patients.* Intensive Care Med, 2009. **35**(3): p. 471-9.

160. Bouzat, P., et al., *Cerebral metabolic effects of exogenous lactate supplementation on the injured human brain.* Intensive Care Medicine, 2014. **40**(3): p. 412-421.

161. Carteron, L., et al., *Hypertonic Lactate to Improve Cerebral Perfusion and Glucose Availability After Acute Brain Injury*.* Critical Care Medicine, 2018. **46**(10): p. 1649-1655.

162. Wolahan, S.M., et al., *Lactate supplementation in severe traumatic brain injured adults by primed constant infusion of sodium L-lactate.* Journal of Neuroscience Research, 2018. **96**(4): p. 688-695.

163. Aliasghari, F., et al., *Impact of Premorbid Malnutrition and Dysphagia on Ischemic Stroke Outcome in Elderly Patients: A Community-Based Study.* J Am Coll Nutr, 2019. **38**(4): p. 318-326.

164. Dávalos, A., et al., *Effect of Malnutrition After Acute Stroke on Clinical Outcome.* Stroke, 1996. **27**(6): p. 1028-1032.

165. Aquilani, R., et al., *Protein supplementation may enhance the spontaneous recovery of neurological alterations in patients with ischaemic stroke.* Clin Rehabil, 2008. **22**(12): p. 1042-50.

166. Hashemilar, M., et al., *Effect of Whey Protein Supplementation on Inflammatory and Antioxidant Markers, and Clinical Prognosis in Acute Ischemic Stroke (TNS Trial): A Randomized, Double Blind, Controlled, Clinical Trial.* Advanced pharmaceutical bulletin, 2020. **10**(1): p. 135-140.

167. Aquilani, R., et al., *Effect of calorie-protein supplementation on the cognitive recovery of patients with subacute stroke.* Nutritional Neuroscience, 2008. **11**(5): p. 235-240.

168. de Aguilar-Nascimento, J.E., B.R. Prado Silveira, and D.B. Dock-Nascimento, *Early enteral nutrition with whey protein or casein in elderly patients with acute ischemic stroke: A double-blind randomized trial.* Nutrition, 2011. **27**(4): p. 440-444.

169. Scherbakov, N., et al., *Stroke induced Sarcopenia: muscle wasting and disability after stroke.* Int J Cardiol, 2013. **170**(2): p. 89-94.

170. Ha, L., et al., *Individual, nutritional support prevents undernutrition, increases muscle strength and improves QoL among elderly at nutritional risk hospitalized for acute stroke: A randomized, controlled trial.* Clinical Nutrition, 2010. **29**(5): p. 567-573.

171. Ha, L., T. Hauge, and P.O. Iversen, *Body composition in older acute stroke patients after treatment with individualized, nutritional supplementation while in hospital.* BMC geriatrics, 2010. **10**: p. 75-75.

172. Badjatia, N., et al., *Neuromuscular Electrical Stimulation and High-Protein Supplementation After Subarachnoid Hemorrhage: A Single-Center Phase 2 Randomized Clinical Trial.* Neurocritical Care, 2021. **35**(1): p. 46-55.

173. Cheng, Y.-H., et al., *Effects of protein supplementation on aerobic training-induced gains in cardiopulmonary fitness, muscle mass, and functional performance in chronic stroke: A randomized controlled pilot study.* Clinical Nutrition, 2020. **39**(9): p. 2743-2750.

174. Mohebi-Nejad, A. and B. Bikdeli, *Omega-3 supplements and cardiovascular diseases.* Tanaffos, 2014. **13**(1): p. 6-14.

175. Singh, M., *Essential fatty acids, DHA and human brain.* Indian J Pediatr, 2005. **72**(3): p. 239-42.

176. Lalancette-Hébert, M., et al., *Accumulation of Dietary Docosahexaenoic Acid in the Brain Attenuates Acute Immune Response and Development of Postischemic Neuronal Damage.* Stroke, 2011. **42**(10): p. 2903-2909.

177. Cai, W., et al., *Post-stroke DHA Treatment Protects Against Acute Ischemic Brain Injury by Skewing Macrophage Polarity Toward the M2 Phenotype.* Translational Stroke Research, 2018. **9**(6): p. 669-680.

178. Belayev, L., et al., *Docosahexaenoic Acid therapy of experimental ischemic stroke.* Translational stroke research, 2011. **2**(1): p. 33-41.

179. Eady, T.N., et al., *Docosahexaenoic Acid Signaling Modulates Cell Survival in Experimental Ischemic Stroke Penumbra and Initiates Long-Term Repair in Young and Aged Rats.* PLOS ONE, 2012. **7**(10): p. e46151.

180. Zhu, W., et al., *Docosahexaenoic Acid (DHA) Provides Neuroprotection in Traumatic Brain Injury Models via Activating Nrf2-ARE Signaling.* Inflammation, 2018. **41**(4): p. 1182-1193.

181. Zhu, W., et al., *Effect of docosahexaenoic acid on traumatic brain injury in rats.* Experimental and therapeutic medicine, 2017. **14**(5): p. 4411-4416.

182. Desai, A., K. Kevala, and H.-Y. Kim, *Depletion of Brain Docosahexaenoic Acid Impairs Recovery from Traumatic Brain Injury.* PLOS ONE, 2014. **9**(1): p. e86472.

183. Russell, K.L., N.E.J. Berman, and B. Levant, *Low brain DHA content worsens sensorimotor outcomes after TBI and decreases TBI-induced Timp1 expression in juvenile rats.* Prostaglandins, Leukotrienes and Essential Fatty Acids, 2013. **89**(2): p. 97-105.

184. Song, T.-J., et al., *Low Plasma Proportion of Omega 3-Polyunsaturated Fatty Acids Predicts Poor Outcome in Acute Non-Cardiogenic Ischemic Stroke Patients.* Journal of stroke, 2015. **17**(2): p. 168-176.

185. Suda, S., et al., *Low serum n-3 polyunsaturated fatty acid/n-6 polyunsaturated fatty acid ratio predicts neurological deterioration in Japanese patients with acute ischemic stroke.* Cerebrovasc Dis, 2013. **36**(5-6): p. 388-93.

186. Jackson, P.A., et al., *DHA-rich oil modulates the cerebral haemodynamic response to cognitive tasks in healthy young adults: a near IR spectroscopy pilot study.* British Journal of Nutrition, 2011. **107**(8): p. 1093-1098.

187. Phang, M., L.F. Lincz, and M.L. Garg, *Eicosapentaenoic and docosahexaenoic acid supplementations reduce platelet aggregation and hemostatic markers differentially in men and women.* J Nutr, 2013. **143**(4): p. 457-63.

188. Larson, M.K., et al., *Exogenous modification of platelet membranes with the omega-3 fatty acids EPA and DHA reduces platelet procoagulant activity and thrombus formation.* American journal of physiology. Cell physiology, 2013. **304**(3): p. C273-C279.

189. Terano, T., et al., *Effect of oral administration of highly purified eicosapentaenoic acid on platelet function, blood viscosity and red cell deformability in healthy human subjects.* Atherosclerosis, 1983. **46**(3): p. 321-31.

190. Lee, J.B., et al., *Docosahexaenoic acid reduces resting blood pressure but increases muscle sympathetic outflow compared with eicosapentaenoic acid in healthy men and women.* American Journal of Physiology-Heart and Circulatory Physiology, 2019. **316**(4): p. H873-H881.

191. Ishaq, G.M., et al., *Effects of α-tocopherol and ascorbic acid in the severity and management of traumatic brain injury in albino rats.* J Neurosci Rural Pract, 2013. **04**(03): p. 292-297.

192. Aiguo, W., Y. Zhe, and F. Gomez-Pinilla, *Vitamin E Protects Against Oxidative Damage and Learning Disability After Mild Traumatic Brain Injury in Rats.* Neurorehabilitation and Neural Repair, 2009. **24**(3): p. 290-298.

193. de Sales, K.P.F., et al., *Effects of Vitamin C on the Prevention of Ischemia-Reperfusion Brain Injury: Experimental Study in Rats.* International journal of vascular medicine, 2019. **2019**: p. 4090549-4090549.

194. Conte, V., et al., *Vitamin E reduces amyloidosis and improves cognitive function in Tg2576 mice following repetitive concussive brain injury.* Journal of Neurochemistry, 2004. **90**(3): p. 758-764.

195. Stamford, J.A., et al., *Ascorbic acid is neuroprotective against global ischaemia in striatum but not hippocampus: histological and voltammetric data.* Brain Research, 1999. **835**(2): p. 229-240.

196. Polidori, M.C., P. Mecocci, and B. Frei, *Plasma Vitamin C Levels Are Decreased and Correlated With Brain Damage in Patients With Intracranial Hemorrhage or Head Trauma.* 2001. **32**(4): p. 898-902.

197. Cherubini, A., et al., *Antioxidant Profile and Early Outcome in Stroke Patients.* 2000. **31**(10): p. 2295-2300.

198. cLagowska-Lenard, M., Z. Stelmasiak, and H. Bartosik-Psujek, *Influence of vitamin C on markers of oxidative stress in the earliest period of ischemic stroke.* Pharmacological Reports, 2010. **62**(4): p. 751-756.

199. Rabadi, M.H. and B.S. Kristal, *Effect of vitamin C supplementation on stroke recovery: a case-control study.* Clinical interventions in aging, 2007. **2**(1): p. 147-151.

200. Razmkon, A., et al., *Administration of Vitamin C and Vitamin E in Severe Head Injury: A Randomized Double-blind Controlled Trial.* Neurosurgery, 2011. **58**(CN_suppl_1): p. 133-137.

201. Ullegaddi, R., H.J. Powers, and S.E. Gariballa, *Antioxidant Supplementation With or Without B-Group Vitamins After Acute Ischemic Stroke: A Randomized Controlled Trial.* 2006. **30**(2): p. 108-114.

202. Lee, C.-H., et al., *Dietary profile of folate intake in long-term post-stroke patients.* Nutrition Research, 2005. **25**(5): p. 465-475.

203. Shi, Z., et al., *Elevated Total Homocysteine Levels in Acute Ischemic Stroke Are Associated With Long-Term Mortality.* Stroke, 2015. **46**(9): p. 2419-2425.

204. Lai, W.K. and M.Y. Kan, *Homocysteine-Induced Endothelial Dysfunction.* Ann Nutr Metab, 2015. **67**(1): p. 1-12.

205. Tsybikov, N.N., et al., *Endothelial dysfunction in experimental hyperhomocysteinemia.* Patologicheskaia fiziologiia i eksperimental'naia terapiia, 2016. **60**(3): p. 42-46.

206. Tyagi, N., et al., *Mechanisms of homocysteine-induced oxidative stress.* American Journal of Physiology-Heart and Circulatory Physiology, 2005. **289**(6): p. H2649-H2656.

207. Ullegaddi, R., H.J. Powers, and S.E. Gariballa, *Antioxidant supplementation enhances antioxidant capacity and mitigates oxidative damage following acute ischaemic stroke.* European Journal of Clinical Nutrition, 2005. **59**(12): p. 1367-1373.

208. Almeida, O.P., et al., *B-vitamins reduce the long-term risk of depression after stroke: The VITATOPS-DEP trial.* Ann Neurol, 2010. **68**(4): p. 503-10.

209. Alfieri, D.F., et al., *Vitamin D deficiency is associated with acute ischemic stroke, C-reactive protein, and short-term outcome.* 2017. **32**(2): p. 493-502.

210. Turetsky, A., R.P. Goddeau, and N. Henninger, *Low Serum Vitamin D Is Independently Associated with Larger Lesion Volumes after Ischemic Stroke.* Journal of Stroke and Cerebrovascular Diseases, 2015. **24**(7): p. 1555-1563.

211. Nie, Z., et al., *Serum levels of 25-hydroxyvitamin D predicts infarct volume and mortality in ischemic stroke patients.* Journal of Neuroimmunology, 2017. **313**: p. 41-45.

212. Poole, K.E.S., et al., *Reduced Vitamin D in Acute Stroke.* 2006. **37**(1): p. 243-245.

213. Jamall, O.A., et al., *Prevalence and correlates of vitamin D deficiency in adults after traumatic brain injury.* Clinical Endocrinology, 2016. **85**(4): p. 636-644.

214. Narasimhan, S. and P. Balasubramanian, *Role of Vitamin D in the Outcome of Ischemic Stroke- A Randomized Controlled Trial.* Journal of clinical and diagnostic research : JCDR, 2017. **11**(2): p. CC06-CC10.

215. Sharma, S., et al., *Neuroprotective Role of Oral Vitamin D Supplementation on Consciousness and Inflammatory Biomarkers in Determining Severity Outcome in Acute Traumatic Brain Injury Patients: A Double-Blind Randomized Clinical Trial.* Clinical drug investigation, 2020. **40**(4): p. 327-334.

216. *Pre-Injury Magnesium Treatment Prevents Traumatic Brain Injury–Induced Hippocampal ERK Activation, Neuronal Loss, and Cognitive Dysfunction in the Radial-Arm Maze Test.* 2005. **22**(7): p. 783-792.

217. Hoane, M.R. and T.M. Barth, *The behavioral and anatomical effects of MgCl2 therapy in an electrolytic lesion model of cortical injury in the rat.* Magnesium research, 2001. **14**(1-2): p. 51-63.

218. Hoane, M.R., *Treatment with magnesium improves reference memory but not working memory while reducing GFAP expression following traumatic brain injury.* Restorative Neurology and Neuroscience, 2005. **23**: p. 67-77.

219. Vink, R., et al., *Magnesium attenuates persistent functional deficits following diffuse traumatic brain injury in rats.* Neuroscience Letters, 2003. **336**(1): p. 41-44.

220. Izumi, Y., et al., *Reduction of Infarct Volume by Magnesium after Middle Cerebral Artery Occlusion in Rats.* Journal of Cerebral Blood Flow & Metabolism, 1991. **11**(6): p. 1025-1030.

221. Murata, T., et al., *Mechanisms of magnesium-induced vasodilation in cerebral penetrating arterioles.* Neuroscience research, 2016. **107**: p. 57-62.

222. Clerc, P., et al., *Magnesium sulfate protects against the bioenergetic consequences of chronic glutamate receptor stimulation.* PloS one, 2013. **8**(11): p. e79982-e79982.

223. Saver, J.L., et al., *Prehospital Use of Magnesium Sulfate as Neuroprotection in Acute Stroke.* New England Journal of Medicine, 2015. **372**(6): p. 528-536.

224. Temkin, N.R., et al., *Magnesium sulfate for neuroprotection after traumatic brain injury: a randomised controlled trial.* The Lancet Neurology, 2007. **6**(1): p. 29-38.

225. Pan, W.-H., et al., *Intake of potassium- and magnesium-enriched salt improves functional outcome after stroke: a randomized, multicenter, double-blind controlled trial.* The American Journal of Clinical Nutrition, 2017. **106**(5): p. 1267-1273.

226. Jarosz, M., et al., *Antioxidant and anti-inflammatory effects of zinc. Zinc-dependent NF-κB signaling.* Inflammopharmacology, 2017. **25**(1): p. 11-24.

227. Kitamura, Y., et al., *Protective effect of zinc against ischemic neuronal injury in a middle cerebral artery occlusion model.* J Pharmacol Sci, 2006. **100**(2): p. 142-8.

228. Cope, E.C., et al., *Use of Zinc as a Treatment for Traumatic Brain Injury in the Rat:Effects on Cognitive and Behavioral Outcomes.* Neurorehabilitation and Neural Repair, 2012. **26**(7): p. 907-913.

229. Kim, Y.-H., J.-W. Eom, and J.-Y. Koh, *Mechanism of Zinc Excitotoxicity: A Focus on AMPK.* Frontiers in Neuroscience, 2020. **14**(958).

230. Lobner, D., et al., *Zinc-induced neuronal death in cortical neurons.* Cell Mol Biol (Noisy-le-grand), 2000. **46**(4): p. 797-806.

231. Hellmich, H.L., et al., *Chelation of neurotoxic zinc levels does not improve neurobehavioral outcome after traumatic brain injury.* Neuroscience letters, 2008. **440**(2): p. 155-159.

232. Doering, P., et al., *Chemical blocking of zinc ions in CNS increases neuronal damage following traumatic brain injury (TBI) in mice.* PloS one, 2010. **5**(4): p. e10131-e10131.

233. Munshi, A., et al., *Depletion of serum zinc in ischemic stroke patients.* Methods and findings in experimental and clinical pharmacology, 2010. **32**: p. 433-6.

234. Bhatt, A., et al., *Clinical Significance of Serum Zinc Levels in Cerebral Ischemia.* Stroke Research and Treatment, 2010. **2010**: p. 245715.

235. Levenson, C.W., *Zinc and Traumatic Brain Injury: From Chelation to Supplementation.* Medical sciences (Basel, Switzerland), 2020. **8**(3): p. 36.

236. Aquilani, R., et al., *Normalization of zinc intake enhances neurological retrieval of patients suffering from ischemic strokes.* Nutr Neurosci, 2009. **12**(5): p. 219-25.

237. Young, B., et al., *Zinc Supplementation Is Associated with Improved Neurologic Recovery Rate and Visceral Protein Levels of Patients with Severe Closed Head Injury.* Journal of Neurotrauma, 1996. **13**(1): p. 25-34.

238. Moemeni, H., et al., *Evaluating the Serum Levels of Calcium, Chloride, Potassium and Sodium in the Stroke Patients.* International Journal of Medical Laboratory, 2016. **3**(2): p. 104-110.

239. Liamis, G., et al., *Hyponatremia in Acute Stroke Patients: Pathophysiology, Clinical Significance, and Management Options.* European Neurology, 2019. **82**(1-3): p. 32-40.

240. Chang, C.H., et al., *Recurrent hyponatremia after traumatic brain injury.* Am J Med Sci, 2008. **335**(5): p. 390-3.

241. Agha, A., M. Sherlock, and C.J. Thompson, *Post-traumatic hyponatraemia due to acute hypopituitarism.* QJM: An International Journal of Medicine, 2005. **98**(6): p. 463-464.

242. Buck, B.H., et al., *Association of Higher Serum Calcium Levels With Smaller Infarct Volumes in Acute Ischemic Stroke.* Archives of Neurology, 2007. **64**(9): p. 1287-1291.

243. Liu, J., et al., *A cohort study of relationship between serum calcium levels and cerebral microbleeds (CMBs) in ischemic stroke patients with AF and/or RHD.* Medicine, 2016. **95**(26).

244. Gupta, A., et al., *Effect of Vitamin D and calcium supplementation on ischaemic stroke outcome: a randomised controlled open-label trial.* Int J Clin Pract, 2016. **70**(9): p. 764-70.

245. Arya, A.K. and B. Hu, *Brain-gut axis after stroke.* Brain Circ, 2018. **4**(4): p. 165-173.

246. Rice, M.W., J.D. Pandya, and D.A. Shear, *Gut Microbiota as a Therapeutic Target to Ameliorate the Biochemical, Neuroanatomical, and Behavioral Effects of Traumatic Brain Injuries.* Frontiers in Neurology, 2019. **10**(875).

247. Ma, E.L., et al., *Bidirectional brain-gut interactions and chronic pathological changes after traumatic brain injury in mice.* Brain, behavior, and immunity, 2017. **66**: p. 56-69.

248. Angoa-Pérez, M., et al., *Repetitive, mild traumatic brain injury results in a progressive white matter pathology, cognitive deterioration, and a transient gut microbiota dysbiosis.* Scientific Reports, 2020. **10**(1): p. 8949.

249. Nicholson, S.E., et al., *Moderate Traumatic Brain Injury Alters the Gastrointestinal Microbiome in a Time-Dependent Manner.* Shock, 2019. **52**(2).

250. Celorrio, M., et al., *Gut microbial dysbiosis after traumatic brain injury modulates the immune response and impairs neurogenesis.* Acta Neuropathologica Communications, 2021. **9**(1): p. 40.

251. Bansal, V., et al., *Traumatic brain injury and intestinal dysfunction: uncovering the neuro-enteric axis.* Journal of neurotrauma, 2009. **26**(8): p. 1353-1359.

252. Carabotti, M., et al., *The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems.* Annals of gastroenterology, 2015. **28**(2): p. 203-209.

253. Liu, P., et al., *The role of short-chain fatty acids in intestinal barrier function, inflammation, oxidative stress, and colonic carcinogenesis.* Pharmacological Research, 2021. **165**: p. 105420.

254. Chen, R., et al., *Transplantation of fecal microbiota rich in short chain fatty acids and butyric acid treat cerebral ischemic stroke by regulating gut microbiota.* Pharmacological Research, 2019. **148**: p. 104403.

255. Hamer, H.M., et al., *Review article: the role of butyrate on colonic function.* Alimentary Pharmacology & Therapeutics, 2008. **27**(2): p. 104-119.

256. Huidi, W., et al., *Modulation of the Gut Microbiota of type 2 Diabetic Mice by Sodium Butyrate Attenuates Ischemic Stroke Injury.* Research Square, 2021.

257. Sun, J., et al., *Clostridium butyricum attenuates cerebral ischemia/reperfusion injury in diabetic mice via modulation of gut microbiota.* Brain Research, 2016. **1642**: p. 180-188.

258. Li, H., et al., *Clostridium butyricum exerts a neuroprotective effect in a mouse model of traumatic brain injury via the gut-brain axis.* Neurogastroenterology & Motility, 2018. **30**(5): p. e13260.

259. Liu, J., et al., *Probiotics Exert Protective Effect against Sepsis-Induced Cognitive Impairment by Reversing Gut Microbiota Abnormalities.* J Agric Food Chem, 2020. **68**(50): p. 14874-14883.

260. Tolhurst, G., et al., *Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2.* Diabetes, 2012. **61**(2): p. 364-71.

261. Ma, Y., et al., *Lactobacillus acidophilus Exerts Neuroprotective Effects in Mice with Traumatic Brain Injury.* J Nutr, 2019. **149**(9): p. 1543-1552.

262. Xin, J., et al., *Probiotic alleviate fluoride-induced memory impairment by reconstructing gut microbiota in mice.* Ecotoxicology and Environmental Safety, 2021. **215**: p. 112108.

263. Davani-Davari, D., et al., *Prebiotics: Definition, Types, Sources, Mechanisms, and Clinical Applications.* Foods (Basel, Switzerland), 2019. **8**(3): p. 92.

264. Bothe, M.K., et al., *Dose-Dependent Prebiotic Effect of Lactulose in a Computer-Controlled In Vitro Model of the Human Large Intestine.* Nutrients, 2017. **9**(7): p. 767.

265. Yuan, Q., et al., *Lactulose Improves Neurological Outcomes by Repressing Harmful Bacteria and Regulating Inflammatory Reactions in Mice After Stroke.* Frontiers in Cellular and Infection Microbiology, 2021. **11**(649).

266. Lee, J., et al., *Gut Microbiota-Derived Short-Chain Fatty Acids Promote Poststroke Recovery in Aged Mice.* Circulation research, 2020. **127**(4): p. 453-465.

267. Zhang, Y., et al., *Orally Administered Crocin Protects Against Cerebral Ischemia/Reperfusion Injury Through the Metabolic Transformation of Crocetin by Gut Microbiota.* Frontiers in pharmacology, 2019. **10**: p. 440-440.

268. Chen, R., et al., *Puerariae Lobatae Radix with chuanxiong Rhizoma for treatment of cerebral ischemic stroke by remodeling gut microbiota to regulate the brain-gut barriers.* J Nutr Biochem, 2019. **65**: p. 101-114.

269. Zhang, F., et al., *Protective Effect of Tong-Qiao-Huo-Xue Decoction on Inflammatory Injury Caused by Intestinal Microbial Disorders in Stroke Rats.* Biol Pharm Bull, 2020. **43**(5): p. 788-800.

270. Guo, Y., et al., *Rhubarb anthraquinone glycosides protect against cerebral ischemia-reperfusion injury in rats by regulating brain–gut neurotransmitters.* Biomedical Chromatography, 2021. **35**(5): p. e5058.

271. Shuqin Pang, Z.L., Carol Chunfeng Wang, Xuepei Hong, Jian Zhou, Fang Chen, Li Ge, Xia Li, Yanling Dai, Yilan Wu, Jiahui Zhang, *Effects of Dioscorea polystachya 'yam gruel' on the cognitive function of diabetic rats with focal cerebral ischemia-reperfusion injury via the gut-brain axis.* Journal of Integrative Neuroscience, 2020. **19**(2): p. 273-283.

272. Liu, J., et al., *Baicalin ameliorates neuropathology in repeated cerebral ischemia-reperfusion injury model mice by remodeling the gut microbiota.* Aging, 2020. **12**(4): p. 3791-3806.

273. He, X., et al., *Involvement of brain-gut axis in treatment of cerebral infarction by β-asaron and paeonol.* Neurosci Lett, 2018. **666**: p. 78-84.

274. Li, H., et al., *Low Cerebral Exposure Cannot Hinder the Neuroprotective Effects of Panax Notoginsenosides.* 2018. **46**(1): p. 53-65.

275. Dou, Z., et al., *Neuroprotection of Resveratrol Against Focal Cerebral Ischemia/Reperfusion Injury in Mice Through a Mechanism Targeting Gut-Brain Axis.* Cell Mol Neurobiol, 2019. **39**(6): p. 883-898.

276. Xia, G.-H., et al., *Stroke Dysbiosis Index (SDI) in Gut Microbiome Are Associated With Brain Injury and Prognosis of Stroke.* Frontiers in neurology, 2019. **10**: p. 397-397.

277. Tan, C., et al., *Dysbiosis of Gut Microbiota and Short-Chain Fatty Acids in Acute Ischemic Stroke and the Subsequent Risk for Poor Functional Outcomes.* JPEN J Parenter Enteral Nutr, 2021. **45**(3): p. 518-529.

278. Rice, M.W., J.D. Pandya, and D.A. Shear, *Gut Microbiota as a Therapeutic Target to Ameliorate the Biochemical, Neuroanatomical, and Behavioral Effects of Traumatic Brain Injuries.* Frontiers in neurology, 2019. **10**: p. 875-875.

279. Li, Q., et al., *Protective mechanism of rhubarb anthraquinone glycosides in rats with cerebral ischaemia-reperfusion injury: interactions between medicine and intestinal flora.* Chinese medicine, 2020. **15**: p. 60-60.

280. Yang, S., et al., *Gut Microbiota-Dependent Marker TMAO in Promoting Cardiovascular Disease: Inflammation Mechanism, Clinical Prognostic, and Potential as a Therapeutic Target.* Frontiers in pharmacology, 2019. **10**: p. 1360-1360.

281. Egerod, K.L., et al., *Profiling of G protein-coupled receptors in vagal afferents reveals novel gut-to-brain sensing mechanisms.* Molecular metabolism, 2018. **12**: p. 62-75.

282. Itoh, S. and H. Lal, *Influences of cholecystokinin and analogues on memory processes.* 1990. **21**(4): p. 257-276.

283. Lo, C.-M., et al., *Characterization of mice lacking the gene for cholecystokinin.* 2008. **294**(3): p. R803-R810.

284. Luyer , M.D., et al., *Nutritional stimulation of cholecystokinin receptors inhibits inflammation via the vagus nerve.* Journal of Experimental Medicine, 2005. **202**(8): p. 1023-1029.

285. Crowell, M.D. and S.B. Wessinger, *5-HT and the brain-gut axis: opportunities for pharmacologic intervention.* Expert Opin Investig Drugs, 2007. **16**(6): p. 761-5.

286. Albert, P.R., C. Benkelfat, and L. Descarries, *The neurobiology of depression--revisiting the serotonin hypothesis. I. Cellular and molecular mechanisms.* Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 2012. **367**(1601): p. 2378-2381.

287. Shin, H.C., et al., *Hippocampal activation of 5-HT1B receptors and BDNF production by vagus nerve stimulation in rats under chronic restraint stress.* 2019. **50**(1): p. 1820-1830.

288. Guerriero, R.M., C.C. Giza, and A. Rotenberg, *Glutamate and GABA imbalance following traumatic brain injury.* Current neurology and neuroscience reports, 2015. **15**(5): p. 27-27.

289. Lewerenz, J. and P. Maher, *Chronic Glutamate Toxicity in Neurodegenerative Diseases—What is the Evidence?* Frontiers in Neuroscience, 2015. **9**(469).

290. Duranti, S., et al., *Bifidobacterium adolescentis as a key member of the human gut microbiota in the production of GABA.* Scientific reports, 2020. **10**(1): p. 14112-14112.

291. Iadecola, C., M.S. Buckwalter, and J. Anrather, *Immune responses to stroke: mechanisms, modulation, and therapeutic potential.* The Journal of Clinical Investigation, 2020. **130**(6): p. 2777-2788.

292. Verboon, L.N., H.C. Patel, and A.D. Greenhalgh, *The Immune System's Role in the Consequences of Mild Traumatic Brain Injury (Concussion).* Frontiers in Immunology, 2021. **12**(313).

293. Ma, Q., et al., *Impact of microbiota on central nervous system and neurological diseases: the gut-brain axis.* Journal of Neuroinflammation, 2019. **16**(1): p. 53.

294. Logsdon, A.F., et al., *Gut reactions: How the blood-brain barrier connects the microbiome and the brain.* Experimental biology and medicine (Maywood, N.J.), 2018. **243**(2): p. 159-165.

295. Zhang, P., et al., *Atorvastatin alleviates microglia-mediated neuroinflammation via modulating the microbial composition and the intestinal barrier function in ischemic stroke mice.* Free Radic Biol Med, 2021. **162**: p. 104-117.

296. Amarenco, P., *Atorvastatin in prevention of stroke and transient ischaemic attack.* Expert Opinion on Pharmacotherapy, 2007. **8**(16): p. 2789-2797.

297. López-Lluch, G. and P. Navas, *Calorie restriction as an intervention in ageing.* The Journal of physiology, 2016. **594**(8): p. 2043-2060.

298. Manzanero, S., et al., *Calorie restriction and stroke.* Experimental & translational stroke medicine, 2011. **3**: p. 8-8.

299. Liu, Y., et al., *Short-term caloric restriction exerts neuroprotective effects following mild traumatic brain injury by promoting autophagy and inhibiting astrocyte activation.* Behavioural Brain Research, 2017. **331**: p. 135-142.

300. Loncarevic-Vasiljkovic, N., et al., *Caloric Restriction Suppresses Microglial Activation and Prevents Neuroapoptosis Following Cortical Injury in Rats.* PLOS ONE, 2012. **7**(5): p. e37215.

301. Huang, J.T., et al., *Calorie restriction conferred improvement effect on long-term rehabilitation of ischemic stroke via gut microbiota.* Pharmacol Res, 2021. **170**: p. 105726.

302. Franson, R.C. and M.D. Rosenthal, *Oligomers of prostaglandin B1 inhibit in vitro phospholipase A2 activity.* Biochim Biophys Acta, 1989. **1006**(3): p. 272-7.

303. Sapirstein, A. and J.V. Bonventre, *Phospholipases A2 in ischemic and toxic brain injury.* Neurochem Res, 2000. **25**(5): p. 745-53.

CHAPTER 3

DYNAMIC CHANGES IN THE GUT MICROBIOME AT THE ACUTE STAGE OF ISCHEMIC STROKE IN A PIG MODEL

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1. Jeon J, Lourenco J, Kaiser EE, et al. Dynamic Changes in the Gut Microbiome at the Acute Stage of Ischemic Stroke in a Pig Model. *Front Neurosci*. 2020;14:587986. Reprinted here with permission of the publisher.

Abstract

Stroke is a major cause of death and long-term disability affecting 7 million adults in the US each year. Recently, it has been demonstrated that neurological diseases, associated pathology and susceptibility changes correlated with changes in the gut microbiota. However, changes in the microbial community in stroke has not been well characterized. The acute stage of stroke is a critical period for assessing injury severity, therapeutic intervention, and clinical prognosis. We investigated the changes in the gut microbiota composition and diversity using a middle cerebral artery (MCA) occlusion ischemic stroke pig model. Ischemic stroke was induced by cauterization of the MCA in pigs. Blood samples were collected pre-stroke, 4 hours (hr), 12 hr, 1 and 5 days post-stroke to evaluate circulating pro-inflammatory cytokines. Fecal samples were collected pre-stroke, 1, 3, and 5 days post-stroke to assess gut microbiome changes. Results showed elevated systemic inflammation with increased plasma levels of tumor necrosis factor alpha at 4 hr, and interleukin 6 at 12 hr post-stroke, relative to pre-stroke. Microbial diversity and evenness were reduced at 1 day post-stroke compared to pre-stroke. Microbial diversity at 3 day post-stroke was negatively correlated with lesion volume. Moreover, beta-diversity analysis revealed a trending overall differences over time, with the most significant changes in microbial patterns observed between pre-stroke and 3 days post-stroke. Abundance of the Proteobacteria was significantly increased, while Firmicutes decreased at 3 days post-stroke, compared to pre-stroke populations. Abundance of the lactic acid bacteria *Lactobacillus* was reduced at 3 days post-stroke. By day 5, the microbial pattern returned to similar values as pre-stroke, suggesting the plasticity of gut microbiome in an acute period of stroke in a pig model. These findings provide a basis for characterizing gut microbial changes during the acute stage of stroke, which can be used to assess stroke pathology and the potential development of therapeutic targets.

Keywords: MCAO, swine model, microbial diversity, inflammation

Introduction

An estimated 7 million adults in the United States suffer from stroke each year, making it the 5th leading cause of death and the 1st leading cause of long-term disability (1). The immune response and inflammation are major stroke components effecting severity as they can significantly exacerbate the primary stroke injury and cause further cell death in the brain (2, 3). High levels of systemic inflammation are closely associated with poor stroke outcomes in stroke animal models and patients (4-8). Interestingly, it has recently been demonstrated that the gut microbiome changes in response to stroke (9-11) and that modulating the gut microbiome can alter the post-stroke inflammatory response, leading to improved recovery in rodent models (12-15). Few studies have assessed the changes in the microbial populations during the acute stage of stroke, making it critically important to better characterize these microbial alterations to identify potential biomarkers for injury severity, recovery, and therapeutic targets.

It has been demonstrated that adjustments in the gut microbiome influence ischemic brain injury by altering immune homeostasis (12, 13) and neuroprotective cytokine production (13). This suggests that the gut microbiome is another potential therapeutic target for stroke (12, 13, 16, 17). Studies of gut microbiome changes in stroke have demonstrated decreases in both commensal and beneficial genera, increases in pathogenic genera in human patients (9, 10) and substantial changes in the phylum Firmicutes, Bacteroidetes, and Actinobacteria in stroke mice (12). Imbalances of the intestinal microbiota can lead to gut barrier dysfunction and impairment of stroke outcomes. In a mouse middle cerebral artery occlusion (MCAO) ischemic stroke model, the stroke mouse exhibited an imbalance in microbial communities, resulting in a reduction of intestinal motility and increased protein leakage in the gut (12). These changes correlated with increased brain

invasion of pro-inflammatory T cells from the gut and significantly increased brain infarction (13). These findings in a rodent stroke model indicate that the gut microbiome is drastically affected by stroke and plays a pivotal role in stroke severity. However, recent failures to translate findings in rodent stroke models have led to the desire to study stroke pathophysiology and therapeutic targets in more translational large animal models such as the pig (18-20).

Pigs are a robust translational animal model for biomedical research, especially gut and brain research due to the myriad of similarities to humans in physiology, anatomy, pathology, and eating behavior (21-24). They are omnivorous and have similar intestinal size and length in proportion to humans, contributing comparable transit time to humans (25, 26). The gut bacterial diversity of pigs are similar to that of humans showing higher richness and lower evenness than other animals (27) and 96 % of the functional genes found in the human gut metagenome was present in the pig gut metagenome (28), suggesting the complexity of the pig microbiota is comparable to that of humans. Moreover, pigs have similar brain size, gray and white matter composition and cytoarchitecture having a gyrencephalic brain like humans and unlike rodents (29). These similarities in the brain are predicted to lead to more human like stroke pathology in the brain and brain-gut interactions. Due to gut homology between humans and pigs, a number of experiments have been conducted in pigs to study the interplay between the gut microbiome and the immune system (30-33), yet never in a stroke pig.

In the current study, we investigated the changes in gut microbial diversity and composition in a MCAO stroke pig model developed by our research team (29, 34, 35). The results of this study conducted in a translational large animal model will help characterize patterns of bacterial changes during the acute stage of stroke, potentially providing future insight into stroke severity, recovery, and therapeutic targets.

Materials and Methods

Stroke Induction and Confirmation utilizing Magnetic Resonance Imaging

All experimental procedures were approved by the University of Georgia Institutional Animal Care and Use Committee and the study was conducted in accordance with the recommendations of the NIH's guide for the use and care of Laboratory Animals (AUP approval number: A2017 07-019-Y2-A16). Seven castrated male Landrace pigs (5-6 months old, 48-56 kg) were individually housed in a room in which the temperature was kept at 27ºC, with a 12-hour light/dark cycle.

Ischemic stroke was induced in pigs by middle cerebral artery occlusion (MCAO) as previously described (29, 34, 35). Briefly, pigs were administered Excede (5 mg/kg intramuscularly and fentanyl patch (100 mcg/hour transdermally) one day prior to the stroke surgery to prevent infections and to manage pain. Midazolam (0.2 mg/kg) and xylazine (2 mg/kg) were administered intramuscularly for pre-surgery analgesia and sedation. For anesthesia, propofol was injected intravenously and prophylactic lidocaine (1.0 mL of 2% lidocaine) was administered locally to the laryngeal folds to facilitate intubation. Anesthesia was maintained with 1.5% isoflurane in oxygen. A curvilinear incision began from the superior right orbit and extended to the rostral aspect of the auricle. The temporalis muscle was retracted and a craniectomy was performed at the exposed local dura mater. The middle cerebral artery located at the distal part of the Circle of Willis was permanently occluded using a bipolar electrocautery forceps. After post-operative recovery, pigs were returned to their respective pens and monitored every 4 hours. To reduce post-operative pain and fever, Banamine (2.2 mg/kg intramuscularly) was administered every 12 hours for the first 24 hours, and every 24 hours for the following 3 days post-stroke.

Magnetic resonance imaging (MRI) was conducted one day post-stroke using a General Electric 3.0 Tesla MRI system to confirm ischemic stroke. Pigs were anesthetized using the aforementioned anesthesia protocol and placed in a supine position using an 8-channel torso coil. T2 Fluid Attenuated Inversion Recovery (T2FLAIR) and Diffusion Weighted Imaging (DWI) sequences were used in conjunction with Apparent Diffusion Coefficient (ADC) maps to confirm the presence of ischemic lesions.

Blood Collection and Pro-inflammatory Cytokine Analysis

Peripheral blood was collected pre-stroke, 4 hr, 12 hr, 1 day, and 5 days post-stroke and plasma was separated and stored at -80°C. Circulating tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) were quantified by ELISAs (R&D systems, Minneapolis, MN, USA) to determine changes in inflammatory response.

Fecal Collection and Microbial DNA Extraction

Fecal samples were collected pre-stroke, 1, 3, and 5 days post-stroke. Samples were obtained directly from the rectum using sterilized plastic fecal loops (5 cm within the rectum). To prevent any contamination during fecal collection, all materials were sterilized prior to sample collection. Again, to prevent contamination, fecal matter was collected directly from the anus. Pig anus was stimulated with a sterilized loop and the stool was collected into a sterile sample tube without any contact with the floor or body. Fecal samples were immediately frozen on dry ice and stored at -80°C until further analysis.

Bacterial DNA was extracted from fecal samples using a previously validated approach described by Rothrock et al. (36). In this method, 330 mg of fecal material is subjected to a combination of mechanical and enzymatic processes using a modified version of the FastDNA Spin Kit for Feces (MP Biomedicals, Solon, OH, USA), and the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA). DNA purification was carried out using the DNA Stool-Human Stool-Pathogen Detection Protocol of the QIAcube Robotic Workstation. Following purification, DNA concentrations were determined spectrophotometrically (Synergy H4 Hybrid Multi-Mode Microplate Reader; BioTek, Winooski, VT).

16S rRNA Gene Sequencing and Analysis

The extracted DNA samples were sent to the Georgia Genomics and Bioinformatics Core (https://dna.uga.edu/) to sequence the 16s rRNA gene. The V3-V4 region was amplified using the S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) primer pair (37). Samples were sequenced using an Illumina MiSeq platform (Illumina, San Diego, CA). Sequencing data were provided as FASTQ files, which were merged and converted into FASTA files, and further analyzed using the QIIME pipeline v1.9.1 (38). Sequences were clustered as operational taxonomic units (OTUs) at 97% similarity, and representative sequences were aligned to the Greengenes database (gg_13_8_otus). Singleton OTUs, and OTUs whose representative sequences could not be aligned were excluded from the analysis. The computed alpha-diversity indexes are as follows: number of observed OTUs, Shannon index, and evenness. Beta-diversity was computed using the weighted UniFrac distance matrix. This metric was chosen because it accounts for the phylogenetic relationship when calculating beta-diversity.

Statistical Analysis

Data were analyzed using Graph Pad Prism (Version 8.1.1; GraphPad Software, Inc.;San Diego, CA, USA) and are shown as mean ± S.E.M. Paired t-tests were used to measure the contrasts between pre-stroke and post-stroke time points. Differences in beta-diversity were accessed by two-sample t-tests between each individual time point, and the nonparametric *P*-values generated by the Bonferroni's multiple comparisons test were used for inferences. Regression analysis was performed to evaluate associations between microbiome changes and stroke severity. For all statistical tests, *P*-values ≤ 0.05 were considered significant, and trends were declared when $0.05 < P < 0.10$.

Results

Magnetic Resonance Imaging confirmed Ischemic Lesions 1 day Post-stroke.

Non-invasive MRI allows for real-time, longitudinal assessment of stroke pathophysiology and is a critical clinical tool commonly used to differentiate between stroke type and severity (29, 39). We confirmed ischemic stroke 1 day post-stroke in all animals in the study. Both T2FLAIR and DWI sequences showed edematous lesions with bright hyperintense signal (Figure 1A&B), whereas ADC maps exhibited cytotoxic edema with dark hypointense regions due to restricted water diffusion (Figure 1C). These results are consistent with ischemic stroke. Lesion volume, midline shift and hemorrhage volume of the same cohort of pigs have been recently published (40).

Circulating TNF-α and IL-6 levels were increased during the Acute stage of Stroke in a MCAO Pig Model.

Elevated systemic inflammation has been associated with gut microbiome dysbiosis and correlated with increased brain infarction (4, 5). Elevated systemic inflammation results in poor clinical outcomes and increased mortality in stroke patients (7, 8), making it a key biomarker in stroke (41). In MCAO pigs, plasma TNF-α was increased ~ 28% at 4 hr post-stroke relative to pre-stroke levels (79.75 ± 6.00 pg/ml vs 61.62 ± 6.38 pg/ml, respectively, P = 0.003, Figure 2A). Comparatively, TNF-α levels rapidly dropped following this peak and reached the lowest level at 1 day post-stroke (43.75 ± 4.38 pg/ml, P = 0.003). TNF-α levels returned to pre-stroke levels by 5 days post-stroke (53.36 ± 5.57 pg/ml). Similar to TNF-α, plasma IL-6 levels were significantly increased ~20% at 12 hr post-stroke compared to pre-stroke (52.46 ± 2.44 pg/ml vs. 44.01± 0.85 pg/ml, respectively P = 0.01) and returned to pre-stroke levels by 5 days post-stroke (44.94 ± 1.07 pg/ml, Figure 2B), confirming an elevated inflammatory response during the acute stage of stroke in the pig model.

Diversity of Fecal Microbiota was altered during the Acute Stage of Ischemic Stroke.

Changes in diversity of gut microbiome are often an indicator of dysbiosis associated with disease pathology (42). In MCAO pigs, microbial diversity and evenness were altered during the acute stage of stroke as shown in Table 1. The Shannon and evenness indices were reduced ($P \leq 0.05$) at 1 day post-stroke compared to pre-stroke, but returned to pre-stroke levels at 3 days post-stroke. However, the number of observed OTUs, which is an estimator of microbial richness, was not significantly affected ($P \geq 0.21$) during the course of the study. Consequently, post-stroke values were not significantly different from pre-stroke values (Table 1).

To further investigate the association between gut dysbiosis and stroke severity, the correlations between microbial diversity and MRI results were assessed. Correlative analysis indicated that

Shannon, (r = -0.9715, *P* = 0.0012), Evenness (r = -0.9395, *P* = 0.0054), and Chao 1 (r = -0.8902, *P* = 0.0174) at day 3 post-stroke were negatively associated with lesion volume as determined by MRI (Figure 3), suggesting the lower microbial diversity post-stroke was related to increased stroke severity at the acute stage of stroke.

Beta-diversity was assessed using the weighted UniFrac distance matrix to investigate the similarity of microbial patterns among groups. There was a trend (*P* = 0.07) for overall differences across all time points (Figure 4). The most distinct separation in the UniFrac distance was observed between 3 days post-stroke and pre-stroke (Figure 4C). Taken together, the alpha-diversity was decreased at 1 day post-stroke and beta-diversity was most distinctly different 3 days post-stroke compared to pre-stroke.

Stroke altered Fecal Microbiome Composition.

The composition changes in gut microbiota were evaluated at different taxonomic levels (phylum, family, and genus) during the acute stage of ischemic stroke (Figures 5-7). The most prevalent phyla pre-stroke were Firmicutes (89.94 ± 1.65 %), followed by Bacteroidetes (3.45 ± 1.02 %), Actinobacteria (1.83 ± 0.70 %), and Proteobacteria (1.13 ± 0.65 %, Figure 5). The composition of these four major phyla changed during the acute stage of stroke. At 3 days post-stroke, the abundance of Firmicutes was decreased by 27% (66.08 ± 7.35 % vs 89.94 ± 1.65 %, *P* = 0.01) while Proteobacteria significantly increased 19-fold relative to pre-stroke levels (20.96 ± 5.50 % vs 1.13 ± 0.65 %, *P* = 0.01). At 5 days post-stroke, both phyla returned to pre-stroke levels (Firmicutes: 86.86 ± 3.70 % and Proteobacteria: 0.68 ± 0.19 %). Similar to Proteobacteria, Actinobacteria reached their highest abundance 3 days post-stroke (3.78 ± 0.85 % vs 1.83 ± 0.70 %, *P* = 0.02 compared to pre-stroke) and showed comparable levels to pre-stroke at 5 days post-stroke

(2.20 ± 0.93 %). The second most abundant phylum at pre-stroke, Bacteroidetes, tended to increase 3 days post-stroke (7.63 ± 1.53 % vs 3.45 ± 1.02 %, $P = 0.06$) compared to pre-stroke and remained consistent at 5 days post-stroke (7.13 ± 2.58 %, Figure 5B). The ratio of Firmicutes to Bacteroidetes was decreased ~60% at 1 day post-stroke compared to that of pre-stroke (17.33 ± 4.69 % vs 43.18 ± 12.59 %, $P = 0.04$, Figure 5C), and returned to the levels observed pre-stroke at 3 days post-stroke and remained stable, suggesting a significant microbial shift occurred at the acute stage of stroke. Other significant changes in bacterial phyla were observed in bacteria with relatively low abundance, including TM7, Cyanobacteria, and Fusobacteria, yet their abundance remained below 0.15% during the entire study (Figure 5B).

Consistent with phyla changes, a significant change in abundance was observed at the family level 3 days post-stroke (Figure 6). The most abundant family pre-stroke was *Lactobacillaceae*, making up 33.13 ± 5.66 % of the population. However, the population rapidly dropped to 10.63 ± 2.67 % 3 days post-stroke ($P < 0.001$) and increased to 20.19 ± 10.98 % 5 days post-stroke. The abundance of *Enterobacteriaceae*, *Erysipelotrichaceae*, *Prevotellaceae*, *Coriobacteriaceae*, *Desulfovibrionaceae*, *Peptostreptococcacea*, and *Enterococcaceae* were increased up to 3 days post-stroke, and returned to pre-stroke levels at 5 days post-stroke (Figure 6). Supplementary Figure 1 and Supplementary Figure 2 show changes in the gut microbiome detected at the class and order levels, respectively.

Four bacterial genera were identified with relatively high abundance (> 1%); *Lactobacillus*, *Prevotella*, *Parabacteroides* and *Collinsella* (Figure 7). *Lactobacillus* had the greatest average abundance pre-stroke (33.13 ± 5.66 %), and its presence reached the lowest point (10.63 ± 2.67 %, $P < 0.001$) 3 days post-stroke; however, it tended to return to pre-stroke levels at 5 days post-stroke (20.19 ± 10.98 %). Contrary to what was observed for *Lactobacillus*, the abundance of *Collinsella*

(2.11 ± 0.59 % vs 0.79 ± 0.38 %) and *Prevotella* (3.17 ± 0.81 % vs 0.84 ± 0.31 %) were increased 3-4 times 3 days post-stroke compared to pre-stroke levels ($P \leq 0.03$). *Parabacteroides* was significantly increased at 1 day post-stroke (0.48 ± 0.18 %) compared to pre-stroke (0.08 ± 0.05 %). Supplementary Figure 3 shows the 18 bacterial genera which were significantly altered during the acute stage of stroke in MCAO pigs. Stroke altered microbiota composition in MCAO pigs, with the majority of changes occurring 3 days post-stroke as observed at the phylum, family and genus levels.

Regression analysis conducted to better understand the changes in microbial composition between pre-stroke and 3 days post-stroke and stroke severity at the acute stage of stroke indicates that changes in abundance of phylum Bacteriodetes, Proteobacteria, Fusobacteria were positively correlated with lesion volume, MLS and hemorrhage volume, while Firmicutes was negatively correlated with the stroke severity (Table 2). Moreover, changes in abundance of *Lactobacillaceae* and *Lactobacillus* were negatively related to the stroke severity (Table 2), supporting that the changes in microbial composition at the acute stage of stroke is closely related to stroke severity.

Discussion

In this study, we investigated alterations in microbial composition during the acute stroke phase in a MCAO pig ischemic stroke model. The fecal microbiome was dynamically changed during this stage, as follows: 1) stroke reduced species diversity and evenness 1 day post-stroke and changed bacterial community patterns (beta-diversity) among groups 3 days post-stroke; 2) the ratio of Firmicutes to Bacteroidetes was decreased 1 day post-stroke; 3) high abundance of Proteobacteria and low abundance of the genus *Lactobacillus* were observed 3 days post-stroke. These results showed a dynamic compositional change of bacteria following a stroke event,

particularly 3 days post-stroke, and that this was transient with most microbiome metrics returning to pre-stroke levels by 5 days post-stroke. Interestingly, increases in the systemic inflammatory response measured by circulating TNF-α and IL-6 were observed along with changes in fecal microbiome at the acute stage of stroke. This initial study demonstrates the plasticity of the gut microbiome during the acute stage of stroke, which occurred concurrently with systemic inflammation.

Microbial dysbiosis of the gastrointestinal tract has been reported in a number of neurological injuries and diseases including stroke (9, 10, 12, 13, 43, 44). These compositional changes of gut microflora can be assessed by using alpha-diversity and beta-diversity indices (45). Alpha-diversity, representing the richness and diversity of a community, has been shown to decrease in attention-deficit/hyperactivity disorder (43), autism spectrum (46) as well as brain injury (12, 44). In accordance with the previous studies, our results showed the rapid reduction of alpha-diversity (Shannon index and evenness) responding to MCAO-induced stroke. Likewise, the differences in beta-diversity, representing overall similarity of bacterial communities, have been reported in stroke (10, 12) and brain injury models (43, 44). Consistent with the shifts shown in brain injury rodent models (44), our beta-diversity analysis revealed the greatest difference at 3 days post-stroke compared to pre-stroke. By 5 days post-stroke, the distinctive microbiome pattern overlapped with the pre-stroke pattern, suggesting that beta-diversity recovered during the acute stage of stroke although the microflora profile is not identical to pre-stroke. Overall, our findings demonstrate a reduced species diversity and evenness, and trending changes in beta-diversity between pre- and post-stroke, indicating that stroke alters the gut microbiome during the acute stage.

Firmicutes and Bacteroidetes are the two predominant phyla in human gut bacteria and the ratios between these phyla are often used as a marker of dysbiosis or is seen as indicative of energy availability in the lower gastrointestinal tract (47). A decreases in the ratio of Firmicutes to Bacteroidetes (F/B ratio) has been reported in neurological disease (44, 48, 49) and inflammatory bowel disease (50). Stroked pigs in this study also showed significantly reduced F/B ratio 1 day post-stroke. Previous obesity studies showed increases in F/B ratio (51, 52), which suggested that obese individuals that had a high abundance of Firmicutes may be more efficient at extracting energy from the diet in the form of volatile fatty acids produced in the lower gastrointestinal tract microbial fermentation (53, 54). The reduction in the F/B ratio observed in the current study suggests that stroke pigs may not be able to produce as many volatile fatty acids following stroke. Consequently, the role of the F/B ratio in a disease pathology needs to be further investigated (55). Proteobacteria is a well-known phyla containing opportunistic pathogenic bacteria such as *Escherichia*, *Salmonella*, *Helicobacter* and others (56) and increased abundances have been observed in type 2 diabetes (57), obesity (58), inflammatory bowel disease (59), and neurological conditions including stroke (10, 44). A rapid increase of Proteobacteria was observed 3 days post-stroke in the current study, indicating an increase in the phyla following stroke onset. Stroked pigs had a greater abundance of *Enterobacteriaceae* and *Desulfovibrionaceae* 3 days post-stroke. Enriched levels of the families *Enterobacteriaceae* and *Desulfovibrionaceae* were previously found in patients with a high risk of stroke (60) and higher level of *Desulfovibrionaceae* was detected in patients following stroke (10). The increased abundance of Proteobacteria at the acute stage of stroke may play an important role in the development of systemic inflammation in stroke, potentially leading to more deleterious outcomes.

Lactic acid bacteria (LAB) may help reducing inflammation and controlling pathogen populations through the production of lactic acids. In addition, LAB produce important gut-derived metabolites such as short chain fatty acids (61), which act as signaling molecules in immune responses (62). Talani et al. demonstrated that treatment of the gut with *Bifidobacteria* improved cognitive behavior and hippocampal plasticity with increases in hippocampal BDNF in rats, suggesting probiotics as a potential therapeutic treatment in brain diseases associated with cognitive functions (63). *Lactobacillus* is a common component of commercial human and animal probiotics (64). Low populations of *Lactobacillus* were found in patients with irritable bowel syndrome, HIV, type 1 diabetes, and multiple sclerosis (65). A decrease in relative abundance of *Lactobacillus* was also observed in the present study 3 days post-stroke. Contrary to our findings, Zeng et al. found enrichment of LAB in high risk stroke patients and suggested that the presence of LAB in the gastrointestinal tract compensates for the loss of butyrate-producing bacteria in these individuals (60). Further research is needed to reconcile the discrepancies in the field. The results from this study suggest that the use of probiotics such as *Lactobacillus* at the acute stage may benefit stroke patients.

Increased populations of specific microflora may result in an increase of end products that are risk factors for stroke such as trimethylamine-N-oxide (TMAO). Trimethylamine, a precursor of TMAO, is produced by gut microbiota from dietary choline and is further metabolized to TMAO in the liver. Circulating TMAO has been reported to increase the buildup of atherosclerotic plaques in coronary vasculature, increasing the risks of stroke (66, 67). Specifically, the abundance of *Peptostreptococcaceae* and *Prevotella* are positively associated with circulating TMAO (68). In the present study, pigs with stroke had increased abundances of both *Peptostreptococcaceae* and *Prevotella*, suggesting that the dysbiosis during the acute stage of stroke is potentially related to

increased TMAO production. Additionally, a dysregulation of lipid profiles is considered as another risk factor for stroke. In stroke pigs, an increased abundance of *Coriobacteriaceae* was found, which was negatively correlated with blood triglycerides and low-density lipoprotein cholesterol in hyperlipidemia patients (69). The increase of *Coriobacteriaceae* observed in this study may be the result of compensatory mechanisms in response to stroke induced changes in gastrointestinal conditions that alter the microbial ecology throughout the gut. Understanding the role of microflora on the regulation of metabolites associated with stroke may provide insight into the development of novel therapeutic targets, as reviewed by Tonomura et al, including the role of bacterial metabolites such as TAMO and short chain fatty acids in stroke (70).

There is mounting evidence highlighting the interactions between the gut microbiota and stroke outcome in humans and animal models. In humans, stroke dysbiosis was closely linked to severe stroke and unfavorable outcome (10, 71). In stroke mouse models, perturbation of the gut microbiota lead to increased intestinal pro-inflammatory T cells and larger ischemic brain lesions (13). In the current study, changes in microbial diversity and microbiota composition were associated with stroke severity measured by high resolution structural MRI at the acute stage of stroke. The lesion volume was negatively related with alpha diversity indexes suggesting that reduced microbial richness and evenness in stroke pigs are related to increased stroke severity. Moreover, we found potential pathogenic bacteria Proteobacteria, *Enterobacteriaceae*, and *Desulfovibrionaceae* were increased and beneficial bacteria *Lactobacillaceae* and *Lactobacillus* were decreased 3 days post-stroke. Interestingly, the abundance of the pathogenic bacteria was positively related to the lesion volume, MLS and hemorrhage volume, while that of beneficial bacteria were negatively related to the stroke severity, suggesting that gut dysbiosis may be a

potential biomarker for injury severity and that the gut microbiota may be a stroke therapeutic target.

The role of inflammation in neurological diseases has been widely recognized (4-8) and alterations in the bacterial community due to disease have been correlated with changes in inflammatory responses (13-16, 72). Consistent with previous findings, increased plasma levels of pro-inflammatory cytokines were observed in the current study, concurrent with the changes in gut microbiome composition. Increased gut permeability and translocation of bacteria to host tissues have been previously reported in stroke conditions (73, 74), supporting the involvement of the gut microbial population in stroke pathophysiology. It is well known that lipopolysaccharides (LPS) from the cell wall of gram-negative bacteria (75) trigger an immune response by binding to Toll-like receptor 4 in endothelial cells, activating monocytes/macrophages, and nuclear factor kappa B signaling cascades, resulting in the production of pro-inflammatory cytokines such as TNF-α (76, 77). Pro-inflammatory cytokines contribute to the disruption of tight junction proteins between the epithelial cells, leading to an increase in gut permeability (78) as well as translocation of bacteria and microbial-derived end products into the blood stream in what is known as “leaky gut syndrome”. Leaky gut syndrome results in a cycle of increasing inflammation that is detrimental to stroke patients which could potentially be mitigated by therapeutic treatments that alter the composition of the gut microbial composition.

Conclusion

The present study demonstrated, for the first time using a large translational animal model such as swine, the plasticity of the gut microbiome during the acute stage of stroke. These changes included significant shifts in microbial diversity, the ratio of Firmicutes to Bacteroidetes, and the abundance of Proteobacteria and *Lactobacillus*. Importantly, the microbial changes in richness and evenness were inversely correlated with brain lesion size as measured by MRI. Given the significant degree of physiologic similarities between swine and humans, findings from the current study contribute to increasing our understanding of the pathophysiology of stroke in human patients. Future studies investigating the role of the microbiome and its effect on the stroke immune response are warranted to understand the effect of therapeutic treatments on the gut microbiota in stroke patients.

References

1. Benjamin EJ, Muntner P, Alonso A, Bittencourt MS, Callaway CW, Carson AP, et al. Heart Disease and Stroke Statistics-2019 Update: A Report From the American Heart Association. Circulation. 2019;139(10):e56-e528.

2. Deb P, Sharma S, Hassan KM. Pathophysiologic mechanisms of acute ischemic stroke: An overview with emphasis on therapeutic significance beyond thrombolysis. Pathophysiology. 2010;17(3):197-218.

3. Borgens RB, Liu-Snyder P. Understanding secondary injury. Q Rev Biol. 2012;87(2):89-127.

4. McColl BW, Rothwell NJ, Allan SM. Systemic inflammatory stimulus potentiates the acute phase and CXC chemokine responses to experimental stroke and exacerbates brain damage via interleukin-1- and neutrophil-dependent mechanisms. J Neurosci. 2007;27(16):4403-12.

5. McColl BW, Rothwell NJ, Allan SM. Systemic inflammation alters the kinetics of cerebrovascular tight junction disruption after experimental stroke in mice. J Neurosci. 2008;28(38):9451-62.

6. Audebert Heinrich J, Rott Michaela M, Eck T, Haberl Roman L. Systemic Inflammatory Response Depends on Initial Stroke Severity but Is Attenuated by Successful Thrombolysis. Stroke. 2004;35(9):2128-33.

7. Elkind MS, Cheng J, Rundek T, Boden-Albala B, Sacco RL. Leukocyte count predicts outcome after ischemic stroke: the Northern Manhattan Stroke Study. J Stroke Cerebrovasc Dis. 2004;13(5):220-7.

8. Di Napoli M, Papa F, Bocola V. C-reactive protein in ischemic stroke: an independent prognostic factor. Stroke. 2001;32(4):917-24.

9. Swidsinski A, Loening-Baucke V, Krüger M, Kirsch S. Central Nervous System and the Colonic Bioreactor: Analysis of Colonic Microbiota in Patients with Stroke Unravels Unknown Mechanisms of the Host Defense after Brain Injury. Intest Res. 2012;10(4):332-42.

10. Yin J, Liao SX, He Y, Wang S, Xia GH, Liu FT, et al. Dysbiosis of Gut Microbiota With Reduced Trimethylamine-N-Oxide Level in Patients With Large-Artery Atherosclerotic Stroke or Transient Ischemic Attack. J Am Heart Assoc. 2015;4(11).

11. Durgan DJ, Lee J, McCullough LD, Bryan RM, Jr. Examining the Role of the Microbiota-Gut-Brain Axis in Stroke. Stroke. 2019;50(8):2270-7.

12. Singh V, Roth S, Llovera G, Sadler R, Garzetti D, Stecher B, et al. Microbiota Dysbiosis Controls the Neuroinflammatory Response after Stroke. J Neurosci. 2016;36(28):7428-40.

13. Benakis C, Brea D, Caballero S, Faraco G, Moore J, Murphy M, et al. Commensal microbiota affects ischemic stroke outcome by regulating intestinal gammadelta T cells. Nat Med. 2016;22(5):516-23.

14. Yamashiro K, Tanaka R, Urabe T, Ueno Y, Yamashiro Y, Nomoto K, et al. Gut dysbiosis is associated with metabolism and systemic inflammation in patients with ischemic stroke. PLOS ONE. 2017;12(2):e0171521.

15. Spychala MS, Venna VR, Jandzinski M, Doran SJ, Durgan DJ, Ganesh BP, et al. Age-related changes in the gut microbiota influence systemic inflammation and stroke outcome. Ann Neurol. 2018;84(1):23-36.

16. Winek K, Dirnagl U, Meisel A. The Gut Microbiome as Therapeutic Target in Central Nervous System Diseases: Implications for Stroke. Neurotherapeutics. 2016;13(4):762-74.

17. Singh V, Sadler R, Heindl S, Llovera G, Roth S, Benakis C, et al. The gut microbiome primes a cerebroprotective immune response after stroke. Journal of cerebral blood flow and metabolism :

official journal of the International Society of Cerebral Blood Flow and Metabolism. 2018;38(8):1293-8.

18. Albers GW, Goldstein LB, Hess DC, Wechsler LR, Furie KL, Gorelick PB, et al. Stroke Treatment Academic Industry Roundtable (STAIR) recommendations for maximizing the use of intravenous thrombolytics and expanding treatment options with intra-arterial and neuroprotective therapies. Stroke. 2011;42(9):2645-50.

19. Fisher M, Feuerstein G, Howells DW, Hurn PD, Kent TA, Savitz SI, et al. Update of the stroke therapy academic industry roundtable preclinical recommendations. Stroke. 2009;40(6):2244-50.

20. Saver JL, Albers GW, Dunn B, Johnston KC, Fisher M, Consortium SV. Stroke Therapy Academic Industry Roundtable (STAIR) recommendations for extended window acute stroke therapy trials. Stroke. 2009;40(7):2594-600.

21. Swindle MM, Makin A, Herron AJ, Clubb FJ, Jr., Frazier KS. Swine as models in biomedical research and toxicology testing. Vet Pathol. 2012;49(2):344-56.

22. Lind NM, Moustgaard A, Jelsing J, Vajta G, Cumming P, Hansen AK. The use of pigs in neuroscience: modeling brain disorders. Neuroscience and biobehavioral reviews. 2007;31(5):728-51.

23. Watanabe H, Andersen F, Simonsen CZ, Evans SM, Gjedde A, Cumming P, et al. MR-based statistical atlas of the Gottingen minipig brain. NeuroImage. 2001;14(5):1089-96.

24. Kaiser EE, West FD. Large animal ischemic stroke models: replicating human stroke pathophysiology. Neural regeneration research. 2020;15(8):1377-87.

25. Heinritz SN, Mosenthin R, Weiss E. Use of pigs as a potential model for research into dietary modulation of the human gut microbiota. Nutr Res Rev. 2013;26(2):191-209.

26. Clouard C, Meunier-Salaun MC, Val-Laillet D. Food preferences and aversions in human health and nutrition: how can pigs help the biomedical research? Animal : an international journal of animal bioscience. 2012;6(1):118-36.

27. Lamendella R, Santo Domingo JW, Ghosh S, Martinson J, Oerther DB. Comparative fecal metagenomics unveils unique functional capacity of the swine gut. BMC Microbiology. 2011;11(1):103.

28. Xiao L, Estelle J, Kiilerich P, Ramayo-Caldas Y, Xia Z, Feng Q, et al. A reference gene catalogue of the pig gut microbiome. Nat Microbiol. 2016;1:16161.

29. Baker EW, Platt SR, Lau VW, Grace HE, Holmes SP, Wang L, et al. Induced Pluripotent Stem Cell-Derived Neural Stem Cell Therapy Enhances Recovery in an Ischemic Stroke Pig Model. Sci Rep. 2017;7(1):10075.

30. Foster N, Lovell MA, Marston KL, Hulme SD, Frost AJ, Bland P, et al. Rapid protection of gnotobiotic pigs against experimental salmonellosis following induction of polymorphonuclear leukocytes by avirulent Salmonella enterica. Infect Immun. 2003;71(4):2182-91.

31. Scharek L, Guth J, Reiter K, Weyrauch KD, Taras D, Schwerk P, et al. Influence of a probiotic Enterococcus faecium strain on development of the immune system of sows and piglets. Vet Immunol Immunopathol. 2005;105(1-2):151-61.

32. Wilson S, Norton P, Haverson K, Leigh J, Bailey M. Development of the palatine tonsil in conventional and germ-free piglets. Dev Comp Immunol. 2005;29(11):977-87.

33. Zhang Q, Widmer G, Tzipori S. A pig model of the human gastrointestinal tract. Gut Microbes. 2013;4(3):193-200.

34. Duberstein KJ, Platt SR, Holmes SP, Dove CR, Howerth EW, Kent M, et al. Gait analysis in a pre- and post-ischemic stroke biomedical pig model. Physiol Behav. 2014;125:8-16.

35. Platt SR, Holmes SP, Howerth EW, Duberstein KJJ, Dove CR, Kinder HA, et al. Development and characterization of a Yucatan miniature biomedical pig permanent middle cerebral artery occlusion stroke model. Exp Transl Stroke Med. 2014;6(1):5.

36. Rothrock MJ, Jr., Hiett KL, Gamble J, Caudill AC, Cicconi-Hogan KM, Caporaso JG. A hybrid DNA extraction method for the qualitative and quantitative assessment of bacterial communities from poultry production samples. Journal of visualized experiments : JoVE. 2014(94):52161.

37. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic acids research. 2013;41(1):e1-e.

38. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nature methods. 2010;7(5):335-6.

39. González RG. Clinical MRI of acute ischemic stroke. Journal of magnetic resonance imaging : JMRI. 2012;36(2):259-71.

40. Kaiser EE, Waters ES, Fagan MM, Scheulin KM, Platt SR, Jeon JH, et al. Characterization of tissue and functional deficits in a clinically translational pig model of acute ischemic stroke. Brain Research. 2020;1736:146778.

41. Di Napoli M, Papa F. Systemic inflammation, blood pressure, and stroke outcome. J Clin Hypertens (Greenwich). 2006;8(3):187-94.

42. Kriss M, Hazleton KZ, Nusbacher NM, Martin CG, Lozupone CA. Low diversity gut microbiota dysbiosis: drivers, functional implications and recovery. Curr Opin Microbiol. 2018;44:34-40.

43. Prehn-Kristensen A, Zimmermann A, Tittmann L, Lieb W, Schreiber S, Baving L, et al. Reduced microbiome alpha diversity in young patients with ADHD. PLoS One. 2018;13(7):e0200728.

44. Nicholson SE, Watts LT, Burmeister DM, Merrill D, Scroggins S, Zou Y, et al. Moderate Traumatic Brain Injury Alters the Gastrointestinal Microbiome in a Time-Dependent Manner. Shock. 2019;52(2):240-8.

45. Wagner B, K. Grunwald G, O. Zerbe G, Mikulich-Gilbertson S, E. Robertson C, Zemanick E, et al. On the Use of Diversity Measures in Longitudinal Sequencing Studies of Microbial Communities. Frontiers in Microbiology. 2018;9.

46. Ma B, Liang J, Dai M, Wang J, Luo J, Zhang Z, et al. Altered Gut Microbiota in Chinese Children With Autism Spectrum Disorders. Front Cell Infect Microbiol. 2019;9:40.

47. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. Science (New York, NY). 2005;308(5728):1635-8.

48. Vogt NM, Kerby RL, Dill-McFarland KA, Harding SJ, Merluzzi AP, Johnson SC, et al. Gut microbiome alterations in Alzheimer's disease. Scientific Reports. 2017;7(1):13537.

49. Rowin J, Xia Y, Jung B, Sun J. Gut inflammation and dysbiosis in human motor neuron disease. Physiological reports. 2017;5(18):e13443.

50. Kabeerdoss J, Jayakanthan P, Pugazhendhi S, Ramakrishna BS. Alterations of mucosal microbiota in the colon of patients with inflammatory bowel disease revealed by real time polymerase chain reaction amplification of 16S ribosomal ribonucleic acid. The Indian journal of medical research. 2015;142(1):23-32.

51. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Human gut microbes associated with obesity. Nature. 2006;444(7122):1022-3.

52. Koliada A, Syzenko G, Moseiko V, Budovska L, Puchkov K, Perederiy V, et al. Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. BMC microbiology. 2017;17(1):120-.

53. Munukka E, Wiklund P, Pekkala S, Volgyi E, Xu L, Cheng S, et al. Women with and without metabolic disorder differ in their gut microbiota composition. Obesity (Silver Spring). 2012;20(5):1082-7.

54. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444(7122):1027-31.

55. Liang D, Leung RK-K, Guan W, Au WW. Involvement of gut microbiome in human health and disease: brief overview, knowledge gaps and research opportunities. Gut Pathogens. 2018;10(1):3.

56. Rizzatti G, Lopetuso LR, Gibiino G, Binda C, Gasbarrini A. Proteobacteria: A Common Factor in Human Diseases. BioMed Research International. 2017;2017:7.

57. Lambeth SM, Carson T, Lowe J, Ramaraj T, Leff JW, Luo L, et al. Composition, Diversity and Abundance of Gut Microbiome in Prediabetes and Type 2 Diabetes. Journal of diabetes and obesity. 2015;2(3):1-7.

58. Fei N, Zhao L. An opportunistic pathogen isolated from the gut of an obese human causes obesity in germfree mice. The Isme Journal. 2012;7:880.

59. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biology. 2012;13(9):R79.

60. Zeng X, Gao X, Peng Y, Wu Q, Zhu J, Tan C, et al. Higher Risk of Stroke Is Correlated With Increased Opportunistic Pathogen Load and Reduced Levels of Butyrate-Producing Bacteria in the Gut. Frontiers in cellular and infection microbiology. 2019;9:4-.

61. George F, Daniel C, Thomas M, Singer E, Guilbaud A, Tessier FJ, et al. Occurrence and Dynamism of Lactic Acid Bacteria in Distinct Ecological Niches: A Multifaceted Functional Health Perspective. Frontiers in Microbiology. 2018;9(2899).

62. Rizzetto L, Fava F, Tuohy KM, Selmi C. Connecting the immune system, systemic chronic inflammation and the gut microbiome: The role of sex. J Autoimmun. 2018;92:12-34.

63. Talani G, Biggio F, Mostallino MC, Locci V, Porcedda C, Boi L, et al. Treatment with gut bifidobacteria improves hippocampal plasticity and cognitive behavior in adult healthy rats. Neuropharmacology. 2020;165:107909.

64. Salvetti E, Torriani S, Felis GE. The Genus Lactobacillus: A Taxonomic Update. Probiotics and Antimicrobial Proteins. 2012;4(4):217-26.

65. Heeney DD, Gareau MG, Marco ML. Intestinal Lactobacillus in health and disease, a driver or just along for the ride? Current Opinion in Biotechnology. 2018;49:140-7.

66. Bennett BJ, de Aguiar Vallim TQ, Wang Z, Shih DM, Meng Y, Gregory J, et al. Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. Cell Metab. 2013;17(1):49-60.

67. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med. 2013;368(17):1575-84.

68. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nature medicine. 2013;19(5):576-85.

69. Liu Y, Song X, Zhou H, Zhou X, Xia Y, Dong X, et al. Gut Microbiome Associates With Lipid-Lowering Effect of Rosuvastatin in Vivo. Frontiers in microbiology. 2018;9:530-.

70. Tonomura S, Ihara M, Friedland RP. Microbiota in cerebrovascular disease: A key player and future therapeutic target. Journal of Cerebral Blood Flow & Metabolism. 2020;40(7):1368-80.

71. Xia G-H, You C, Gao X-X, Zeng X-L, Zhu J-J, Xu K-Y, et al. Stroke Dysbiosis Index (SDI) in Gut Microbiome Are Associated With Brain Injury and Prognosis of Stroke. Frontiers in neurology. 2019;10:397-.

72. Zeraati M, Enayati M, Kafami L, Shahidi SH, Salari AA. Gut microbiota depletion from early adolescence alters adult immunological and neurobehavioral responses in a mouse model of multiple sclerosis. Neuropharmacology. 2019;157:107685.

73. Crapser J, Ritzel R, Verma R, Venna VR, Liu F, Chauhan A, et al. Ischemic stroke induces gut permeability and enhances bacterial translocation leading to sepsis in aged mice. Aging. 2016;8(5):1049-63.

74. Stanley D, Mason L, Mackin K, Srikhanta Y, Lyras D, Prakash MD, et al. Translocation and dissemination of commensal bacteria in post-stroke infection. Nature Medicine. 2016;22.

75. Hurley JC. Endotoxemia: methods of detection and clinical correlates. Clin Microbiol Rev. 1995;8(2):268-92.

76. Mafra D, Lobo JC, Barros AF, Koppe L, Vaziri ND, Fouque D. Role of altered intestinal microbiota in systemic inflammation and cardiovascular disease in chronic kidney disease. Future Microbiology. 2014;9(3):399-410.

77. Cario E, Brown D, McKee M, Lynch-Devaney K, Gerken G, Podolsky DK. Commensal-associated molecular patterns induce selective toll-like receptor-trafficking from apical membrane to cytoplasmic compartments in polarized intestinal epithelium. The American journal of pathology. 2002;160(1):165-73.

78. Katzenberger RJ, Ganetzky B, Wassarman DA. The gut reaction to traumatic brain injury. Fly. 2015;9(2):68-74.

Table 3.1. Alpha-diversity was changed during the acute stage of ischemic stroke in a pig model.

Alpha-diversity Indexes		Pre-stroke (n=7)	Post-stroke		
			1 day (n=7)	3 days (n=6)	5 days (n=4)
Number of observed OTUs	Mean	7110	5824	6140	5744
	S.E.M	1130	407	222	344
	P-value	-	NS	NS	NS
Shannon	Mean	8.14	7.46	7.92	7.93
	S.E.M	0.40	0.27	0.18	0.31
	P-value	-	0.05	NS	NS
Evenness	Mean	0.638	0.596	0.630	0.635
	S.E.M	0.022	0.017	0.013	0.022
	P-value	-	0.02	NS	NS

P-value: Paired t-test comparing the mean pre-stroke values vs. each time point post-stroke.

S.E.M = Standard error of mean; NS. Not significant, $P>0.05$

Table 3.2. Changes in gut microbiota composition at 3 days post-stroke were correlated with lesion volume, midline shift, and hemorrhage volume in the ischemic stroke pig model (n=6).

		Lesion volume	MLS	Hemorrhage Volume
Phylum	Bacteroidetes	r = 0.6318 *P* = 0.0205	r = 0.5920 *P* = 0.0330	r = 0.6310 *P* = 0.0207
	Firmicutes	r = -0.7999 *P* = 0.0010	r = -0.7415 *P* = 0.0037	r = -0.8202 *P* = 0.0006
	Proteobacteria	r = 0.8500 *P* = 0.0002	r = 0.7837 *P* = 0.0015	r = 0.8856 *P* < 0.0001
	Fusobacteria	r = 0.6817 *P* = 0.0103	r = 0.5170 NS	r = 0.4576 NS
Family	*Lactobacillaceae*	r = -0.7057 *P* = 0.0070	r = -0.7150 *P* = 0.0060	r = -0.6911 *P* = 0.0089
	Enterobacteriaceae	r = 0.8440 *P* = 0.0003	r = 0.7715 *P* = 0.0020	r = 0.8802 *P* < 0.0001
	Prevotellaceae	r = 0.7355 *P* = 0.0042	r = 0.7120 *P* = 0.0063	r = 0.7905 *P* = 0.0013
	Desulfovibrionaceae	r = 0.7141 *P* = 0.0061	r = 0.7966 *P* = 0.0011	r = 0.7107 *P* = 0.0065
	Enterococcaceae	r = 0.8270 *P* = 0.0005	r = 0.7174 *P* = 0.0058	r = 0.8710 *P* = 0.0001
Genus	*Lactobacillus*	r = -0.7056 *P* = 0.0071	r = -0.7150 *P* = 0.0060	r = -0.6910 *P* = 0.0089
	Prevotella	r = 0.7355 *P* = 0.0042	r = 0.7120 *P* = 0.0063	r = 0.7904 *P* = 0.0013
	Parabacteroides	r = 0.6110 *P* = 0.0265	r = 0.5803 *P* = 0.0376	r = 0.5573 *P* = 0.0479

r = Pearson correlation coefficient; NS. Not significant, *P*>0.05

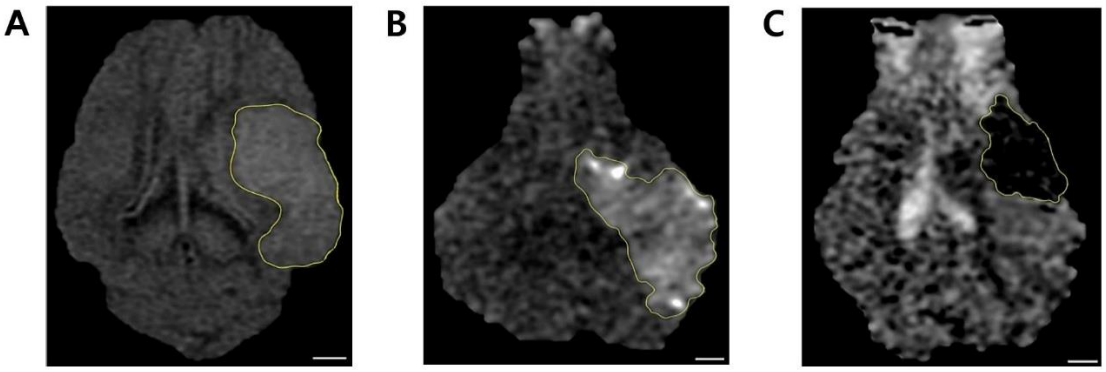


Figure 3.1. MRI confirmed ischemic stroke in pigs. The hyperintense regions seen in **(A)** T2 FLAIR and **(B)** DWI sequences corresponded to **(C)** ADC map hypointense regions thus confirming the presence of ischemic infarction 1 day post-MCAO surgery (n=7). A scale bar is provided in each picture (6 mm) and the lesion area was outlined by a yellow line. These are representative images from an individual animal.

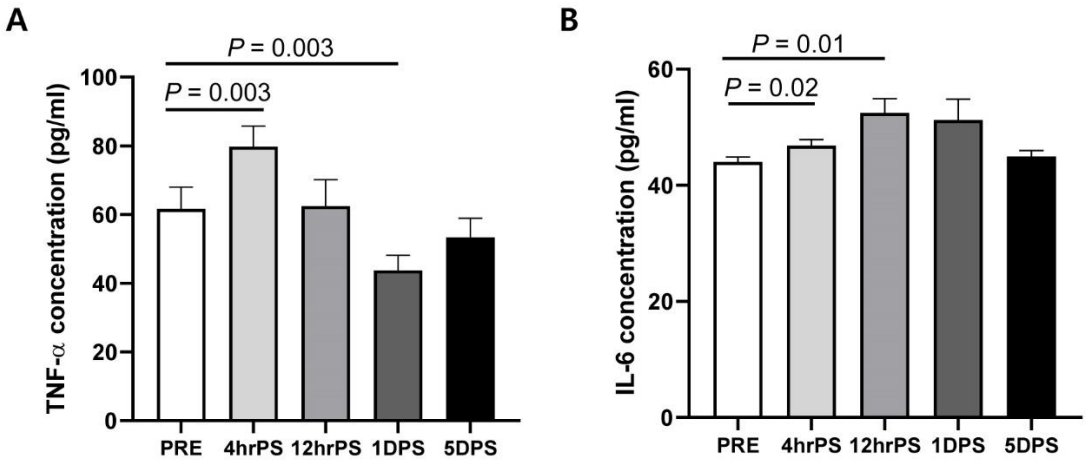


Figure 3.2. Plasma concentrations of pro-inflammatory cytokines were increased during the acute stage of ischemic stroke in pigs. Plasma concentration (pg/ml) of **(A)** TNF-α and **(B)** IL-6 were measured pre-stroke (PRE, n=7), 4 hours post-stroke (4hrPS, n=7 and 6 respectively), 12 hours post-stroke (12hrPS, n=7), 1 day post-stroke (1DPS, n=7), and 5 days post-stroke (5DPS, n=5). *P*-value: Paired t-test comparing the mean pre-stroke values vs. each time point post-stroke.

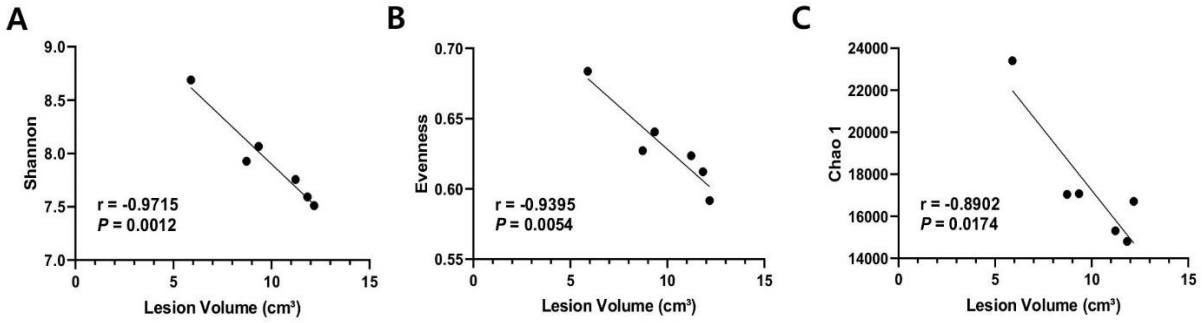


Figure 3.3. **Microbial diversity was negatively correlated with brain lesion volume.** Brain lesion volume measured 1 day post-stroke was negatively correlated with alpha-diversity indexes including **(A)** Shannon, **(B)** Evenness, and **(C)** Chao1 at 3 days post-stroke (n=6). Regression analysis was performed to evaluate associations between microbial composition and stroke lesion size. Pearson correlation coefficient and P-values shown.

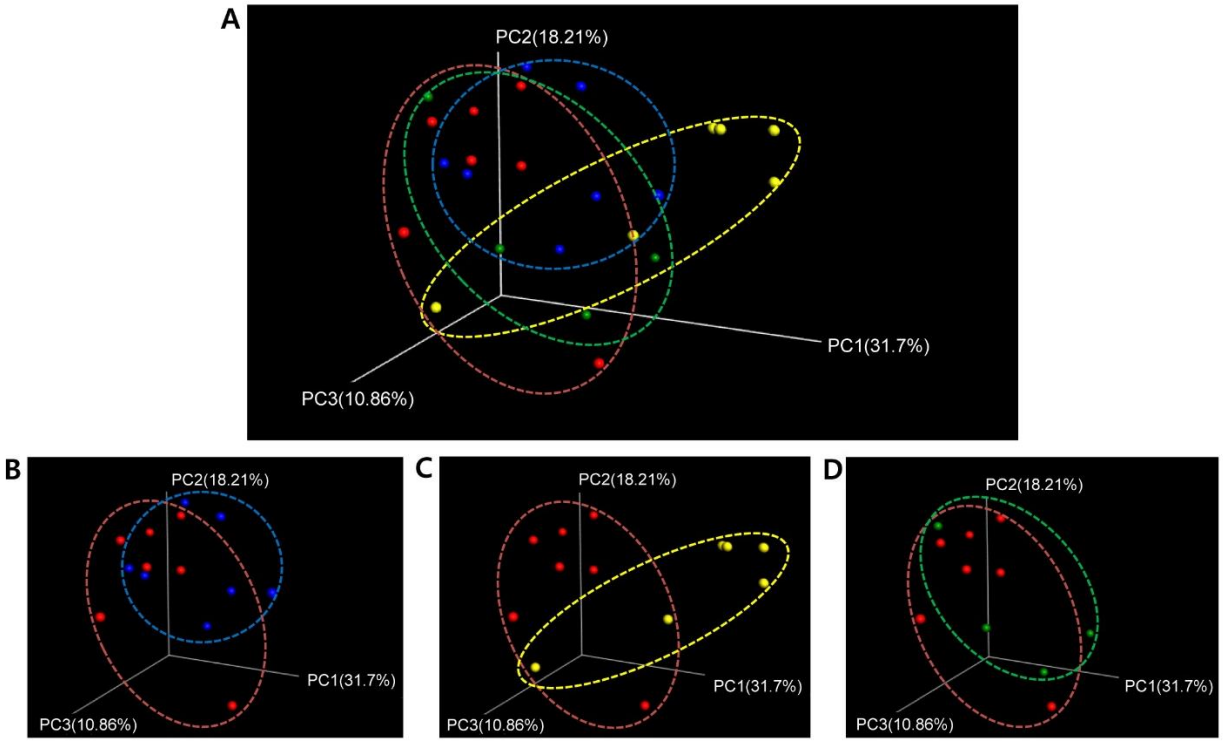


Figure 3.4. Beta-diversity (Weighted UniFrac PCoA plots) showed trending differences between pre- and post-stroke in MCAO pig model. (A) Beta-diversity changes during the acute stage of stroke were shown by PCoA plots. Different bacterial communities were compared between **(B)** pre-stroke (n=7) vs 1 day post-stroke (n=7), **(C)** pre-stroke vs 3 days post-stroke (n=6), and **(D)** pre-stroke vs 5 days post-stroke (n=4). Trending differences were observed between pre-stroke and 3 days post-stroke (Nonparametric *P*-values generated by the Bonferroni's multiple comparisons test were used for inferences).

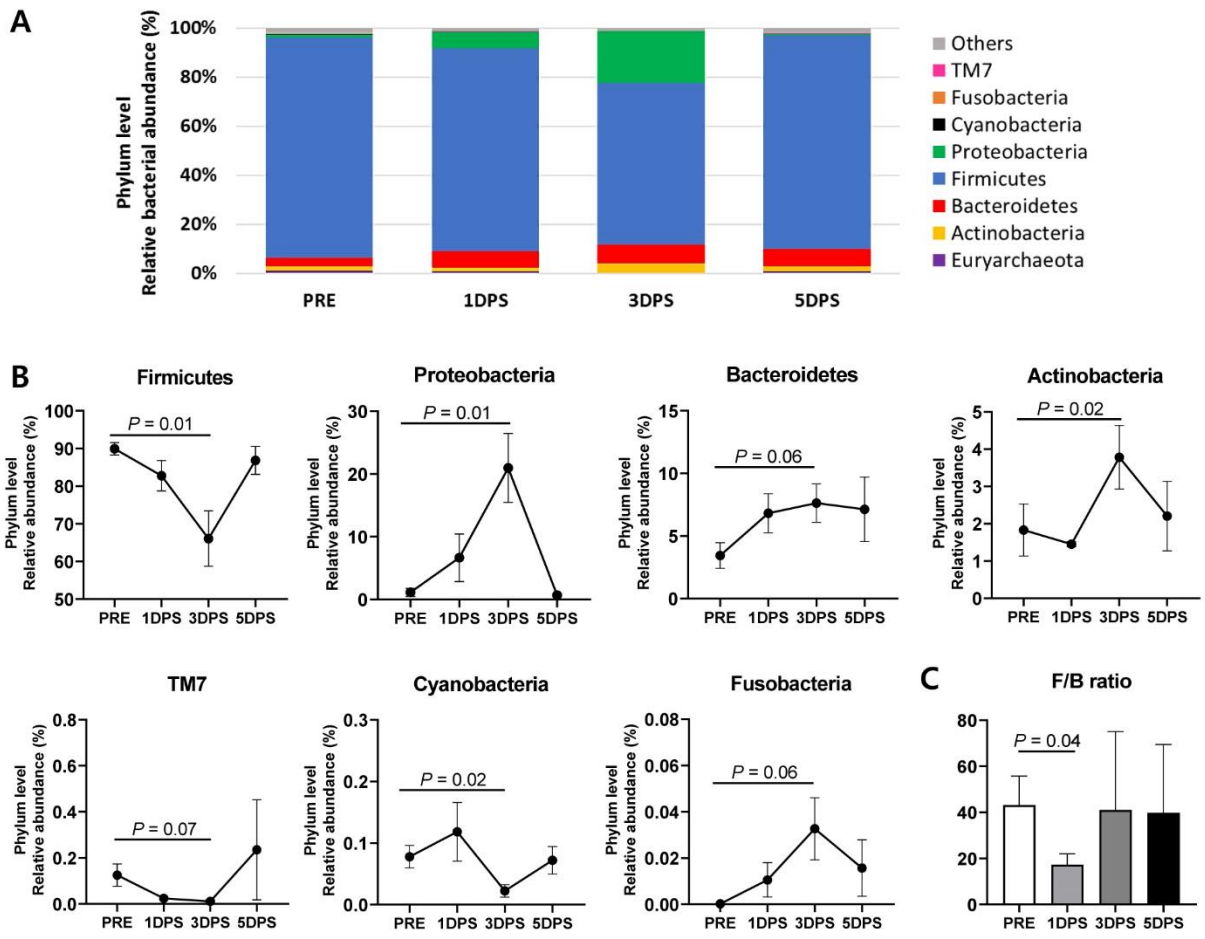


Figure 3.5. Stroke resulted in phylum level perturbations in gut microbiota between pre- and post-stroke in MCAO pig model. (A) Mean relative abundances of bacterial phylum pre-stroke (PRE, n=7), 1 day post-stroke (1DPS, n=7), 3 days post-stroke (3DPS, n=6), and 5 days post-stroke (5DPS, n=4) are shown. **(B)** Bacterial phyla showed changes in the abundance ($P < 0.10$) during acute stage of stroke. **(C)** The ratio of Firmicutes to Bacteroidetes (F/B) at 1 day post-stroke (1DPS) was lower than pre-stroke (PRE), 3 days post-stroke (3DPS) and 5 days post-stroke (5DPS). *P*-value: Paired t-test comparing the mean pre-stroke values vs. each time point post-stroke.

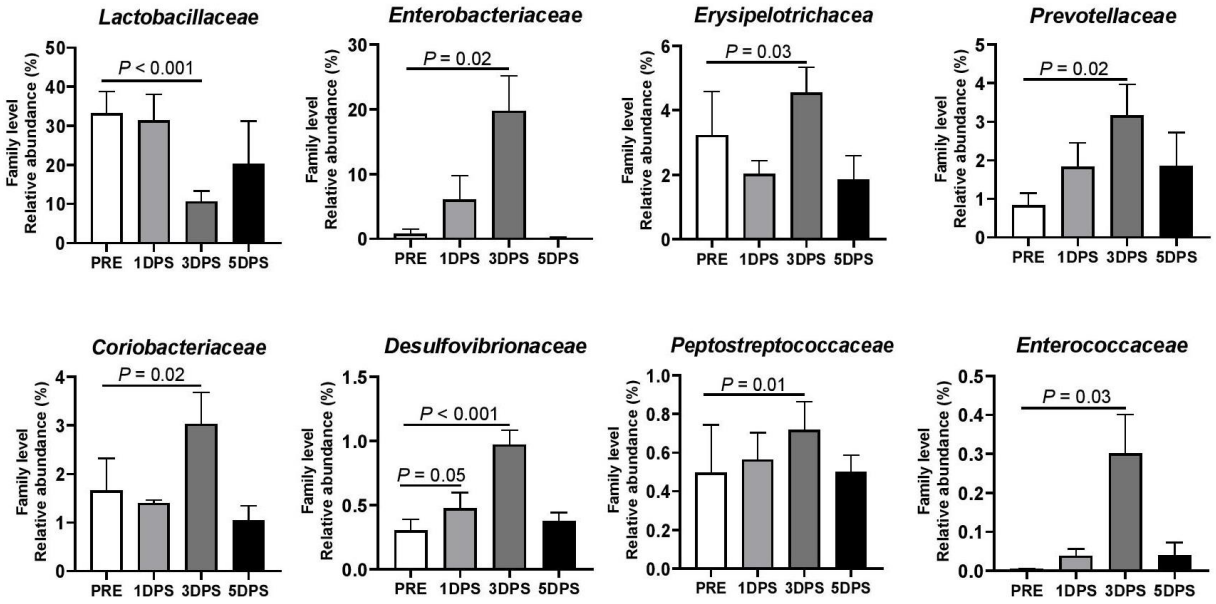


Figure 3.6. Stroke resulted in family level perturbations in gut microbiota between pre- and post-stroke in MCAO pig model. Bacterial families showed changes in abundance ($P \leq 0.05$) during the acute stage of stroke. *P*-value: Paired t-test comparing the mean pre-stroke values vs. each time point post-stroke. Pre-stroke (PRE, n=7), 1 day post-stroke (1DPS, n=7), 3DPS (n=6), 5DPS (n=4).

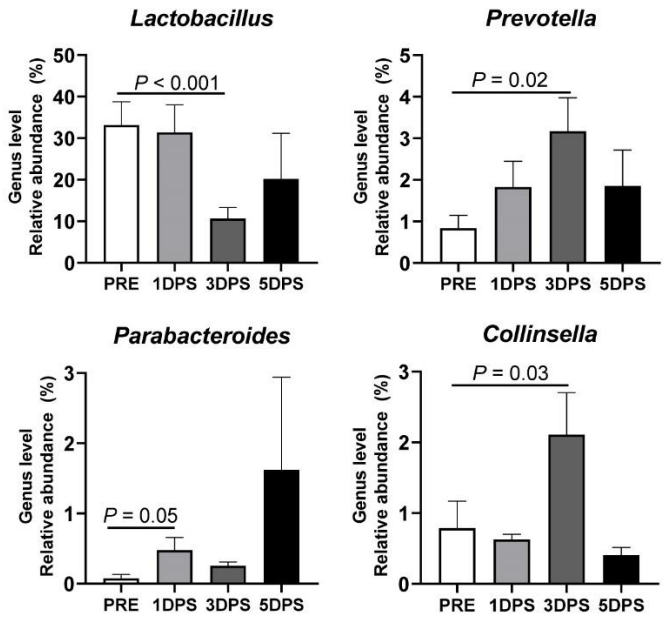
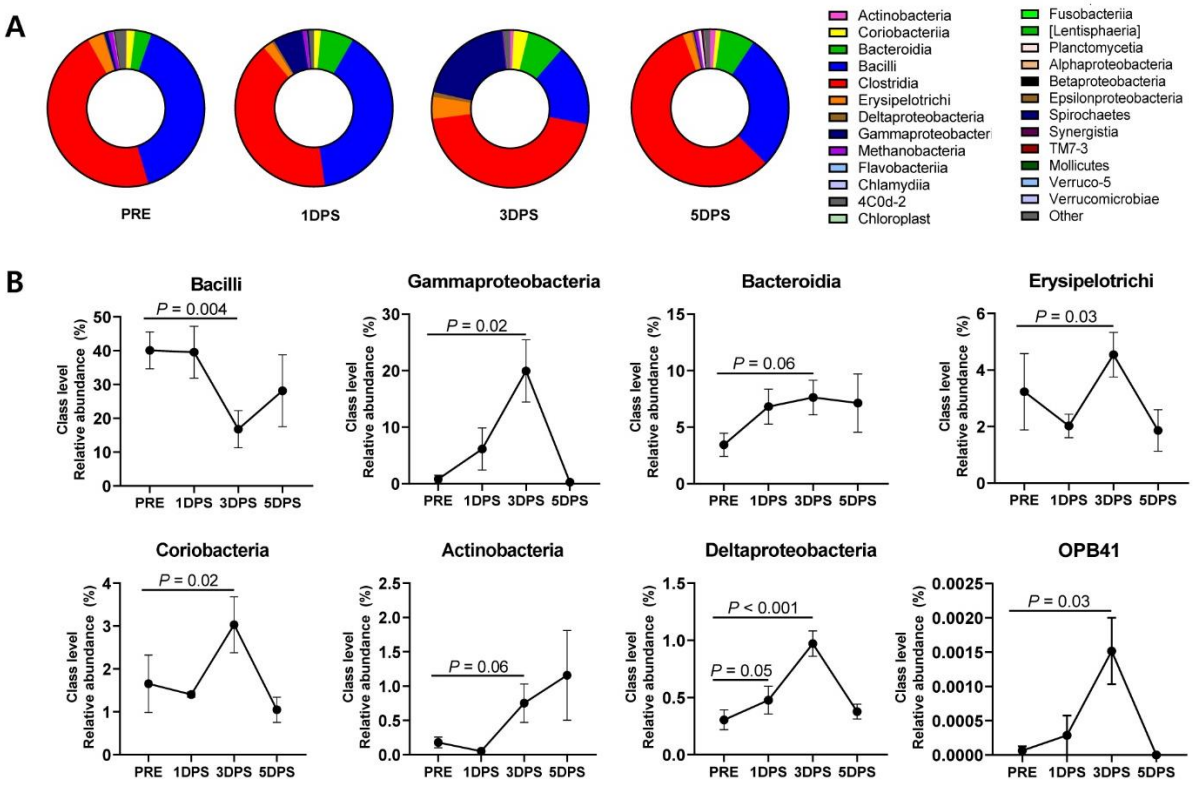
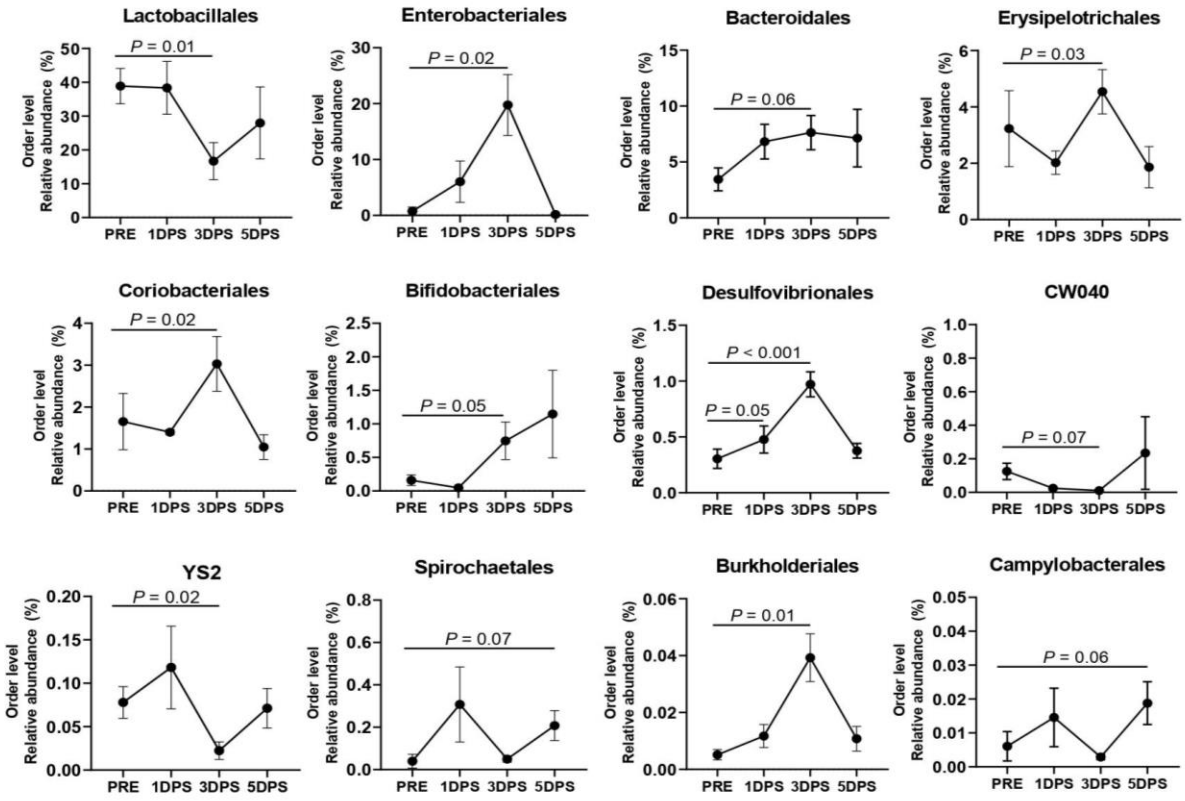


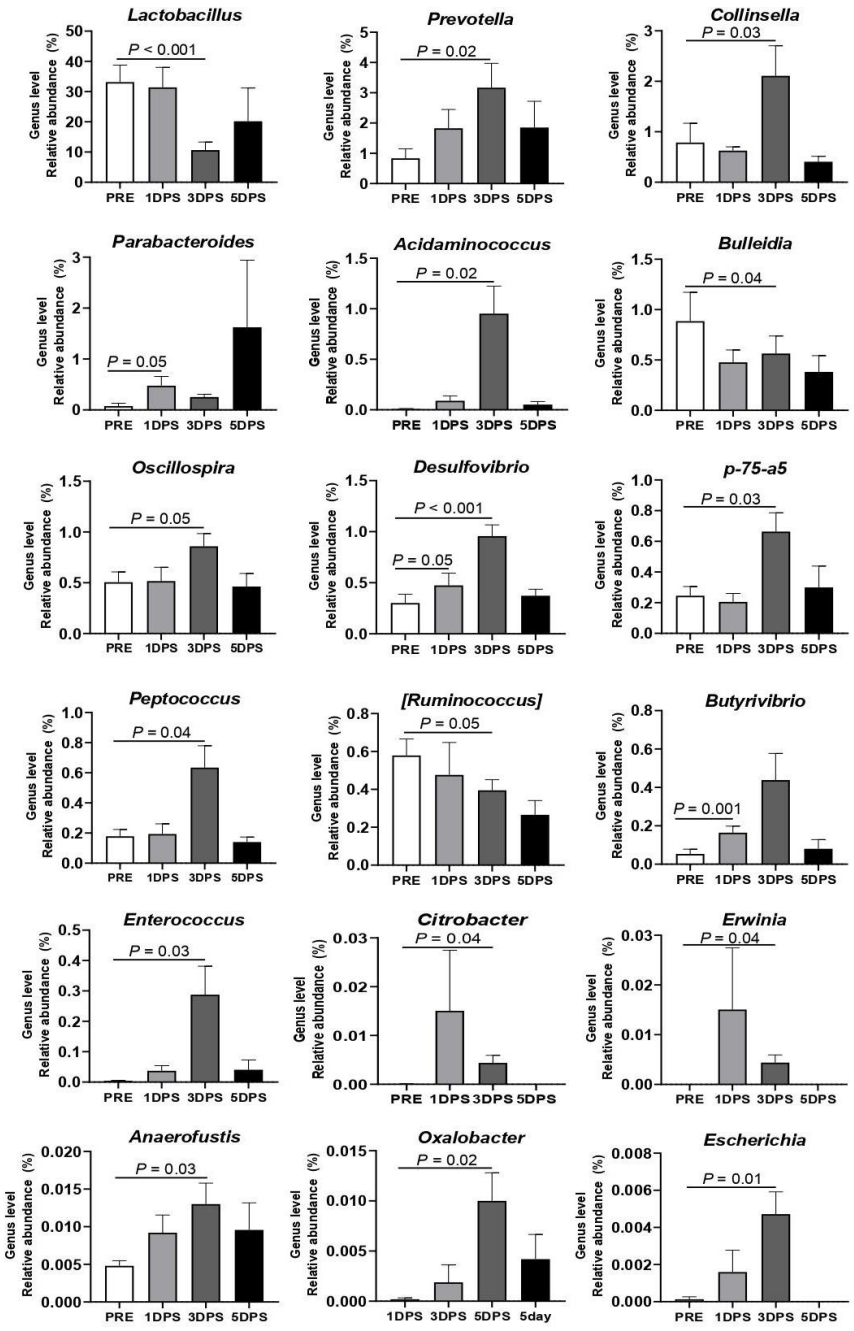
Figure 3.7. Stroke resulted in genus level perturbations in gut microbiota between pre- and post-stroke in MCAO pig model. The abundance of *Lactobacillius*, *Prevotella*, *Parabacteroides*, *Collinsella* showed >1% relative abundance and demonstrated a significant change in abundance during acute stroke. *P*-value: Paired t-test comparing the mean pre-stroke values vs. each time point post-stroke. Pre-stroke (PRE, n=7), 1 day post-stroke (1DPS, n=7), 3DPS (n=6), 5DPS (n=4).



Supplementary Figure 3.S1. Changes in gut microbiome were detected at the class level pre- and post-stroke. (A) Circle plots showed changes in mean relative abundance of all bacterial classes pre-stroke (PRE, n=7), 1 day post-stroke (1DPS, n=7), 3 days post-stroke (3DPS, n=6), and 5 days post-stroke (5DPS, n=4). (B) Bacterial classes showed changes in abundance ($P < 0.10$) during the acute stage of stroke. P-value: Paired t-test comparing the mean pre-stroke values vs. each time point post-stroke.



Supplementary Figure 3.S2. Changes in gut microbiome were detected at the order level pre- and post-stroke. Bacterial orders showed changes in abundance ($P < 0.10$) during the acute stroke stage. P-value: Paired t-test comparing the mean pre-stroke values vs. each time point post-stroke. PRE (n=7), 1DPS (n=7), 3DPS (n=6), 5DPS (n=4).



Supplementary Figure 3.S3. Changes in gut microbiome were detected at the genus level pre- and post-stroke. Bacterial genus showed changes in abundance ($P \leq 0.05$) during the acute stroke stage. P-value: Paired t-test comparing the mean pre-stroke values vs. each time point post-stroke. PRE (n=7), 1DPS (n=7), 3DPS (n=6), 5DPS (n=4).

CHAPTER 4[1]

TANSHINONE IIA-LOADED NANOPARTICLES AND NEURAL STEM CELL COMBINATION THERAPY IMPROVES GUT HOMEOSTASIS AND RECOVERY IN A PIG ISCHEMIC STROKE MODEL.

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1. Jeon JH, Kaiser EE, Waters ES, Yang X, Lourenco JM, Fagan MM, Scheulin KM, Sneed SE, Shin SK, Kinder HA, Kumar A, Platt SR, Ahn J, Duberstein KJ, Rothrock Jr M, Callaway TR, Xie J, West FD, and Park HJ. To be submitted to *Scientific Reports.*

Abstract

Impaired gut homeostasis is often associated with stroke with leaky gut syndrome and increased gut, brain, and systemic inflammation further exacerbating brain damage. We previously reported that intracisternal administration of Tanshinone IIA-loaded nanoparticles (Tan IIA-NPs) and transplantation of induced pluripotent stem cell-derived neural stem cells (iNSCs) lead to enhanced neuroprotective and regenerative activity and results in improved recovery in a pig stroke model. We hypothesized that the Tan IIA-NP+iNSC combination therapy mediated stroke recovery may also have an impact on gut inflammation and integrity in stroke pigs. Yucatan pigs were induced ischemic stroke and either received PBS (Control, n=6) or Tan IIA-NP+iNSC (Treatment, n=6) treatment. The Tan IIA-NP+iNSC treatment reduced protein expression of jejunal TNF-α, TNF-α receptor1, and phosphorylated IkBα and increased expression of jejunal occludin, claudin1, and ZO-1 at 12 weeks post-treatment (PT). Treated pigs had higher fecal SCFAs levels than their counterparts throughout the study period and fecal SCFAs levels were negatively correlated with jejunal inflammation. Interestingly, fecal SCFAs levels were negatively correlated with brain lesion volume and midline shift at 12 weeks PT. Collectively, the anti-inflammatory and neuroregenerative treatment at the brain level resulted in increased SCFAs levels and decreased inflammation and membrane permeability in the gut.

Introduction

Ischemic stroke is a major causes of morbidity and mortality in the United States, accounting for 150,005 deaths in 2019 [1]. Ischemic stroke not only directly effects the brain but has broad reaching effects including on the gastrointestinal (GI) system. In human stroke patients, up to 50% experience GI complications, including dysphagia, constipation, and GI dysmotility, which correlate with poor stroke outcomes such as increased hospital length of stay, mortality, and poor neurological outcomes [2, 3]. In preclinical animal model studies, ischemic stroke impairs gut motility [4] and increases gut inflammation [5] and mucosal damage, leading to elevated gut permeability and translocation of bacteria to the circulatory system [6, 7], as well as increased systemic inflammation [8]. These results highlight the strong relationship between the gut and the stroked brain.

The communication system between the gut and brain, the gut-brain axis, has proven to play a critical role in regulating brain function and gut homeostasis in neurological diseases such as stroke [9]. This bidirectional axis functions through neural pathways, the immune system, and the endocrine system [10, 11]. The stimulated vagus nerve and activated hypothalamus-pituitary-adrenal axis interact with enteric macrophage and dendritic cells, alter the gut function and integrity, leading to disruption of microbial composition [10, 11]. Microbes in the gut produce neurotransmitters, including serotonin, tryptophan, γ-aminobutyric acid (GABA), and microbial metabolites such as short-chain fatty acids (SCFAs), which enter the bloodstream and interact with the host's immune system, enteric nervous system and vagus nerve, transmitting the information to the brain [10, 11]. Previous studies have shown systemic administration of anti-inflammatory agents in stroke rodent models not only reduced brain lesion volume and neurological deficit, but also attenuated gut-, systemic- and neuro-inflammation [12, 13]. Moreover, administration of

healthy fecal microbiota to stroke mice significantly reduces infarct volume, brain edema and neurological impairment [14]. These findings highlight the dynamic interplay between the brain and gut through the gut-brain axis and the potential of this axis to be a novel therapeutic target to improve stroke outcomes.

The primary stroke injury leads to significant cell death and a secondary injury cascade of free radical formation and activation of a robust immune response that causes a cycle of increasing tissue damage in the brain [15, 16]. Targeting this secondary injury cascade has been a major focus of stroke therapeutic development. Recently, our research team [17] demonstrated that Tanshinone IIA (Tan IIA), an antioxidant and anti-inflammatory agent [18, 19], administration in a poly lactic-co-glycolic acid nanoparticle (Tan IIA-NPs) to neural cells suppressed oxidative stress and inflammation [17]. In an ischemic stroke pig model, the intracisternal administration of Tan IIA-NPs reduced hemispheric swelling, midline shift, and ischemic lesion volumes and improved functional deficits including changes in key spatiotemporal and kinetic gait parameters during the acute stroke stage [17]. Our research team also recently demonstrated that induced pluripotent stem cell derived neural stem cells (iNSCs) transplanted into the stroke brain can replace damaged and dead neurons and glia, improving white matter integrity, cerebral blood perfusion and brain metabolism in ischemic stroke pig model [20]. Interestingly, combination of intracisternal administration of Tan IIA-NPs and iNSCs therapy improved efficacy and engraftment of iNSCs, and enhanced brain tissue recovery and neurological function during the chronic stages of ischemic stroke in the porcine model (Kaiser et al., under review, 2022). However, questions remain as to how brain-targeted stroke therapies alter the GI system and potentially inhibit GI induced systemic and secondary inflammation in the stroke brain.

In the present study, we aim to investigate how ischemic stroke and local treatment of Tan IIA-NP+iNSC combination therapy in the brain alters gut homeostasis and gut integrity in a porcine ischemic stroke model. The pig is a robust translational animal model with similar brain and GI anatomy and physiology to humans, making pigs an ideal model for future clinical studies [21-24]. The findings from the translational pig model will provide a better understanding of the bidirectional communication between the brain and gut and inform future efforts to develop stroke therapeutics.

Materials and Methods

Animal information

All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals guidelines and were approved by the University of Georgia (UGA) Institutional Animal Care and Use Committee (IACUC; Protocol Number: 2017–07–019Y1A0). Castrated male Yucatan pigs (9-month-old, 31-41 kg body weight) were enrolled in this study. Pigs were individually housed in a 12 hour light-dark cycle with a room temperature of approximately 27 ºC. All pigs had free access to water and were fed standard grower diets.

Treatment and group information

The experimental study design is summarized in **Figure 1.** Pigs underwent middle cerebral artery occlusion surgery (MCAO) and received either PBS (Control group n=6) or Tan IIA loaded PLGA nanoparticle (Tan IIA-NPs) and iNSCs treatment (Treatment group, n=6). PBS or Tan IIA-NPs were administered intracisternal at 1 hour post-stroke and PBS or iNSCs were transplanted into the perilesional region at 5 days post-stroke. Tan IIA-NPs were treated before the administration

of iNSCs treatment to reduce the cytotoxic environment following stroke, thus increasing the survivability and effectiveness of iNSCs in the brain.

Preoperative, perioperative, and post-operative procedures

1 day prior to surgery, Excede (5mg/kg, intramuscular (IM)) and fentanyl patches (100mg/kg/hr, transdermal (TD)) were provided to pigs to prevent infection and treat pain. On the day of surgery, analgesia and sedation were obtained using xylazine (2mg/kg, IM) and midazolam (0.2mg/kg, IM). Propofol (10mg/ml, intravenous (IV)) and prophylactic lidocaine (2%, topically to the laryngeal folds) were applied to allow endotracheal intubation. Lactated Ringer's solution (5mL/kg/hour, IV) was given to maintain hydration. Isoflurane (Abbott Laboratories) in oxygen was used to maintain anesthesia. MCAO surgery was performed as described previously [27]. A curvilinear incision was made from the superior right orbit to the rostral side of the auricle. The temporalis muscle was retracted, and the exposed local dura mater was excised. Using bipolar electrocautery forceps, the middle cerebral artery at the distal part of the Circle of Willis was permanently occluded. After the stroke operation, pigs were returned to their pen, and tube intubation was removed. Pigs were closely watched every 15 mins until heart rate, respiration, and the temperature returned to normal. Pigs were then monitored every 4 hours for 24 hours and twice a day during the experiment thereafter. Flunixin meglumine (2.2mg/kg, IM) was given every 12 hours for the first 24 hours and every 24 hours for the next three days for post-stroke pain and fever control.

Brain volumetric changes

MRI was conducted (General Electric 3.0 Tesla MRI system) 1 day post-stroke to confirm the ischemic stroke (Data not shown) and brain volumetric changes including lesion volume and

midline shift were measured at 12 weeks post-treatment as indicator of stroke recovery. The MRI results of the same cohort of pigs have been recently published by Kaiser et al (Kaiser et al., under review, 2022).

Fecal collection

Fecal samples were collected at pre-stroke (Pre-stroke), 1, 3, 5 days post-stroke (Acute1), 1, 3 days post-treatment (Acute2), 1, 2, 4 weeks post-treatment (Subacute), and 6, 9, 12 weeks post-treatment (Chronic). Each stroke stage was designated based on previously identified and classified human stroke stages [25, 26]. To avoid any contamination during fecal collection, all materials were sterilized before sample collection with 70% alcohol. The pig anus was softly stimulated by a sterilized fecal loop and the feces were collected into a 50ml tube without touching the floor or body. After aliquoting the feces into three 2ml tubes, they were immediately frozen on dry ice and stored at -80°C before further analysis.

Short-chain fatty acids (SCFAs) analysis

Fecal sample SCFAs were analyzed as before [28]. Fecal samples were suspended in water (1:3 = fecal mg : water ml) and mixed using a vortex. The fecal suspension was centrifuged at 10,000g for 10 minutes and 1mL of supernatant was pipetted into a new centrifuge tube containing 200ul of a metaphosphoric acid solution (25% wt/vol) before overnight freezing. Samples were then thawed and centrifuged at 10,000g for 10 minutes. The supernatant was transferred to a polypropylene tube and mixed with ethyl acetate (2:1 = ethyl acetate : supernatant). The tubes were vortexed for 15 seconds before settling for 5 minutes. Following that, 500ul of the upper suspension was transferred to screw-thread vials for SCFAs analysis with a Shimadzu GC-2010

Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) with a flame ionization detector and a capillary column (Zebron ZB-FFAP; 30m x 0.32mm x 0.25μm; Phenomenex Inc., Torrance, CA, USA). The injection volume of the sample was 1.0ug and helium was used as the carrier gas. The column temperature was initially set at 110°C and raised to 200°C. The injector and detector temperatures were set at 250°C and 350°C, respectively.

Tissue collection

At 12 weeks post-treatment, distal jejunum (about 200cm before the end of the ileum) tissues were collected and scraped using glass slides. The samples were immediately frozen in liquid nitrogen and then stored at -80°C until further analysis.

ELISA assay

The proinflammatory cytokine tumor necrosis factor-alpha (TNF-α) was quantified by Porcine Quantikine ELISA (R&D company, Minneapolis, MN, USA, Cat. PTA00) in jejunal homogenate samples according to the manufacturer's instructions.

Western blot analysis

The distal jejunum tissue was collected and scraped with a glass slide. 50 ~ 100mg of scraped jejunal mucosa was added in 500ul of Radioimmunoprecipitation Assay (RIPA) lysis buffer (Santa Cruz Biotechnology, CA, USA) and homogenized. Samples were then centrifuged at 10,000g for 20 minutes at 4°C twice to collect the clear supernatant. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Thermo Fisher, Rockford, IL, USA) following the manufacturer's protocol. 25ug protein was loaded on either 10% or 16% Tris-glycine gel

(Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA). Membranes were blocked in either LICOR blocking buffer (LI-COR, Lincoln, NE, USA) or 5% non-fat skim milk for 1 hour at room temperature prior to the addition of primary antibodies: Occludin (1:1000, Abcam, ab31721), Claudin1 (1:1000, Abcam, ab15098), Zonula occludens-1 (ZO-1) (1:1000, Abcam, ab214228), TNF-α receptor 1 (TNFR1) (1:1000, Abcam, ab19139), IkBα (1:1000, Cell signaling, #4814), p-IkBα (1:200, Cell signaling, #9246), and beta-actin (1:10,000, Sigma Aldrich, #A5441-100UL) overnight at 4°C. After washing the membrane with tris-buffered saline (TBS) with tween T20 (TBS-T) buffer 4 times for 5 minutes, the secondary antibody, IRDye 800CW goat anti-rabbit IgG (1:10,000, LI-COR, #926-32211) and IRDye 680RD goat anti-mouse IgG (1:10,000, LI-COR, #926-68070) were incubated at room temperature for 1 hour. Then, the membrane was washed with TBS-T 4 times for 5 minutes and stored in PBS until the image detection. The membrane was imaged using the Odyssey imaging system (LI-COR, Lincoln, NE, USA) and the images were analyzed by ImageJ software (v8.4.3, San Diego, CA, USA). Beta-actin was used as a housekeeping gene and the relative abundance of target proteins to beta-actin was shown.

NF-kB P65 binding activity assay

Nuclear protein extraction was performed in jejunum tissue using a Nuclear Extract Kit (Active Motif, Carlsbad, CA) and NF-kB P65 binding activity was measured using TransAM^{TM} NF-kB P65 transcription factor assay kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions.

Statistical analysis

Longitudinal changes in fecal SCFAs levels were evaluated with a multivariable quadratic regression model to investigate the differences between the groups (MATLAB). Western blot, ELISA, and NF-kB P65 binding activity data were analyzed by GraphPad Prism software (v8.4.3, San Diego, CA, USA) using unpaired t-test between the control and the treatment group. Data are presented as mean ± standard error of the mean (S.E.M). Pearson correlations was performed to evaluate associations between fecal SCFAs level, gut inflammation, expressions of gut tight junction protein, and brain volumetric changes.

Results

Tan IIA-NP+iNSC treatment reduced inflammation and improved expression of tight junction proteins in jejunal mucosa 12 weeks post-treatment.

To evaluate the effect of the Tan IIA-NP+iNSC combination therapy on gut inflammation, protein expression of TNF-α, TNF-α receptor1 (TNFR1), and binding activity of NF-kB P65 were examined in jejunal scrapings at 12 weeks post-treatment (PT) (**Figure 2**). The Tan IIA-NP+iNSC group showed reduced protein levels of proinflammatory cytokine TNF-α by 54.91% (Control 4.68 ± 0.99 vs Treatment 2.11 ± 0.48, $P = 0.042$, **Figure 2A**) and its receptor TNFR1 by 28.57% (Control 0.70 ± 0.08 vs Treatment 0.50 ± 0.04, $P = 0.045$, **Figure 2B**) compared to the control group. NF-kB plays an important role in regulating the inflammatory response and it induces inflammation through IkBα phosphorylation, an NF-kB inhibitor, and activation of transcriptional factors such as P65 [29]. Interestingly, Tan IIA-NP+iNSC treatment also reduced protein levels of phosphorylated IkBα (Control 0.33 ± 0.01 vs Treatment 0.25 ± 0.02, $P = 0.002$, **Figure 2C**)

compared to the control group. Binding activity of NF-kB P65 showed a decreasing trend in the treated group relative to the control group (Control 0.80 ± 0.12 vs Treatment 0.56 ± 0.04, 0.1 > *P* > 0.05, **Figure 2D)**. These results suggest that Tan IIA-NP+iNSC therapy reduces the levels of proinflammatory cytokine and activation of NF-kB signaling in the pig ischemic stroke model.

To investigate the effect of the Tan IIA-NP+iNSC combination therapy on gut integrity, protein expressions of gut tight junction proteins occludin, claudin1, and ZO-1 were measured in jejunal mucosa at 12 weeks PT (**Figure 2E**). The results showed that Tan IIA-NP+iNSC treated animals had 1.5-fold higher protein expression of occludin (Control 0.28 ± 0.06 vs Treatment 0.43 ± 0.03, *P* = 0.043), a 1.6-fold higher expression level of claudin 1 (0.56 ± 0.07 vs 0.92 ± 0.08, *P* = 0.008), and a 2.4-fold higher expression level of ZO-1 (0.11 ± 0.03 vs 0.26 ± 0.05, *P* = 0.016) compared to that of the control group. These results indicate that the Tan IIA-NP+iNSC treatment preserves gut tight junction protein expression following stroke.

Gut inflammation negatively correlated with gut tight junction protein expression at 12 weeks post-treatment.

To further evaluate the association between the gut inflammatory response and gut integrity, correlations were assessed between proinflammatory cytokines TNF-α and TNFR1 and gut tight junction proteins occludin, claudin1, and ZO-1 12 weeks PT in jejunal mucosa samples (**Figure 2F**). Increased protein levels of TNF-α (claudin1: r = - 0.5886, *P* = 0.044, ZO-1: r = - 0.7495, *P* = 0.005) and TNFR1 (occludin: r = - 0.7878, *P* = 0.002, claudin1: r = - 0.7237, *P* = 0.008 , ZO-1: r = - 0.5201, *P* = 0.083) were negatively correlated with gut tight junction protein expression, suggesting the detrimental effects of TNF-α on gut integrity. Increased protein levels of phosphorylated IkBα (p-IkBα/total IkBα) (claudin1: r = -0.5081, *P* = 0.092, ZO-1: r = -0.5645, *P*

= 0.056) and NF-kB P65 binding activity (ZO-1: r = - 0.5332, *P* = 0.074) showed a trending negative correlation with tight junction protein expression although not statistically significant. Interestingly, protein levels of TNF-α were positively correlated with TNFR1 (r = 0.5751, *P* = 0.050), and high TNFR1 levels were associated with elevated NF-kB P65 binding activity (r = 0.5692, *P* = 0.053). NF-kB P65 binding activity had a trending positive correlation with phosphorylation of IkBα (r = 0.4993, *P* = 0.098), indicating an association between the TNF-α, TNFR1, and NF-kB signaling. Collectively, these results suggest that the decrease in tight junction protein expressions in gut epithelial cells following stroke may be mediated by the proinflammatory cytokine TNF-α and NF-kb pathway in ischemic stroke.

Tan IIA-NP+iNSC therapy altered fecal SCFAs levels in an ischemic stroke pig model.

Longitudinal changes in fecal SCFAs level were analyzed pre-stroke and up to 12 weeks PT. Changes in fecal SCFAs levels from baseline (pre-stroke values) at each time point of stroke including Acute1 (1-5 days post-stroke), Acute2 (1-3 days PT), Subacute (1-4 weeks PT), and Chronic (6-12 weeks PT) were calculated and shown in **Figure 3**). There were significant differences in SCFAs between the Tan IIA-NP+iNSC treated animals and control animals with the Tan IIA-NP+iNSC treated animals consistently demonstrating higher levels of SCFAs relative to non-treated control animals (**Figure 3**). Particularly, the levels of fecal total SCFAs (*P* = 0.003) and acetate (*P* = 0.002) were significantly higher in the treatment group compared to the control group up to 12 weeks PT. The changes in fecal propionate levels (*P* = 0.059) showed trending higher levels in the treatment group than in the control group, whereas the changes in fecal butyrate, isobutyrate, isovalerate, and valerate levels had no significant differences between groups post-

stroke ($P > 0.05$). These results show that Tan IIA-NP+iNSC combination therapy leads to increased intestinal levels of SCFAs during post-stroke recovery.

Changes in fecal SCFAs levels during stroke recovery correlated with jejunal inflammation and ZO-1 expression 12 weeks post-treatment.

To assess the association between the gut inflammatory response and gut integrity with SCFAs levels, protein levels of TNF-α, TNFR1, phosphorylation of IkBα, gut tight junction proteins, and binding activity of NF-kB P65 at 12 weeks PT were correlated with changes in fecal SCFAs levels post-stroke. Changes in fecal total SCFAs and acetate levels at Subacute (1-4 weeks PT) were negatively correlated with the protein levels of TNF-α and phosphorylated IkBα (**Table 1**), and positively correlated with the expression of gut tight junction protein ZO-1 at 12 weeks PT (**Figure 4**). Changes in fecal isobutyrate and isovalerate levels at Acute 2 (1-3 days PT) and Subacute (1-4 weeks PT) stages were also negatively correlated with the protein levels of phosphorylated IkBα at 12 weeks PT (**Table 1**). These results indicate that increased SCFA levels in the gut at early stages PT (1 day to 4 weeks PT) are related to reduced gut inflammation and improved gut integrity in later stroke stages. Interestingly, changes in fecal butyrate levels at the Chronic stage (6-12 weeks PT) had a significant positive correlation with protein levels of TNF-α, TNFR1, and NF-kB P65 binding activity at 12 weeks PT. Collectively, changes in SCFAs levels in the gut following stroke may be related to levels of gut inflammation and membrane permeability over an extended period.

The jejunal protein levels of phosphorylated IkBα correlate with lesion volume and midline shift changes 12 weeks post-treatment.

To investigate the association between the gut inflammatory response and stroke brain volumetric changes, protein levels of TNF-α, TNFR1, phosphorylated IkBα, gut tight junction proteins, and binding activity of NF-kB P65 at 12 weeks PT were correlated with MRI lesion volume and midline shift results at 12 weeks PT (**Figure 5**). Lesion volume ($r = 0.7944$, $P = 0.0020$) and midline shift ($r = 0.5353$, $P = 0.0729$) were positively correlated with the protein levels of phosphorylated IkBα in gut at 12 weeks PT (**Figure 5**). This result suggests that elevated gut inflammation may be associated with cerebral tissue damage.

Changes in fecal SCFAs levels post-stroke correlated with lesion volume and midline shift changes 12 weeks post-treatment.

Previously, our research team reported that intracisternal administration of Tan IIA-NPs and iNSCs transplantation therapy reduced brain tissue damage 12 weeks PT compared to the non-treated control group (Kaiser et al, under review, 2022). In this study, we investigated how changes in SCFAs levels in the gut following treatment are linked to intracerebral stroke damage. To examine the relationship between SCFA levels in the gut and stroke brain volumetric changes, changes in fecal SCFAs levels post-stroke were correlated with stroke lesion volume and midline shift MRI results at 12 weeks PT (**Table 2**). The changes in fecal total SCFAs, acetate, isobutyrate, isovalerate, and valerate levels at Acute 2 (1-3 days PT) had a negative correlation with lesion volume at 12 weeks PT (**Table 2**). The changes in fecal total SCFAs, acetate, isobutyrate, and isovalerate levels at Acute 2 (1-3 days PT) also had an inverse correlation with midline shift at 12

weeks PT. Collectively, these results show that changes in fecal SCFAs levels post-stroke may affect stroke recovery.

Discussion

This current study demonstrated that the Tan IIA-NP+iNSC combination therapy in the stroke brain modulated gut homeostasis in a MCAO ischemic stroke pig model, indicated by reduced pro-inflammatory cytokine level, improved tight junction protein expression in the jejunum, and increased fecal SCFAs levels. Moreover, higher fecal SCFAs levels post-stroke were associated with decreased brain lesion volume and midline shift. The findings from this study suggest that the modulation of stroke brain with the anti-inflammatory and neuroregenerative Tan IIA-NP+iNSC treatment resulted in an improved gut inflammatory status and integrity via the brain-gut axis interactions in a pig model of ischemic stroke.

The pro-inflammatory immune response is a key contributor to stroke sequelae and resulting pathology [30]. During stroke, damaged and dying neural cells release damaged-associated molecular patterns (DAMPs) that activate microglia and a astrocytes in the brain lesion site and enhance recruitment and parenchymal invasion of leukocytes from peripheral circulation [31]. Recently, our research team demonstrated that local treatment with Tan IIA-NP+iNSC in the stroke brain reduced the number of microglial and infiltrating immune cells in the brain, maintained endogenous neurons, and enhanced populations of endogenous neural stem cells in the brain in the pig model (Kasier et al., under review, 2022). Activated microglia and macrophages release proinflammatory cytokines such as TNF-α and IL-1β in the brain [32]. The released DAMPs and these cytokines can enter systemic circulation through the impaired blood-brain

barrier and the cerebrospinal fluid (CSF) drainage system [33]. Therefore, the anti-inflammatory effect of Tan IIA-NP+iNSC on the intracerebral immune response may prevent increased systemic and gut inflammation. This is supported by the present study as the Tan IIA-NP+iNSC treatment led to reduced protein levels of proinflammatory cytokines (e.g. TNF-α) and improved tight junction protein expression levels in the gut.

Inflammatory processes are closely related to the gastrointestinal dysfunction, such as compromised gut permeability [34]. Increased gut permeability can allow harmful antigens and microorganisms to enter systemic circulation from the intestinal lumen, resulting in abnormal immune reactions, therefore changes in intestinal permeability is an important indicator of gut health [35]. Previous studies showed that administration of proinflammatory cytokines IL-1β and TNF-α to Caco-2 cells *in vitro* increased epithelial permeability in does- and time- dependent manner [36, 37]. Therefore, these results suggest a crucial role for proinflammatory cytokine in regulating intestinal barrier function. Interestingly, the present study reported that treatment with Tan IIA-NP+iNSC reduced the protein levels of TNF-α and TNFR1 and increased the protein expression of occludin, claudin1, and ZO-1 in the gut. It has been suggested that TNF-α mediates the expression of tight junction proteins through a specific molecular mechanism [39]. Briefly, TNF-α binds to the TNF- α receptor in the epithelial cells and stimulates the NF-kB signaling pathway by increasing myosin light chain kinase (MLCK) gene expression [39, 40]. MLCK then phosphorylates MLC and phosphorylated MLC induces contraction of the cytoskeletal structure attached to the tight junction proteins, increasing the gut permeability [39-41]. Similar with this finding, our study reported that protein levels of jejunal TNF-α were positively correlated with the protein TNFR1, both of which were negatively correlated with gut tight junction protein

expression. Therefore, the findings from this study indicate that intestinal TNF-α levels will be an important factor in regulating tight junction-mediated gut permeability in this MCAO pig model.

The transcriptional factor NF-kB is a critical mediator of inflammatory stimuli [29]. NF-kB is a family of inducible transcription factors such as NF-B1 (p50), NF-B2 (p52), and RelA (p65), and they bind to specific region of DNA known as enhancer and influence the gene expression [42]. In general, NF-kB proteins are sequestered in the cytoplasm by IkB family, an inhibitory proteins; however, when the NF-kB pathway is activated and IkB is phosphorylated, the NF-KB transcriptional factors are released and enter the cytoplasm to promote gene expression [43]. The present study showed that the intestinal binding activity of NF-kB P65 was lower in Tan IIA-NP+iNSC treated animals than in non-treated animals, although it was not statistically significant. The NF-kB P65 binding activity had a trending positive correlation with the protein levels of phosphorylated IkB-α, suggesting the involvement of NF-kb pathway in this study. As mentioned in the paragraph above, the activated NF-kb pathway can be induced by proinflammatory cytokines such as TNF-α and elevate MLCK gene expression, thereby reducing the expression of gut tight junction proteins in epithelial cells [44, 45]. Similarly, the present study found that the binding activity of NF-kB P65 also tended to correlate positively with the protein levels of TNFR1, and negatively with ZO-1 in the gut. These results propose that TNF-α induced NF-kB signaling may be a main pathway for modulating gut integrity in this ischemic stroke pig model.

SCFAs are a major metabolite produced by the gut microbe when undigested carbohydrate are fermented and mainly consists of acetate, propionate, and butyrate. In the intestinal tract, SCFAs regulate gut epithelial barrier function, integrity, and immune response [46], and also play a crucial role as a mediator in gut-brain interaction [10, 11]. The present study showed that pigs

who received Tan IIA-NP+iNSC therapy had increased changes in fecal SCFAs levels throughout the study period, and these changes were not only correlated with reduced gut inflammation and improved gut integrity, but also associated with reduced lesion volumes and midline shift 12 weeks post-treatment. SCFAs can be absorbed by colonocytes and interact with intestinal immune cells, as well as cross the blood brain barrier through the circulatory system and modulate astrocyte activation and microglia maturation in the brain [47-49]. Previous studies reported that increased SCFAs levels in the gut, brain, and plasma through fecal transplantation reduced post-stroke neurological deficit and neuroinflammation, suggesting that intestinal SCFAs may affect the brain through blood circulation [50]. Moreover, SCFAs supplementation in drinking water improved post-stroke recovery by increasing synaptic plasticity and cortical reorganization and reducing microglial activation and frequency of T cells in the brain ischemic stroke mice [51], indicating a significant effect of SCFAs on recovery mechanisms in the brain. These previous studies support the findings of the current study that changes in SCFAs levels following Tan IIA-NP+iNSC treatment are likely associated with improved stroke pathological recovery.

The most abundant SCFAs in all groups are acetate, propionate, and butyrate, and have been widely investigated on their effects on host health [46]. Branched short-chain fatty acids (BCFAs), such as isobutyrate and isovalerate are produced in lower amounts by gut microbes that ferment branched chain amino acids [52]. The impact of BCFAs on host health is largely unknown. Interestingly, the present study found that changes in fecal levels of isobutyrate and isovalerate levels during the acute stage of stroke were negatively correlated with lesion volume and midline shift changes in the chronic stroke stage. Similarly, Zhang and Chen reported that stroke rodent models showed increased fecal isobutyrate and isovalerate levels in the early stage of stroke [12, 14], while decreased fecal levels of butyrate [12] or acetate and propionate [14] post-stroke.

Increased BCFAs levels in the early stage of stroke may be due to a compensation mechanism after the depletion of the major SCFAs caused by gut dysbiosis. Therefore, these findings suggest that BCFAs may play an important role in regulating stroke recovery, however, given the lack of studies evaluating BCFAs changes and their effect on stroke, further studies are needed to investigate a potential beneficial role of BCFAs in human health.

Conclusion

The findings of this study demonstrate that Tan IIA-NP+iNSC therapy decreased jejunal inflammation and membrane permeability and increased fecal SCFAs levels in the gut. This foundational study indicates that brain-targeted neuroprotective and regenerative stroke therapies enhance gut homeostasis through gut-brain interaction and results in decreased stroke pathology that is associated with improved stroke recovery in human patients.

References

1. Tsao, C.W., et al., *Heart Disease and Stroke Statistics-2022 Update: A Report From the American Heart Association.* 2022. **145**(8): p. e153-e639.
2. Schaller, B.J., R. Graf, and A.H. Jacobs, *Pathophysiological Changes of the Gastrointestinal Tract in Ischemic Stroke.* Official journal of the American College of Gastroenterology | ACG, 2006. **101**(7).
3. Camara-Lemarroy, C.R., B.E. Ibarra-Yruegas, and F. Gongora-Rivera, *Gastrointestinal complications after ischemic stroke.* Journal of the Neurological Sciences, 2014. **346**(1): p. 20-25.
4. Singh, V., et al., *Microbiota Dysbiosis Controls the Neuroinflammatory Response after Stroke.* J Neurosci, 2016. **36**(28): p. 7428-40.
5. Ye, D., et al., *Exploratory Investigation of Intestinal Structure and Function after Stroke in Mice.* Mediators of Inflammation, 2021. **2021**: p. 1315797.
6. Crapser, J., et al., *Ischemic stroke induces gut permeability and enhances bacterial translocation leading to sepsis in aged mice.* Aging (Albany NY), 2016. **8**(5): p. 1049-63.
7. Stanley, D., et al., *Translocation and dissemination of commensal bacteria in post-stroke infection.* Nat Med, 2016. **22**(11): p. 1277-1284.
8. Chen, Y., et al., *Persistence of Gut Microbiota Dysbiosis and Chronic Systemic Inflammation After Cerebral Infarction in Cynomolgus Monkeys.* Frontiers in Neurology, 2019. **10**(661).
9. Ma, Q., et al., *Impact of microbiota on central nervous system and neurological diseases: the gut-brain axis.* Journal of Neuroinflammation, 2019. **16**(1): p. 53.

10. Cryan, J.F. and T.G. Dinan, *Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour.* Nature Reviews Neuroscience, 2012. **13**(10): p. 701-712.

11. Morais, L.H., H.L. Schreiber, and S.K. Mazmanian, *The gut microbiota–brain axis in behaviour and brain disorders.* Nature Reviews Microbiology, 2021. **19**(4): p. 241-255.

12. Zhang, P., et al., *Atorvastatin alleviates microglia-mediated neuroinflammation via modulating the microbial composition and the intestinal barrier function in ischemic stroke mice.* Free Radical Biology and Medicine, 2021. **162**: p. 104-117.

13. Zhang, F., et al., *Protective Effect of Tong-Qiao-Huo-Xue Decoction on Inflammatory Injury Caused by Intestinal Microbial Disorders in Stroke Rats.* Biological and Pharmaceutical Bulletin, 2020. **43**(5): p. 788-800.

14. Chen, R., et al., *Transplantation of fecal microbiota rich in short chain fatty acids and butyric acid treat cerebral ischemic stroke by regulating gut microbiota.* Pharmacological Research, 2019. **148**: p. 104403.

15. Carmichael, S.T., *Cellular and molecular mechanisms of neural repair after stroke: Making waves.* Annals of Neurology, 2006. **59**(5): p. 735-742.

16. Arai, K., et al., *Cellular mechanisms of neurovascular damage and repair after stroke.* Journal of child neurology, 2011. **26**(9): p. 1193-1198.

17. Waters, E.S., et al., *Intracisternal administration of tanshinone IIA-loaded nanoparticles leads to reduced tissue injury and functional deficits in a porcine model of ischemic stroke.* IBRO neuroscience reports, 2021. **10**: p. 18-30.

18. Song, Z., et al., *Tanshinone IIA Protects Against Cerebral Ischemia Reperfusion Injury by Regulating Microglial Activation and Polarization via NF-κB Pathway.* Frontiers in Pharmacology, 2021. **12**.

19. Liu, C., *Improvement effects of tanshinone on the impaired cognition and motor function in MCAO mice with ischemic penumbra.* Pak J Pharm Sci, 2021. **34**(3(Special)): p. 1211-1216.

20. Baker, E.W., et al., *Induced Pluripotent Stem Cell-Derived Neural Stem Cell Therapy Enhances Recovery in an Ischemic Stroke Pig Model.* Scientific reports, 2017. **7**(1): p. 10075-10075.

21. Tanaka, Y., et al., *Experimental model of lacunar infarction in the gyrencephalic brain of the miniature pig: neurological assessment and histological, immunohistochemical, and physiological evaluation of dynamic corticospinal tract deformation.* Stroke, 2008. **39**(1): p. 205-12.

22. Gieling, E.T., et al., *The Pig as a Model Animal for Studying Cognition and Neurobehavioral Disorders*, in *Molecular and Functional Models in Neuropsychiatry*, J.J. Hagan, Editor. 2011, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 359-383.

23. Heinritz, S.N., R. Mosenthin, and E. Weiss, *Use of pigs as a potential model for research into dietary modulation of the human gut microbiota.* Nutrition Research Reviews, 2013. **26**(2): p. 191-209.

24. Gonzalez, L.M., A.J. Moeser, and A.T. Blikslager, *Porcine models of digestive disease: the future of large animal translational research.* Transl Res, 2015. **166**(1): p. 12-27.

25. Allen, L.M., et al., *Sequence-specific MR Imaging Findings That Are Useful in Dating Ischemic Stroke.* 2012. **32**(5): p. 1285-1297.

26. Iyer, K.K., *Effective assessments of electroencephalography during stroke recovery: contemporary approaches and considerations.* 2017. **118**(5): p. 2521-2525.

27. Platt, S.R., et al., *Development and characterization of a Yucatan miniature biomedical pig permanent middle cerebral artery occlusion stroke model.* Exp Transl Stroke Med, 2014. **6**(1): p. 5.

28. Lourenco, J.M., et al., *Comparison of the ruminal and fecal microbiotas in beef calves supplemented or not with concentrate.* PloS one, 2020. **15**(4): p. e0231533-e0231533.

29. Liu, T., et al., *NF-κB signaling in inflammation.* Signal Transduction and Targeted Therapy, 2017. **2**(1): p. 17023.

30. Iadecola, C., M.S. Buckwalter, and J. Anrather, *Immune responses to stroke: mechanisms, modulation, and therapeutic potential.* The Journal of Clinical Investigation, 2020. **130**(6): p. 2777-2788.

31. Dabrowska, S., et al., *Neuroinflammation as a target for treatment of stroke using mesenchymal stem cells and extracellular vesicles.* Journal of neuroinflammation, 2019. **16**(1): p. 178-178.

32. Clausen, B.H., et al., *Interleukin-1beta and tumor necrosis factor-alpha are expressed by different subsets of microglia and macrophages after ischemic stroke in mice.* Journal of neuroinflammation, 2008. **5**: p. 46-46.

33. Anrather, J. and C. Iadecola, *Inflammation and Stroke: An Overview.* Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics, 2016. **13**(4): p. 661-670.

34. Peuhkuri, K., H. Vapaatalo, and R. Korpela, *Even low-grade inflammation impacts on small intestinal function.* World journal of gastroenterology, 2010. **16**(9): p. 1057-1062.

35. Vancamelbeke, M. and S. Vermeire, *The intestinal barrier: a fundamental role in health and disease.* Expert review of gastroenterology & hepatology, 2017. **11**(9): p. 821-834.

36. Ma, T.Y., et al., *Mechanism of TNF-{alpha} modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression.* Am J Physiol Gastrointest Liver Physiol, 2005. **288**(3): p. G422-30.

37. Al-Sadi, R.M. and T.Y.J.T.J.o.I. Ma, *IL-1β causes an increase in intestinal epithelial tight junction permeability.* 2007. **178**(7): p. 4641-4649.

38. Van Itallie, C.M. and J.M. Anderson, *Architecture of tight junctions and principles of molecular composition.* Seminars in Cell & Developmental Biology, 2014. **36**: p. 157-165.

39. Wadgaonkar, R., et al., *Endothelial cell myosin light chain kinase (MLCK) regulates TNFalpha-induced NFkappaB activity.* J Cell Biochem, 2005. **94**(2): p. 351-64.

40. Al-Sadi, R., M. Boivin, and T. Ma *Mechanism of cytokine modulation of epithelial tight junction barrier*. Frontiers in bioscience (Landmark edition), 2009. **14**, 2765-2778 DOI: 10.2741/3413.

41. He, W.-Q., et al., *Contributions of Myosin Light Chain Kinase to Regulation of Epithelial Paracellular Permeability and Mucosal Homeostasis.* 2020. **21**(3): p. 993.

42. Oeckinghaus, A. and S.J.C.S.H.p.i.b. Ghosh, *The NF-κB family of transcription factors and its regulation.* 2009. **1**(4): p. a000034.

43. Yu, H., et al., *Targeting NF-κB pathway for the therapy of diseases: mechanism and clinical study.* Signal Transduction and Targeted Therapy, 2020. **5**(1): p. 209.

44. Ye, D., I. Ma, and T.Y. Ma, *Molecular mechanism of tumor necrosis factor-alpha modulation of intestinal epithelial tight junction barrier.* Am J Physiol Gastrointest Liver Physiol, 2006. **290**(3): p. G496-504.

45. Ma, T.Y., et al., *TNF-α-induced increase in intestinal epithelial tight junction permeability requires NF-κB activation.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2004. **286**(3): p. G367-G376.

46. Parada Venegas, D., et al., *Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases.* Frontiers in Immunology, 2019. **10**.

47. Wang, Y., et al., *The Gut-Microglia Connection: Implications for Central Nervous System Diseases.* Frontiers in Immunology, 2018. **9**.

48. Silva, Y.P., A. Bernardi, and R.L. Frozza, *The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication.* Frontiers in endocrinology, 2020. **11**: p. 25-25.

49. Wenzel, T., et al., *Short-chain fatty acids (SCFAs) alone or in combination regulate select immune functions of microglia-like cells.* Molecular and Cellular Neuroscience, 2020. **105**: p. 103493.

50. Lee, J., et al., *Gut Microbiota-Derived Short-Chain Fatty Acids Promote Poststroke Recovery in Aged Mice.* Circulation research, 2020. **127**(4): p. 453-465.

51. Sadler, R., et al., *Short-Chain Fatty Acids Improve Poststroke Recovery via Immunological Mechanisms.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2020. **40**(5): p. 1162-1173.

52. Diether, N.E. and B.P. Willing, *Microbial Fermentation of Dietary Protein: An Important Factor in Diet–Microbe–Host Interaction.* Microorganisms, 2019. **7**(1): p. 19.

53. Heimann, E., et al., *Branched short-chain fatty acids modulate glucose and lipid metabolism in primary adipocytes.* Adipocyte, 2016. **5**(4): p. 359-368.

54. Kaiser, E.E. and F.D. West, *Large animal ischemic stroke models: replicating human stroke pathophysiology.* Neural Regen Res, 2020. **15**(8): p. 1377-1387.

55. Lind, N.M., et al., *The use of pigs in neuroscience: modeling brain disorders.* Neuroscience & Biobehavioral Reviews, 2007. **31**(5): p. 728-751.

56. Swindle, M.M., et al., *Swine as models in biomedical research and toxicology testing.* Veterinary pathology, 2012. **49**(2): p. 344-356.

57. Cullen, D.K., et al., *A Porcine Model of Traumatic Brain Injury via Head Rotational Acceleration.* Methods Mol Biol, 2016. **1462**: p. 289-324.

58. Dickerson, J.W.T., J. Dobbing, and R.A. McCance, *Prenatal and postnatal growth and development of the central nervous system of the pig.* Proceedings of the Royal Society of London. Series B. Biological Sciences, 1967. **166**(1005): p. 384-395.

59. Sciascia, Q., G. Daş, and C.C. Metges, *REVIEW: The pig as a model for humans: Effects of nutritional factors on intestinal function and health1.* Journal of Animal Science, 2016. **94**(suppl_3): p. 441-452.

60. Miller, E.R. and D.E. Ullrey, *The Pig as a Model for Human Nutrition.* Annual Review of Nutrition, 1987. **7**(1): p. 361-382.

61. Xiao, L., et al., *A reference gene catalogue of the pig gut microbiome.* Nat Microbiol, 2016. **1**: p. 16161.

62. Ley, R.E., et al., *Obesity alters gut microbial ecology.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 11070-5.

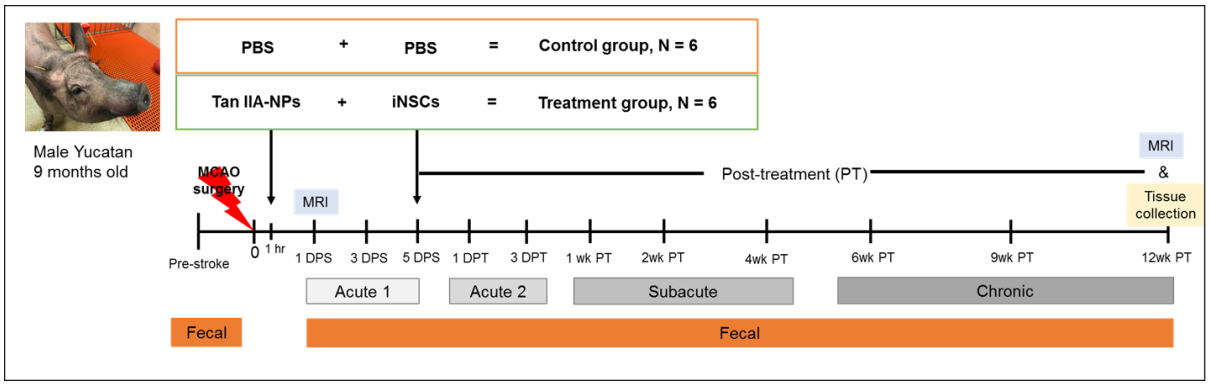

Figure 4.1. Experimental study design.

Castrated male Yucatan pigs (9-month-old, 31–41 kg body weight) underwent middle cerebral artery occlusion (MCAO) and received either PBS (Control group, n=6) or Tan IIA-NPs and iNSCs (Treatment group, n=6). Either PBS or Tan IIA-NPs were administered intracisternal at 1 hour post-stroke (PS) and PBS or iNSCs were transplanted into the perilesional region at 5 days PS. Ischemic stroke was confirmed at 1 day PS by MRI and lesion volume and midline shift were measured 12 weeks (wk) post-transplant (PT). Post-stroke stages were categorized into Actue (1–5 days PS), Acute 2 (1–3 days PT), Subacute (1–4 wk PT), and Chronic (6–12 wk PT) [25, 26]. Fecal samples were collected pre-stroke and at multiple time point post-stroke to measure fecal short-chain fatty acids levels. All pigs were sacrificed at 12 wk PT and distal jejunal samples were scraped and collected for further analysis. Abbreviation; MCAO: Middle cerebral artery occlusion; Tan IIA-NPs: Tanshinone IIA loaded PLGA nanoparticle; iNSCs: Induced pluripotent stem cell-derived neural stem cells; MRI: magnetic resonance imaging; DPS: days post-stroke; DPT: days post-treatment; wk PT: weeks post-treatment.

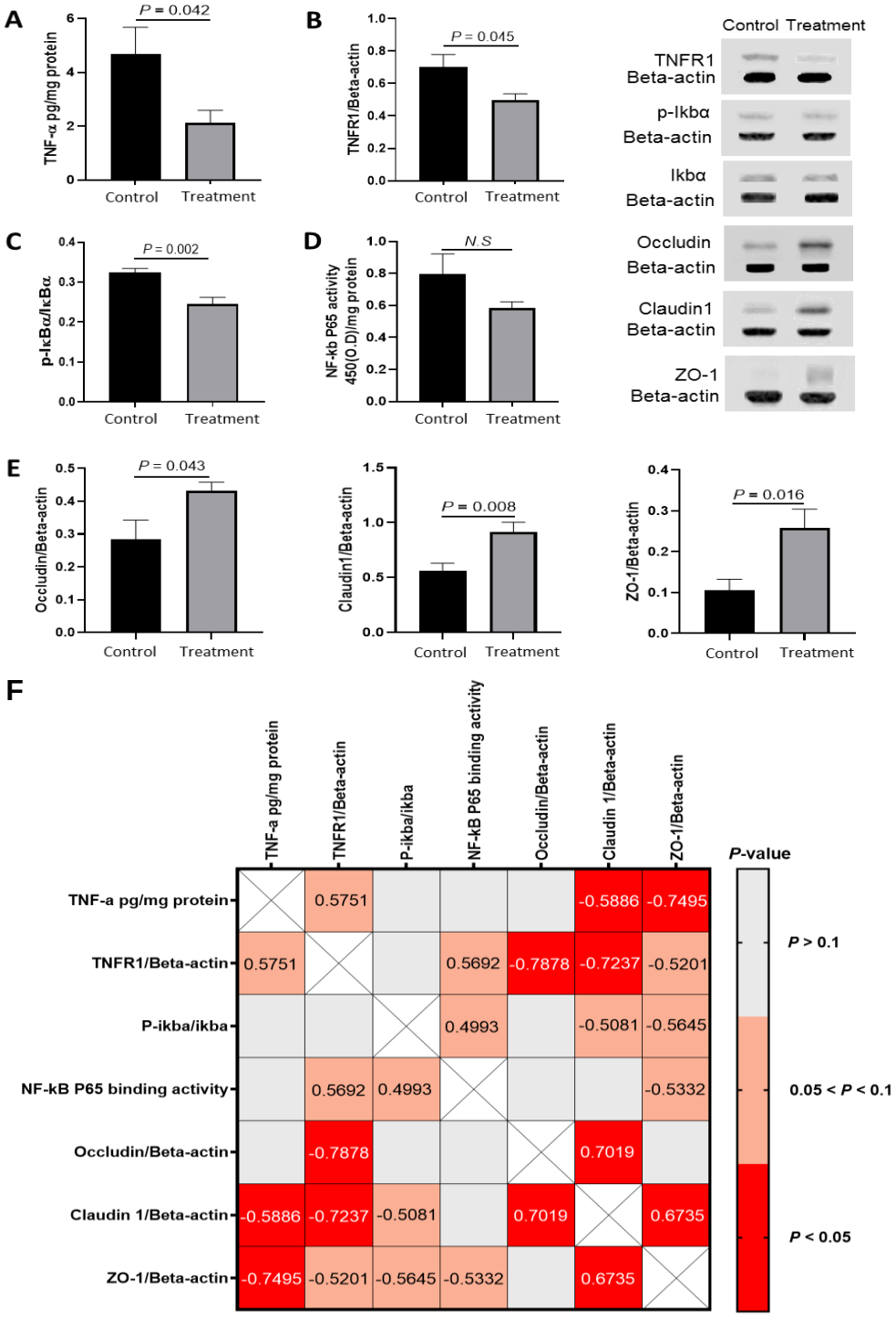

Figure 4.2. Tan IIA-NP+iNSC treatment reduced inflammation and improved expression of tight junction proteins in jejunal mucosa at 12 weeks post-treatment. At 12 weeks post-

treatment, treated pigs showed decreased protien levels of **(A)** TNF-α, **(B)** TNFR1, **(C)** phosphorylation of IkBα relative to the control. **(D)** Treatment decreased binding activity of NF-kB P65 compared to control although not statistically significant. **(E)** Treatment significantly increased protein expression of gut tight junction proteins occludin, claudin1, and ZO-1 compared to control. Unpaired t-test. N = 6 each group, * $P < 0.5$, ** $P < 0.01$. **(F)** Protein levels of TNF-α, TNFR1, phosphorylated IkBα, and binding activity of NF-kB P65 were correlated with the expression of gut tight junction proteins occludin, claudin1, and ZO-1. Significant correlations (*P*-value < 0.05) are highlighted in red, while trending correlations are represented in orange. The number inside each box represents the r-value, r = Pearson correlation coefficient.

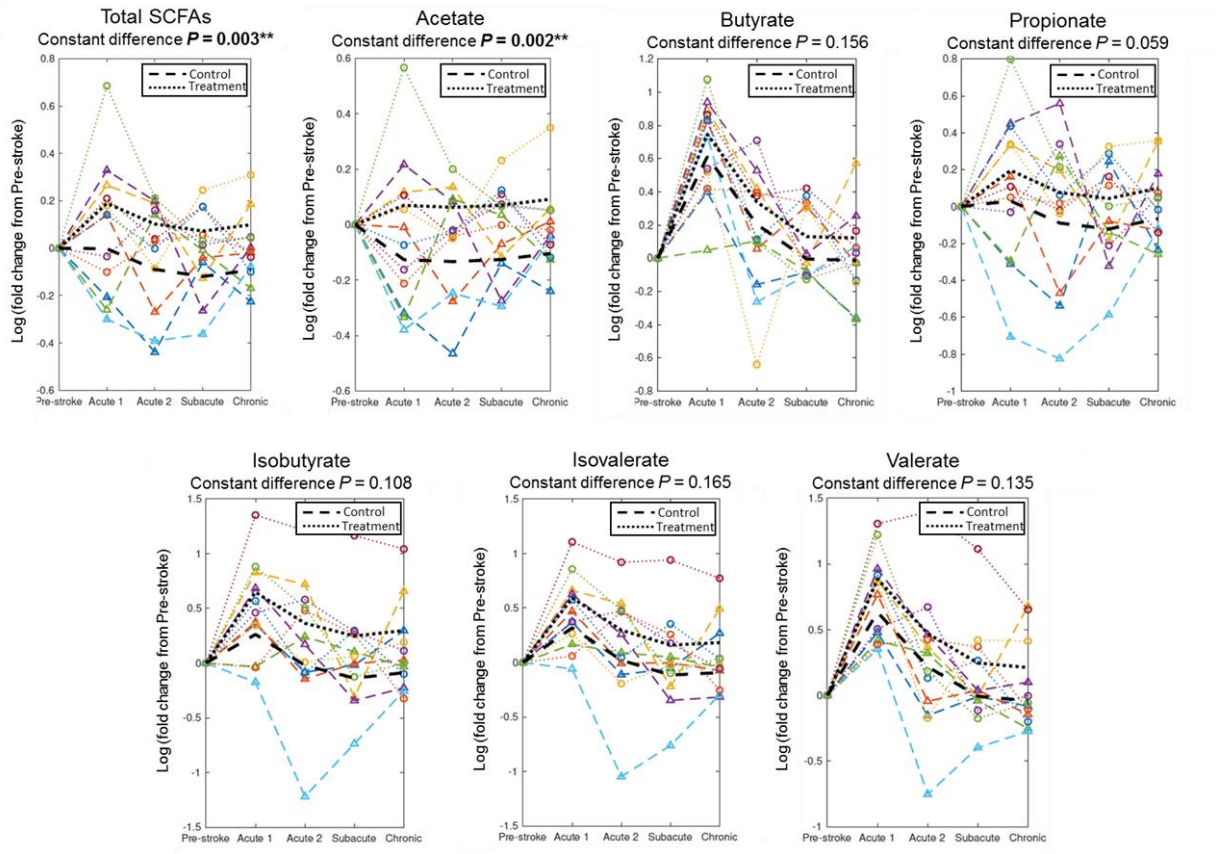

Figure 4.3. Tan IIA-NPs+iNSCs combination therapy increased fecal SCFAs levels in ischemic stroke pigs. Longitudinal changes in fecal SCFAs levels were examined by a multivariate quadratic regression model. Colored lines represent the individual changes in fecal SCFAs of pigs used in this study. The two black lines represent the changes in fecal SCFAs in the control group (n = 6) and the treatment group (n = 6). Changes in fecal total SCFA and acetate levels from baseline were significantly higher in the treatment group compared to the control group post-stroke. ** $P < 0.01$

Table 4.1. Correlations between changes in fecal SCFAs levels post-stroke and jejunal inflammation at 12 weeks post-treatment in an ischemic stroke pig model.

12 weeks PT / Post-stroke	TNF-α	TNF-α receptor 1	Ikb-α phosphorylation	NF-kB P65 binding activity
Total SCFAs	*P* = 0.032[c] r = -0.619	N.S	*P* = 0.041[c] r = -0.595	N.S
Acetate	*P* = 0.031[c] r = -0.622	N.S	*P* = 0.031[c] r = -0.623	*P* = 0.029[c] r = -0.628
Butyrate	*P* = 0.030[d] r = 0.624	*P* = 0.031[d] r = 0.622	N.S	*P* = 0.014[d] r = 0.075
Propionate	N.S	N.S	N.S	N.S
Isobutyrate	N.S	N.S	*P* = 0.031[b,] 0.011[c] r = -0.623, -0.700	N.S
Isovalerate	N.S	N.S	*P* = 0.039[b,] 0.009[c] r = -0.600, -0.716	N.S
Valerate	N.S	N.S	*P* = 0.024[b] r = -0.644	N.S

Time point of fecal SCFAs showing significant correlation: a = Acute1, b = Acute2, c = Subacute, d = Chronic. r = Pearson correlation coefficient. PT: post-treatment

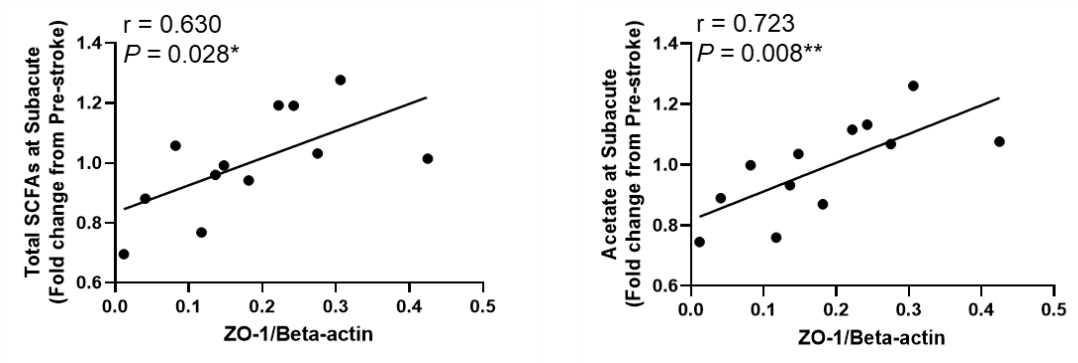

Figure 4.4. Total SCFAs and acetate levels at the subacute stage were positively correlated with ZO-1 protein expression in the jejunal mucosa at 12 weeks post-treatment. Fecal total SCFAs and acetate levels at the subacute (1-4 weeks PT) was positively correlated with ZO-1 protein expression at 12 weeks PT (All $P < 0.05$). r = Pearson correlation coefficient and *P*-values are shown.* $P < 0.05$, ** $P < 0.01$, PT: post-treatment

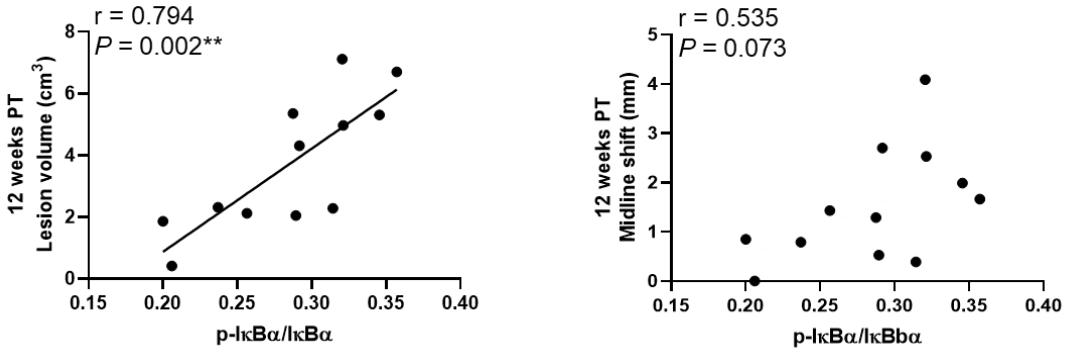

Figure 4.5. Protein levels of phosphorylated IkB*α* correlated with the brain lesion volume and midline shift changes 12 weeks post-treatment. Lesion volume and midline shift at 12 weeks PT were positively correlated with jejunal protein levels of phosphorylated IkBα at 12 weeks PT. r = Pearson correlation coefficient and *P*-values are shown. * $P < 0.05$, ** $P < 0.01$, PT: post-treatment

Table 4.2. Correlations between changes in fecal SCFA levels post-stroke and lesion volume and midline shift changes 12 weeks post-treatment.

12 weeks PT / Post-stroke	Lesion volume (cm^3)	Midline shift (mm)
Total SCFAs	$P = 0.027^b$ r = -0.634	$P = 0.037^b$ r = -0.605
Acetate	$P = 0.038^b$ r = -0.602	$P = 0.037^b$ r = -0.604
Butyrate	N.S	N.S
Propionate	N.S	N.S
Isobutyrate	$P = 0.006^b$ r = -0.741	$P = 0.042^a, 0.012^b$ r = -0.593, -0.694
Isovalerate	$P = 0.004^b, 0.038^c$ r = -0.763, -0.604	$P = 0.007^b$ r = -0.730
Valerate	$P = 0.020^b$ r = -0.659	$P = 0.039^a$ r = -0.600

Time point of fecal SCFAs showing significant correlation: a = Acute1, b = Acute2, c = Subacute, d = Chronic. r = Pearson correlation coefficient. PT: post-treatment

CHAPTER 5[1]

CHANGES IN ORAL MICROBIAL DIVERSITY IN A PIGLET MODEL OF TRAUMATIC BRAIN INJURY

1. Jeon JH, Lourenco JM, Fagan MM, Welch CB, Sneed SE, Stephanie D, Duberstein KJ, Callaway TR, West FD, and Park HJ. To be submitted to *Brain Sciences*.

Abstract

Dynamic changes in the oral microbiome have gained attention due to their potential diagnostic role in neurological diseases such as Alzheimer's disease and Parkinson's disease. Traumatic brain injury (TBI) is a leading cause of death and disability in the United States, but no studies have examined the changes in oral microbiome during the acute stage of TBI using a clinically translational pig model. Crossbred piglets (4-5 weeks old, male) underwent either a controlled cortical impact (TBI, n=6) or sham surgery (Sham, n=6). The oral microbiome parameters were quantified from the upper and lower gingiva, both buccal mucosa, and floor of the mouth pre-surgery and 1, 3, and 7 days post-surgery (PS) using the 16S rRNA gene. Faith's Phylogenetic Diversity was significantly lower in the TBI piglets at 7 days PS compared to those of Sham, and beta-diversity at 1, 3, and 7 days PS was significantly different between TBI and Sham piglets. However, no significant changes in the taxonomic composition of the oral microbiome were observed following TBI compared to Sham. Further studies are needed to investigate the potential diagnostic role of the oral microbiome during the chronic stage of TBI with a larger number of subjects.

Keywords

Oral microbiome, neurological disease, porcine model

1. Introduction

The oral microbiome is the second most diverse in the human body after the gut and is usually stable and nonpathogenic in healthy individuals [1]. However, alterations in microbial composition or loss of diversity can cause an imbalance in the oral microbiome, which is detrimental to oral health and linked to increased risk of systemic diseases [2]. Recently, researchers have shown a potential link between oral microbes and neurological diseases with studies highlighting dynamic changes in the oral microbiome of Alzheimer's disease (AD) and Parkinson's disease (PD) patients. Wu et al [3] reported that AD patients had significant increases in Firmicutes, Lactobacillales, and *Streptococcus* levels in dental plaque compared to that in healthy controls. *Lactobacillus*, which is known as a probiotic in the gut microbiome is considered pathogenic bacteria in the oral cavity since it causes root caries and tooth loss [4, 5]. Similarly, *Streptococcus*, the most commonly found in the oral microbiome, also contains some pathogenic species such as *Streptococcus mutans*, which is an acid-producing bacteria that causes carious lesions [6]. Consistent with these observations, more missing teeth and higher dental plaque weight were found in AD patients compared to healthy controls, and poor oral condition was correlated with cognitive deficits [3], suggesting a potential link between the existence of periodontal pathogens and AD development. Similarly, patients with PD had increased oral microbial levels of *Streptococcus* (*Streptococcus mutans* [7] and *Streptococcus pneumonia* [8]), *Lactobacillaceae* [7, 9], and *Lactobacillus* [8, 10] and showed a positive correlation between *Lactobacillus reuteri* and slower movement in PD patients [10]. These studies suggest that there may be a relationship between cognitive and functional decline in neurological conditions and increased pathogenic oral bacteria.

Traumatic brain injury (TBI) is one of the most common types of brain injuries and often leads to major disability and death in global populations [11]. Despite the devastating effects of TBI, there are a number of challenges including detection of mild TBI, discerning TBI severity, and predicting potential patient outcomes. Understanding the dynamic interplay between TBI and the oral microbiome may provide unique insight into using the oral microbiome as a biomarker to detect TBI. However, the majority of published studies exploring the relationship between TBI and microbiome have almost exclusively focused on the lower intestine or fecal samples [12-14]. To the best of our knowledge, there are no studies that have explored the oral microbiome changes after TBI. Collecting fecal samples can be challenging, as patients with brain injury commonly experience bowel dysfunction, such as constipation [15]. Therefore, the highly accessible mucosal surface of the oral cavity is potentially a preferable microbiome for evaluating TBI patient injury.

In the current study, we used a well-established piglet model [16-19] to assess changes in oral microbial diversity and composition during the acute stage of TBI. The pig is a robust translational model due to its comparable brain and gastrointestinal (GI) anatomy, physiology, and pathophysiological traits relative to humans; thus, making the pig an ideal large animal model for clinical research [20, 21]. The findings of this study may support the potential role of the oral microbiome as a diagnostic and prognostic biomarker for TBI and provides the basis for future oral microbiome studies in TBI patients.

2. Materials and Methods

2.1 Animals TBI induction

Castrated crossbred male piglets (n = 12; 4-5 weeks old) were acquired from the University of Georgia swine unit and randomly selected and assigned into two groups; 1) TBI surgery group (TBI, n=6) and 2) Sham surgery group (Sham, n=6). Moderate/severe TBI was induced using a controlled cortical impact (CCI) model as previously described by our group [19] with the following parameters velocity: 4m/s, depth: 9mm, dwell: 400ms. The sham surgery group was sedated using the same protocol as the TBI group and underwent a craniectomy but did not receive a CCI. All animals were carefully monitored post-surgery every 30 minutes until vitals (heart rate, respiration rate, temperature) returned to normal, then every 4 hours for 8 hours, every 8 hours for following 24 hours, and twice daily thereafter. Induced TBI was confirmed by magnetic resonance imaging (MRI) 1 day post-surgery (Data not shown). All work in this study was conducted in accordance with the guidelines established by the University of Georgia Institutional Animal Care and Use Committee.

2.2 Oral mucosa collection

Oral mucosa was collected with sterile cotton swabs by rubbing the oral cavity. Two swabs were applied to the upper and lower gingiva and both buccal mucosa for 3 minutes (mins). Another two swabs were applied to the floor of the mouth for 3 mins. Each one of the two swabs were placed in the same 15ml tube (two tubes per sample) and immediately frozen in dry ice. The oral mucosa collection was performed while animals were under anesthesia pre-surgery and 1, 3, and 7 days post-surgery (PS).

2.3 Oral microbial DNA extraction

Oral DNA extraction was processed according to a modified protocol of the QIAamp Fast DNA Stool Mini Kit (Qiagen; Germantown, MD, United States). Frozen swabs in a 15ml tube were thawed on ice and 1.2mls of sterile PBS was added. Samples were thoroughly vortexed to release the oral mucosa from the swab into the PBS for 5 mins and centrifuged at maximum speed (3901g) for 1 min at 4℃. Supernatant was aliquoted into a 2ml tubes, and 450ul of warmed inhibit Ex buffer was added, and mixed. Tubes were vortexed for 10 mins, then put into a 95℃ water bath for 5 mins, and vortexed 15 sec. After centrifuging for 1 min at 20,000g at room temperature (RT), 670ul of sample were added into a 2ml tube containing 50uls of proteinase K. 670uls of AL buffer was then added and vortexed for 20 secs. After a 10 min incubation in a 70℃ water bath, tubes were centrifuged for 1 min at RT. Then, 670uls of ethanol was added, samples were vortexed for 20 secs, and centrifuged for 1 min at 20,000g, RT. Supernatant was added to the spin column, centrifuged for 1 min, and then AW1 buffer was added and centrifuged for 1 min. AW2 buffer was then added, and samples were centrifuged for 3 mins. Samples were centrifuged again for 1 min to ensure that no AW2 buffer remained in the tubes. To elute DNA, 30uls of ATE buffer was added, incubated for 3 mins, and centrifuged for 1 min. Then, 50uls of ATE buffer was added, incubated for 3 mins, and samples were centrifuged for 1 min. The DNA concentration of each sample was quantified spectrophotometrically utilizing the Take3 plate and Synergy H4 multimode plate reader (BioTek, Winooski, VT).

2.4 16s rRNA gene sequencing analysis

DNA samples were sent to LC Sciences (Houston, Texas, USA) for 16s ribosomal RNA (rRNA) gene sequencing. The V3-V4 region was amplified with primer pairs S-D-Bact-0341-b-S-17 (5′-

CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) and sequenced using the Illumina NovaSeq platform. Sequencing data were first demultiplexed before being converted into FASTQ files, and the paired-end sequences imported into QIIME 2 [22]. The non-biological nucleotides were then removed, and sequences were denoised, dereplicated, and chimera-filtered using DADA2 [23]. Taxonomies were assigned to the sequences by using a pre-trained Naive Bayes classifier trained on the SILVA 138 SSU database [24], and reads were classified by taxon using the fitted classifier [25]. For alpha and beta-diversity analyses, all samples were rarefied to a common sequencing depth. Alpha-diversity was determined by the observed number of observed features (number of ASVs), Faith's phylogenetic Diversity (total length of phylogenetic branches), Shannon index (species richness and evenness), and Pielou's Evenness (species evenness). Beta-diversity was assessed using the unweighted UniFrac distance matrix, which considers phylogenetic connections and was visualized by principal coordinate analysis (PCoA). Oral microbial composition was measured at phylum (Relative abundance >1%), family (>1%), genus (>1%), and species (>0.5%) levels. All indices of microbial diversities and composition were examined using QIIME2 plugins.

2.5 Statistical analysis

Mixed effects ANOVA was used to compare differences between groups and time points. The statistical results showed the main effect of time (Time effect) and group (Group effect) and the interaction effect between time and group (Time-by-Group interaction effect). Data are shown as fitted mean ± standard error of the mean (SEM). Differences in beta-diversity were evaluated by Bonferroni-corrected multiple comparisons (corrected *p*-value) between each time point and between the groups. *P*-values under 0.05 were regarded as significant for all statistical tests.

3. Results

The alpha-diversity indexes were analyzed to examine changes in oral microbial diversity in TBI and Sham piglets during the acute stage of TBI (**Figure 1**). Significant time effects were found in Number of Observed Features (Time effect $P = 0.00$), Shannon (Time effect $P = 0.008$), Evenness (Time effect $P = 0.043$), and Faith's Phylogenetic Diversity (Time effect $P = 0.00$) up to 7 days post-surgery. However, there were no group or time-by-group interaction effects in alpha-diversity indexes except for Faith's Phylogenetic diversity. Significant group ($P = 0.003$) and time-by-group interaction effects ($P = 0.001$) were observed in Faith's Phylogenetic Diversity, showing significantly lower levels in the TBI group compared to the Sham group at 7 days PS (**Figure 1D**). These results suggest that species diversity and evenness were not significantly altered after TBI, but the phylogenetic diversity differed 7 days post-surgery compared to the Sham group.

Beta-diversity of the oral microbiome was assessed using an unweighted UniFrac distance matrix to examine the similarity or dissimilarity of microbial patterns between the TBI and sham groups (**Figure 2**). Interestingly, both the TBI and Sham groups had significantly different beta-diversity between time points (**Figure 2A**), showing altered beta-diversity at 1, 3, and, 7 days post-surgery compared to pre-surgery. The sham group also showed different beta-diversity at 7 days post-surgery compared to 1- and 3 days post-surgery. These results indicate that the oral microbial structure was altered post-surgery in both the TBI and Sham groups. Between the TBI and Sham group, distinct microbial patterns were observed at 1, 3, and 7 days post-surgery, with the most apparent difference at 7 days post-surgery (**Figure 2B**, All Bonferroni corrected P-value < 0.05).

This finding implies that TBI surgery altered oral microbial structure, aside from the effects of surgical stress, and that the changes persisted up to 7 days post-surgery.

Oral microbial composition (**Figure 3**) was measured at the phylum (P, relative abundance >1%), family (F, relative abundance >1%), genus (G, relative abundance > 1%), and species level (S, relative abundance > 0.5%) in both TBI and Sham groups pre-surgery and 1, 3, and 7 days PS. The oral microflora in this study showed a similar predominant bacterial composition compared to previous human and porcine oral microbiome studies [26-28]. The top 5 most abundant phyla pre-surgery were Firmicutes (Mean relative abundance ± SEM, 50.56 ± 4.08%), Proteobacteria (34.10 ± 4.09%), Actinobacteria (8.66 ± 1.72%), Bacteroidetes (4.6 ± 0.78%), and Fusobacteria (1.67 ± 0.20%). The top 5 most prevalent bacterial families pre-surgery were *Streptococcaceae* (27.03 ± 3.11%), *Pasteurellaceae* (16.39 ± 1.98%), *Moraxellaceae* (10.66 ± 1.96%), *Lactobacillaceae* (6.45 ± 1.91%), and *Veillonellaceae* (5.58 ± 1.12%). The top 5 most abundant bacterial genera pre-surgery were *Streptococcus* (27.03 ± 3.11%), *Actinobacillus* (14.68 ± 1.77%), *Moraxella* (6.95 ± 1.39%), *Lactobacillus* (6.44 ± 1.91%), and *Veillonella* (4.97 ± 1.04%). Lastly, the top 5 most prevalent bacterial species pre-surgery were *Streptococcus suis* (6.42 ± 0.95%), *Actinobacillus indolicus* (3.59 ± 0.74%), *Actinomyces denticolens* (3.49 ± 1.60%), *Actinobacillus porcitonsillarum* (3.08 ± 0.70%), and *Actinomyces howellii* (1.11 ± 0.49%).

Significant time effects were observed in several microbial taxonomic compositions including P_Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, F_*Streptococcaceae*, *Pasteurellaceae*, *Lactobacillaceae*, *Lachnospiraceae*, *Micrococcaceae*, *Ruminococcaceae*, *Leptotrichiaceae*, G_*Streptococcus*, *Actinobacillus*, *Moraxella*, *Lactobacillus*, *Rothia*, *Blautia*, *Leptotrichia*, S_*Streptococcus suis*, *Actinobacillus indolicus*, and *Bergeyella porcorum* in both TBI and Sham

groups (Data not shown, all $P < 0.05$). Among oral bacteria, significant Time-by-Group interaction effects were found in P_Proteobacteria ($P = 0.026$), F_*Leptotrichiaceae* ($P = 0.037$), F_*Micrococcaceae* ($P = 0.019$), and G_*Rothia* ($P = 0.023$) in both TBI and Sham groups; however, the post hoc Tukey HSD (Honestly Significant Difference) comparison did not show any differences between TBI and Sham groups at any time point post-surgery. Therefore, the oral microbial composition did not differ between TBI and Sham groups at the phylum (1>%), family (1>%), genus (1>%), or species (0.5>%) level during the acute stage of TBI, despite showing some differences in phylogenetic diversity and microbial patterns by alpha- and beta-diversity, respectively.

4. Discussion

Here, we investigated for the first time changes in the oral microbiome during the acute stage of TBI in a piglet model. Moderate/severe TBI induced differences in Faith's phylogenic diversity and beta-diversity between the TBI and sham piglets. The Faith's phylogenetic diversity was lower in the TBI group at 7 days post-surgery compared to the sham group, and different beta-diversity (microbial patterns) was found between the groups post-surgery, with the most distinct alterations at 7 days post-surgery. However, the taxonomic composition of the oral microbiome did not significantly change following TBI. This translational study provides novel insight into the oral microbiome TBI response and suggests that further studies are warranted to investigate changes in the oral microbiome during the chronic stage of TBI.

Dysbiosis, an indicator of an imbalanced microbiome, is characterized by a reduction in microbial diversity, compositional changes, and is associated with various disease states [25].

Previous studies showed that AD patients had decreased microbial richness and diversity with lower levels of oral microbial number of operational taxonomic units (OTUs) [3], Chao1 (species richness), Shannon index, and phylogenetic diversity whole tree as compared to healthy controls patients [26]. Similarly, PD patients had lower alpha-diversity with reduced species richness and evenness as well as different microbial communities (beta-diversity) compared to the healthy controls [7]. In this study, we examined the oral microbiota profile in a translational piglet TBI model and only found Faith's phylogenetic diversity was lower in the TBI group 7 days post-surgery compared to the sham group, while Number of Observed Features, Shannon, and Evenness only had significant time effects. This suggests that TBI may have a limited effect on oral microbial diversity during the acute stage of TBI. Faith's Phylogenetic Diversity represents species richness, computing the sum of the branches of phylogenetic trees connecting all species of a given taxonomic group [27]. Faith's Phylogenetic Diversity is known to be more sensitive in distinguishing disease factors in humans as this diversity measures phylogenetic differences between species, whereas traditional species diversity does not differentiate between species [28, 29]. Previous studies showed that phylogenetic diversity was reduced in the oral microbiome of patients with AD [26] and nasopharyngeal carcinoma [30] compared to the healthy controls, which was also shown in the gut microbiome of patients with AD [31], inflammatory bowel disease [32], and autism [33]. These results suggest that decreased phylogenetic diversity may be linked to poor microbial resilience and health. Therefore, reduced phylogenetic diversity can be a potential indicator of TBI, but further studies are needed to clarify its role during the acute stage of TBI. Interestingly, the most distinct differences in beta-diversity were also found at 7 days post-surgery between the TBI and Sham groups. Both Faith's Phylogenetic Diversity and UniFrac matrix (beta-diversity) measure phylogenetic distances within and between the communities, respectively;

therefore, the changes in Faith's phylogenetic diversity between the groups likely has a relationship with differential microbial patterns (beta-diversity) at 7 days post-surgery.

Although changes were observed in oral microbial diversity following TBI, the present study did not find significant differences in taxonomic composition between TBI and Sham animals. There are a number of potential reasons as to why oral microbiota composition changes were not observed between TBI and the Sham groups in the current study. First, dysbiosis induced by TBI may cause higher inter-individual variation in microbial composition than sham piglets. According to the Anna Karenina principle, the microbial community varies more in dysbiotic individuals than that of healthy individuals [34]. High variability may reduce the ability of bacteria to regulate microbial composition, preventing the establishment of unique microbial communities [34]. This theory was also applied in a study of relapse/refractory multiple myeloma patients, who did not have a distinct oral microbial composition compared to the general population [35]. To overcome this challenge, it may be necessary to increase the number of animals to enhance the statistical power. Second, surgical stress may have had a major influence on microbial changes in both TBI and Sham groups. It is expected that the significant time effects in the oral microbial composition may have been induced by surgical or environmental stress, as both can alter microbial diversity and microbial composition [36]. Existing studies reported that the Fusobacteria level was increased in response to treatment of stress hormone, cortisol in subgingival dental plaque samples cultured *in vitro* [37], and its genus level of oral *Fusobacterium* and *Leptotrichia* spp. were positively associated with cortisol levels in humans [38]. The present study also showed an increased Fusobacteria post-surgery and *Leptotrichia* at 3 days PS in both TBI and Sham groups compared to pre-surgery (data not shown). This suggests a potential impact of

surgical stress on microbial changes in both the TBI and Sham groups that may mask the compositional differences between groups. The precise mechanisms by which cortisol alters the oral microbiome are still unknown but several stress hormones have been related to both stimulatory and inhibitory effects on oral microbial growth [39, 40]. Future studies should consider this complexity of stress effect on oral microbiome changes. Third, significant changes in the oral microbiome may not be apparent at the acute TBI stage. Neurodegenerative diseases such as AD and PD are chronic diseases that develop slowly over time and affect primarily elderly people [41, 42]. Studies of chronic AD and PD patients showed individuals possessed altered oral microbial diversity or abundance compared to healthy controls [3, 7-10, 26]. Similarly, diseases that have been reported to have altered oral microbiome are chronic inflammatory disorders (ex. Rheumatoid arthritis) or chronic diseases such as cancer, diabetes, and cardiovascular disease [43]. Therefore, the acute stage of TBI may have been too early to detect robust quantitative changes in alpha-diversity and differences in phylum, family, genus, and species. It will be intriguing to investigate changes in the oral microbiota during the chronic stage of TBI. Finally, the changes in the oral microbiome composition may differ based on several factors such as sampling location and the age of the pig. For example, in PD patients, changes in oral microbial diversity and composition were different between samples of soft (tongue dorsum and buccal mucosa) and hard tissue (chewing surfaces of the molars) of the oral cavity [8]. Moreover, the oral microbiota in infant and toddlers was less diverse than that of adults and contained some species not commonly identified in the adults oral cavity [44], suggesting that the oral microbiome dynamically changes and develops as an individual ages. Therefore, future studies should consider these multiple variations that may affect oral microbiome outcomes.

The bidirectional communication between the oral microbiome and brain have not yet been defined. However, it has been proposed that the oral microbiota may affect the brain through direct or indirect means. Interestingly, *Treponema species,* the common oral bacteria, were detected in post-mortem AD brains [45], indicating a direct interaction of oral pathogens with the brain. AD patients also had increased levels of oral *Moraxella, Leptotrichia,* and *Sphaerochaeta* in saliva samples compared to healthy controls [26]. These gram-negative bacteria release lipopolysaccharides (LPS), a strong stimulator of the immune response and inflammation, which are closely associated with worsening AD outcomes [46]. The levels of LPS and K99 pili protein released from gram-negative bacteria were greater in the brains of AD patients than in normal brains [47], and intraperitoneal injection of LPS induced amyloid-beta plaques formation in rodent brain by stimulating neuroinflammation [48]. Gram-negative bacteria such as *Escherichia coli*, *Klebsiella spp*., *Kluyvera spp*., *Serratia spp*., *Proteus spp*., and *Enterobacter spp*. were also isolated in the oral cavity of PD patients [49]. Moreover, increased regional inflammation in the oral cavity was found in PD patients, showing elevated cytokine levels of IL-1 and IL-1RA compared to healthy controls [7]. These results suggest that an increase in oral pathogenic bacteria and their proinflammatory molecules may enter the brain tissue through the bloodstream, cause systemic and neuro-inflammation, and exacerbate disease severity and progression [50]. Therefore, oral dysbiosis may enhance neuropathology via the oral-brain connection. TBI causes significant inflammation in the brain including the activation of microglia, the resident immune cell of the brain, and astrocytes. These activated cells produce a host of inflammatory cytokines (e.g. IL-1β, TNF-α) and chemokines (e.g. CCL2, CXCL10) that attracts peripheral immune cells to the brain leading to an increasing cycle of inflammation [51, 52]. The interconnectivity of the oral cavity and brain through the circulatory and lymphatics/glymphatics systems may potentially enable oral

bacteria and inflammatory molecules to enter the brain particularly since the blood brain barrier is breached.

The top 10 most abundant genera in this study were *Streptococcus, Actinobacillus, Moraxella, Lactobacillus, Veillonella, Actinomyces, Rothia, Neisseria, Blautia,* and *Prevotella,* which is similar to a previous pig salivary microbiome study [24]. Oral and gut bacteria have different habitats, thus they have distinct microbial diversity and structure in both humans [22, 53, 54] and pigs [24]. Maki et al [22] summarized that the human oral microflora has a high abundance of *Streptococcus, Fusobacterium, Neisseria, Prevotella, Actinomyces, Pasteurella* and *Veillonella,* whereas the gut has high abundance of *Alistipes*, *Akkermansia*, *Blautia*, *Faecalibacterium, Roseburia*, *Sutterella,* and etc [22]. The results from this study and others have shown that the pig oral microbiome was also enriched with *Streptococcus*, *Neisseria*, *Moraxella*, *Rothia, Actinobacillus,* and *Fusobacterium,* while earlier studies from our group and others have shown that fecal samples were abundant in *Clostridium, Lactobacillus, Turicibacteri,* and *Prevotella* [24, 55]. This supports that oral and gut microbiome have very different microbial composition. One similarity between the oral and gut microbiome is that different regions of the oral cavity, such as saliva, tonsils, buccal mucosa, gingiva, and plaques have a distinct microbial composition [56], just as different gut segments have different microbial composition [57]. The existence of these various microbiomes suggests that it is important to understand how these diverse microbiomes of the human body interact with its host and affect health.

Conclusion

The findings of this study demonstrated that TBI induced changes in microbial diversity, but did not alter the taxonomic composition of the oral microbiota during the acute stage of moderate/severe TBI in a swine model. This is an early stage study of the oral microbiota, and more research is needed to evaluate the dynamic interplay between TBI and the oral microbiome in longitudinal studies including the chronic TBI recovery stage.

References

1. Deo, P.N. and R. Deshmukh, *Oral microbiome: Unveiling the fundamentals.* Journal of oral and maxillofacial pathology : JOMFP, 2019. **23**(1): p. 122-128.
2. Sudhakara, P., et al., *Oral Dysbiotic Communities and Their Implications in Systemic Diseases.* Dentistry journal, 2018. **6**(2): p. 10.
3. Wu, Y.-F., et al., *Oral Microbiota Changes in Elderly Patients, an Indicator of Alzheimer's Disease.* International journal of environmental research and public health, 2021. **18**(8): p. 4211.
4. Ahirwar, S., M. Gupta, and D.S.K. Snehi, *DENTAL CARIES AND LACTOBACILLUS: ROLE AND ECOLOGY IN THE ORAL CAVITY.* International Journal of Pharmaceutical Sciences and Research, 2019. **10**: p. 4818-4829.
5. Shimada, A., et al., *Oral lactic acid bacteria related to the occurrence and/or progression of dental caries in Japanese preschool children.* Bioscience of microbiota, food and health, 2015. **34**(2): p. 29-36.
6. Forssten, S.D., M. Björklund, and A.C. Ouwehand, *Streptococcus mutans, caries and simulation models.* Nutrients, 2010. **2**(3): p. 290-298.
7. Fleury, V., et al., *Oral Dysbiosis and Inflammation in Parkinson's Disease.* Journal of Parkinson's disease, 2021. **11**(2): p. 619-631.
8. Rozas, N.S., G.D. Tribble, and C.B. Jeter, *Oral Factors That Impact the Oral Microbiota in Parkinson's Disease.* Microorganisms, 2021. **9**(8): p. 1616.
9. Pereira, P.A.B., et al., *Oral and nasal microbiota in Parkinson's disease.* Parkinsonism Relat Disord, 2017. **38**: p. 61-67.

10. Mihaila, D., et al., *The oral microbiome of early stage Parkinson's disease and its relationship with functional measures of motor and non-motor function.* PloS one, 2019. **14**(6): p. e0218252-e0218252.

11. Hyder, A.A., et al., *The impact of traumatic brain injuries: A global perspective.* NeuroRehabilitation, 2007. **22**: p. 341-353.

12. Zhu, C.S., et al., *A Review of Traumatic Brain Injury and the Gut Microbiome: Insights into Novel Mechanisms of Secondary Brain Injury and Promising Targets for Neuroprotection.* Brain sciences, 2018. **8**(6): p. 113.

13. Howard, B.M., et al., *Characterizing the gut microbiome in trauma: significant changes in microbial diversity occur early after severe injury.* Trauma Surg Acute Care Open, 2017. **2**(1): p. e000108.

14. Celorrio, M., et al., *Gut microbial dysbiosis after traumatic brain injury modulates the immune response and impairs neurogenesis.* Acta Neuropathologica Communications, 2021. **9**(1): p. 40.

15. Li, J., et al., *Incidence of constipation in stroke patients: A systematic review and meta-analysis.* Medicine (Baltimore), 2017. **96**(25): p. e7225.

16. Kinder, H.A., et al., *Traumatic Brain Injury Results in Dynamic Brain Structure Changes Leading to Acute and Chronic Motor Function Deficits in a Pediatric Piglet Model.* J Neurotrauma, 2019. **36**(20): p. 2930-2942.

17. Kinder, H.A., E.W. Baker, and F.D. West, *The pig as a preclinical traumatic brain injury model: current models, functional outcome measures, and translational detection strategies.* 2019. **14**(3): p. 413-424.

18. Baker, E.W., et al., *Scaled traumatic brain injury results in unique metabolomic signatures between gray matter, white matter, and serum in a piglet model.* PLOS ONE, 2018. **13**(10): p. e0206481.

19. Baker, E.W., et al., *Controlled Cortical Impact Severity Results in Graded Cellular, Tissue, and Functional Responses in a Piglet Traumatic Brain Injury Model.* Journal of Neurotrauma, 2018. **36**(1): p. 61-73.

20. Clouard, C., M.-C. Meunier-Salaün, and D. Val-Laillet, *Food preferences and aversions in human health and nutrition: How can pigs help the biomedical research?* Animal : an international journal of animal bioscience, 2012. **6**: p. 118-36.

21. Kinder, H.A., E.W. Baker, and F.D. West, *The pig as a preclinical traumatic brain injury model: current models, functional outcome measures, and translational detection strategies.* Neural regeneration research, 2019. **14**(3): p. 413-424.

22. Bolyen, E., et al., *Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2.* Nature Biotechnology, 2019. **37**(8): p. 852-857.

23. Callahan, B.J., et al., *DADA2: High-resolution sample inference from Illumina amplicon data.* Nature Methods, 2016. **13**(7): p. 581-583.

24. Quast, C., et al., *The SILVA ribosomal RNA gene database project: improved data processing and web-based tools.* Nucleic acids research, 2013. **41**(Database issue): p. D590-D596.

25. Pedregosa, F., et al., *Scikit-learn: Machine learning in Python.* 2011. **12**: p. 2825-2830.

26. Maki, K.A., et al., *The Oral and Gut Bacterial Microbiomes: Similarities, Differences, and Connections.* Biological Research For Nursing, 2020. **23**(1): p. 7-20.

27. Dewhirst, F.E., et al., *The human oral microbiome.* Journal of bacteriology, 2010. **192**(19): p. 5002-5017.

28. Murase, K., et al., *Characterization of pig saliva as the major natural habitat of Streptococcus suis by analyzing oral, fecal, vaginal, and environmental microbiota.* PLOS ONE, 2019. **14**(4): p. e0215983.

29. Kriss, M., et al., *Low diversity gut microbiota dysbiosis: drivers, functional implications and recovery.* Current opinion in microbiology, 2018. **44**: p. 34-40.

30. Liu, X.-X., et al., *Analysis of Salivary Microbiome in Patients with Alzheimer's Disease.* Journal of Alzheimer's Disease, 2019. **72**: p. 633-640.

31. Faith, D.P., *Conservation evaluation and phylogenetic diversity.* Biological Conservation, 1992. **61**(1): p. 1-10.

32. Armstrong, G., et al., *Efficient computation of Faith's phylogenetic diversity with applications in characterizing microbiomes.* Genome Res, 2021. **31**(11): p. 2131-2137.

33. Faith, D.P., *Phylogenetic Diversity and Conservation Evaluation: Perspectives on Multiple Values, Indices, and Scales of Application*, in *Phylogenetic Diversity: Applications and Challenges in Biodiversity Science*, R.A. Scherson and D.P. Faith, Editors. 2018, Springer International Publishing: Cham. p. 1-26.

34. Zhu, X.-X., et al., *The Potential Effect of Oral Microbiota in the Prediction of Mucositis During Radiotherapy for Nasopharyngeal Carcinoma.* eBioMedicine, 2017. **18**: p. 23-31.

35. Vogt, N.M., et al., *Gut microbiome alterations in Alzheimer's disease.* Scientific Reports, 2017. **7**(1): p. 13537.

36. Bassett, S.A., et al., *Changes in composition of caecal microbiota associated with increased colon inflammation in interleukin-10 gene-deficient mice inoculated with Enterococcus species.* Nutrients, 2015. **7**(3): p. 1798-1816.

37. Kang, D.-W., et al., *Reduced Incidence of Prevotella and Other Fermenters in Intestinal Microflora of Autistic Children.* PLOS ONE, 2013. **8**(7): p. e68322.

38. Zaneveld, J.R., R. McMinds, and R. Vega Thurber, *Stress and stability: applying the Anna Karenina principle to animal microbiomes.* Nature Microbiology, 2017. **2**(9): p. 17121.

39. Ludwig, H., et al., *Reduced alpha diversity of the oral microbiome correlates with short progression-free survival in patients with relapsed/refractory multiple myeloma treated with ixazomib-based therapy (AGMT MM 1, phase II trial).* eJHaem, 2021. **2**(1): p. 102-106.

40. Kelly, L.S., et al., *Stress-related changes in the gut microbiome after trauma.* Journal of Trauma and Acute Care Surgery, 2021. **91**(1).

41. Duran-Pinedo, A.E., J. Solbiati, and J. Frias-Lopez, *The effect of the stress hormone cortisol on the metatranscriptome of the oral microbiome.* npj Biofilms and Microbiomes, 2018. **4**(1): p. 25.

42. Simpson, C.A., et al., *Oral microbiome composition, but not diversity, is associated with adolescent anxiety and depression symptoms.* Physiol Behav, 2020. **226**: p. 113126.

43. Roberts, A., et al., *Stress and the periodontal diseases: effects of catecholamines on the growth of periodontal bacteria in vitro.* Oral Microbiology and Immunology, 2002. **17**(5): p. 296-303.

44. Jentsch, H.F.R., D. März, and M. Krüger, *The effects of stress hormones on growth of selected periodontitis related bacteria.* Anaerobe, 2013. **24**: p. 49-54.

45. Henry, R.G. and B.J. Smith, *Managing older patients who have neurologic disease: Alzheimer disease and cerebrovascular accident.* Dent Clin North Am, 2009. **53**(2): p. 269-94, ix.

46. Hane, F.T., et al., *Recent Progress in Alzheimer's Disease Research, Part 3: Diagnosis and Treatment.* Journal of Alzheimer's disease : JAD, 2017. **57**(3): p. 645-665.

47. Willis, J.R. and T. Gabaldón, *The Human Oral Microbiome in Health and Disease: From Sequences to Ecosystems.* 2020. **8**(2): p. 308.

48. Sulyanto, R.M., et al., *The Predominant Oral Microbiota Is Acquired Early in an Organized Pattern.* Scientific Reports, 2019. **9**(1): p. 10550.

49. Riviere, G.R., K.H. Riviere, and K.S. Smith, *Molecular and immunological evidence of oral Treponema in the human brain and their association with Alzheimer's disease.* Oral Microbiol Immunol, 2002. **17**(2): p. 113-8.

50. Alexander, C. and E.T. Rietschel, *Bacterial lipopolysaccharides and innate immunity.* J Endotoxin Res, 2001. **7**(3): p. 167-202.

51. Zhan, X., et al., *Gram-negative bacterial molecules associate with Alzheimer disease pathology.* Neurology, 2016. **87**(22): p. 2324-2332.

52. Wang, L.-M., et al., *Lipopolysaccharide endotoxemia induces amyloid-β and p-tau formation in the rat brain.* American journal of nuclear medicine and molecular imaging, 2018. **8**(2): p. 86-99.

53. Gosney, M., et al., *The incidence of oral Gram-negative bacteria in patients with Parkinson's disease.* European Journal of Internal Medicine, 2003. **14**(8): p. 484-487.

54. Narengaowa, et al., *The Oral-Gut-Brain AXIS: The Influence of Microbes in Alzheimer's Disease.* Frontiers in cellular neuroscience, 2021. **15**: p. 633735-633735.

55. Pavlova, V., et al., *Pioglitazone Therapy and Fractures: Systematic Review and Meta-Analysis.* Endocrine, metabolic & immune disorders drug targets, 2018. **18**(5): p. 502-507.

56. Gyoneva, S. and R.M. Ransohoff, *Inflammatory reaction after traumatic brain injury: therapeutic potential of targeting cell–cell communication by chemokines.* Trends in Pharmacological Sciences, 2015. **36**(7): p. 471-480.

57. Costello, E.K., et al., *Bacterial community variation in human body habitats across space and time.* Science (New York, N.Y.), 2009. **326**(5960): p. 1694-1697.

58. Huse, S.M., et al., *A Core Human Microbiome as Viewed through 16S rRNA Sequence Clusters.* PLOS ONE, 2012. **7**(6): p. e34242.

59. Jeon, J., et al., *Dynamic Changes in the Gut Microbiome at the Acute Stage of Ischemic Stroke in a Pig Model.* Front Neurosci, 2020. **14**: p. 587986.

60. Sampaio-Maia, B., et al., *Chapter Four - The Oral Microbiome in Health and Its Implication in Oral and Systemic Diseases*, in *Advances in Applied Microbiology*, S. Sariaslani and G. Michael Gadd, Editors. 2016, Academic Press. p. 171-210.

61. Leite, G.G.S., et al., *Mapping the Segmental Microbiomes in the Human Small Bowel in Comparison with Stool: A REIMAGINE Study.* Digestive Diseases and Sciences, 2020. **65**(9): p. 2595-2604.

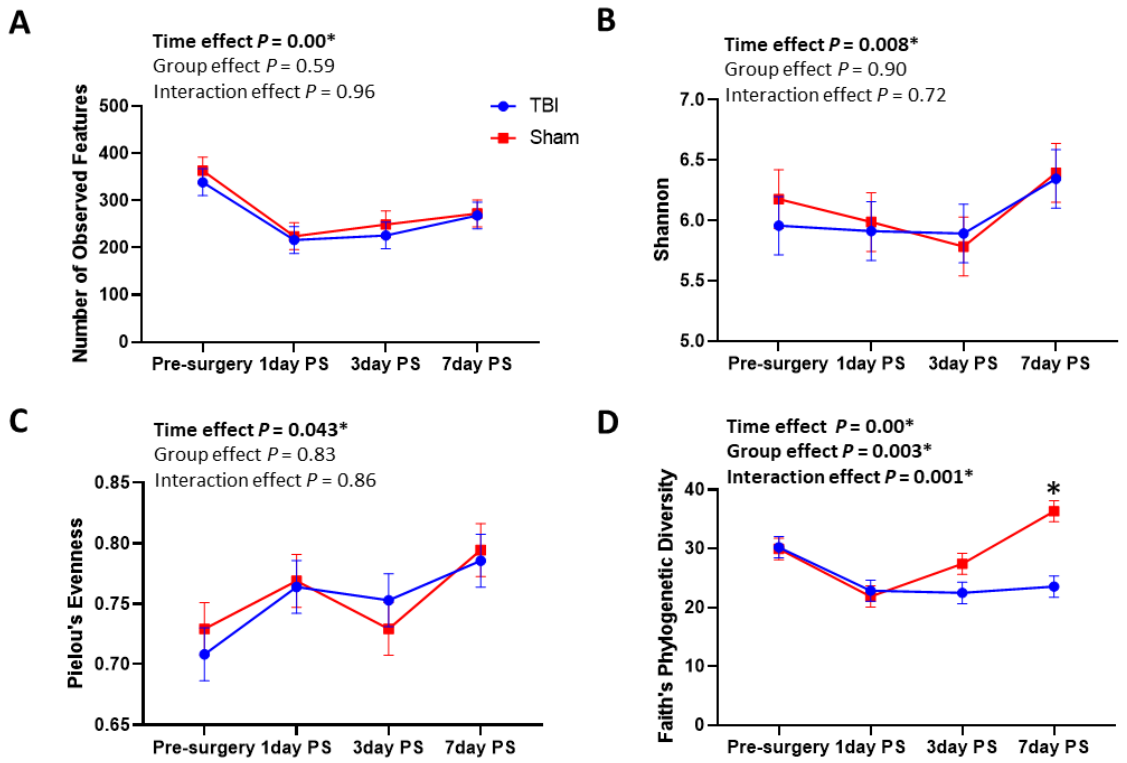

Figure 5.1. TBI did not significantly alter alpha-diversity indexes of the oral microbiome except for Faith's Phylogenetic Diversity. Alpha-diversity indexes were measured by the Number of Observed Features, Shannon, Pielou's Evenness, and Faith's Phylogenetic Diversity. **(A-D)** Significant time effects were found in all alpha-diversity indexes, while no group or time-by-group interaction effects were observed except for Faith's Phylogenetic Diversity. **(D)** Faith's Phylogenetic Diversity was significantly lower in the TBI group (n=6) compared to the Sham group (n=6) at 7 days PS (Time, Group, and Time-by-Group interaction effects $P < 0.05$). ***** : Time-by-Group interaction effect : Tukey post hoc comparison between the TBI and Sham groups. PS : post-surgery

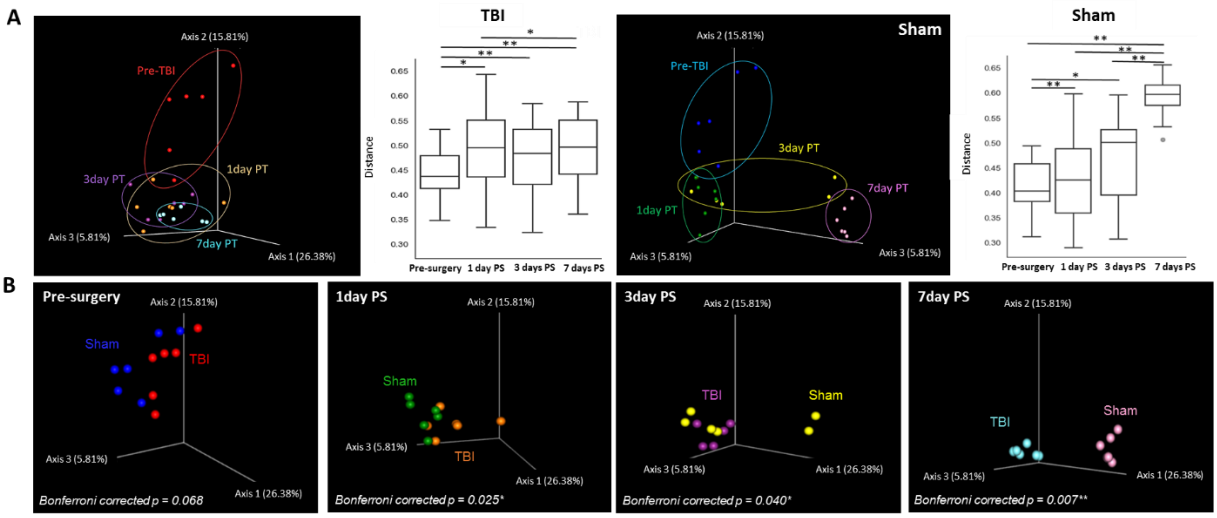

Figure 5.2. Beta-diversity of the oral microbiome was different between TBI and Sham piglets. Unweighted unifrac matrix distance was used to evaluate the microbial pattern within and between groups during the acute stage of TBI. **(A)** Both TBI (n=6) and Sham (n=6) groups showed significant changes in different beta-diversity post-surgery compared to pre-surgery. **(B)** Distinct microbial patterns were observed between the TBI and Sham groups at 1-, 3-, and 7 days PS, with the most apparent difference at 7 days PS. Bonferroni corrected *p*-value : **p* <0.05, ***p* <0.01. TBI : traumatic brain injury, PS : post-surgery.

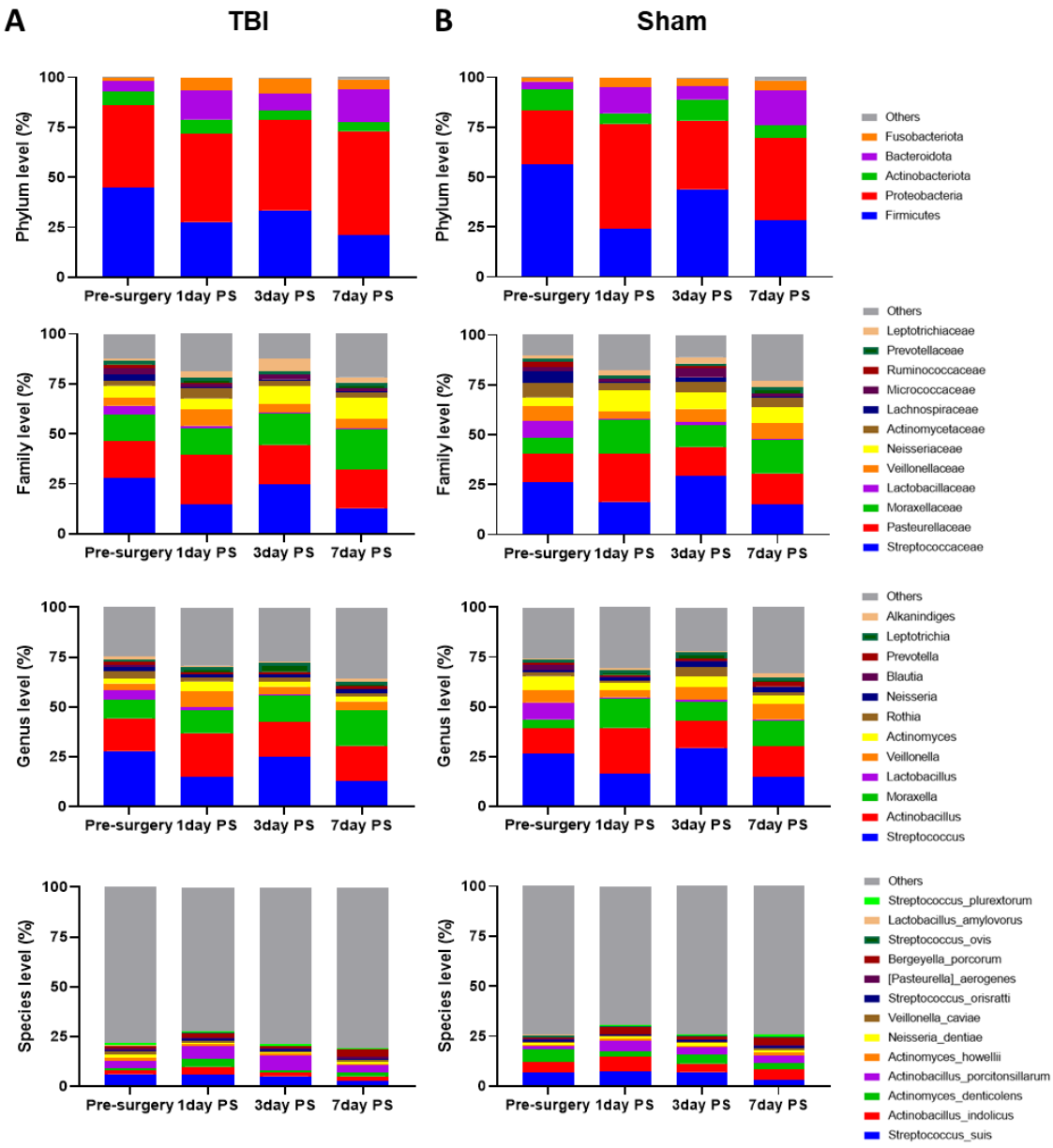

Figure 5.3. TBI did not significantly change the taxonomic composition of the oral microbiome between TBI and Sham piglets. The oral microbial composition was analyzed at the phylum (>1%), family (>1%), genus (>1%), and species (>0.5%) levels pre-surgery and 1, 3, and 7 days post-surgery in **(A)** TBI (n=6) and **(B)** Sham (n=6) groups. There were no significant compositional differences between TBI and Sham groups up to 7 days post-surgery. TBI : Traumatic brain injury, PS : post-surgery

CHAPTER 6

SUMMARY AND CONCLUSIONS

This study has three objectives. First, this study aims to characterize the changes in the gut microbiome during the acute stage of an ischemic stroke and its relationship with stroke severity. Second, it seeks to investigate changes in gut inflammation and membrane integrity in response to brain treatment with intracisternal administration of Tanshinone IIA-loaded nanoparticles (Tan IIA-NPs) and induced pluripotent stem cell-derived neural stem cell (iNSCs) during the long-term ischemic stroke. And third, this study aims to explore the changes in the oral microbiome during the acute stage of traumatic brain injury (TBI).

The study presented in Chapter 3 was conducted with the primary objective to examine the changes in gut microbial composition and diversity during the acute stage of an ischemic stroke, which is up to 5 days post-stroke. In this study, ischemic stroke altered the gut microbial diversity, including alpha- and beta-diversity. The alpha-diversity measured by Shannon and Evenness indexes were lower 1 day post-stroke compared to pre-stroke and the beta-diversity showed an overall tending difference across the time points during acute stage of stroke, with the most distinct changes in microbial pattern 3 days post-stroke compared to pre-stroke. The relative abundance of Firmicutes declined by 27% in stroke, while Bacteroidetes levels tended to increase at 3 days post-stroke compared to pre-stroke. Furthermore, there were perturbations of the gut microbiome at the family and genus levels 3 days post-stroke compared to pre-stroke, with significant increases in opportunistic pathogenic bacteria (Proteobacteria, *Enterobacteriaceae*, and *Desulfovibrionaceae*) and bacteria related to trimethylamine-N-oxide (TMAO) (*Peptostreptococcaceae* and *Prevotella*)

and decreases in beneficial microbes (*Lactobacillaceae* and *Lactobacillus*). Importantly, reduced species diversity, increased pathogenic microbes, and decreased beneficial microbes were correlated with large lesion volume, midline shift, and hemorrhage volume, suggesting that post-stroke gut dysbiosis may negatively affect stroke severity. The study presented in Chapter 3 provides a potential role for the gut microbiome as a useful biomarker for stroke severity.

The study presented in Chapter 4 aimed to examine whether the anti-inflammatory and neuroregenerative treatment delivered to the brain changes gut inflammation and membrane integrity during the 12 weeks of long-term ischemic stroke. The Tan IIA-NPs and iNSCs therapy in the brain improved jejunal inflammation and membrane integrity in the gut of pigs with ischemic stroke, showing the reduced protein levels of TNF-α and TNF-α receptor 1 and increased expression of gut tight junction proteins (occludin, claudin1, and ZO-1) at 12 weeks post-treatment. The treatment also increased the fecal levels of total SCFAs and acetate post-stroke, and those increases were negatively correlated with protein expression of TNF-α, phosphorylated IkB-α, and ZO-1, and binding activity of NF-kB P65 in the gut. The changes in fecal SCFAs post-stroke were also correlated with the brain volumetric changes including lesion volume and midline shift 12 weeks post-treatment. The results of the study presented in Chapter 4 indicate that brain treatment using anti-inflammatory and neuroregenerative agents induced changes in the gastrointestinal system. These findings will provide a better understanding of bidirectional communication between the gut and the brain and suggest the gut-brain axis as a therapeutic target that may affect stroke recovery.

Despite the potential role of the gut microbiome as a biomarker or indicator of brain injuries, sample collection is challenging because about half of the patients admitted to hospital with strokes suffer from swallowing disorders, constipation, loss of bowel control, and fecal impaction. After

the gut, the oral microbiome is the second largest and most diverse microbial community in the human body, and changes in the oral microbiome have recently been observed in patients with Alzheimer's and Parkinson's disease. Therefore, collecting the oral mucosa may allow for easy access to investigating the microbial changes in brain injury patients. The study presented in Chapter 5 examined the changes in the oral microbiome during the acute stage of TBI using a pig model. As a result, TBI induced changes in oral microbial diversity, but no differences were observed in the taxonomical composition of the oral microbiome between TBI and Sham. In alpha-diversity, Faith's phylogenetic diversity was significantly lower following the TBI compared to sham surgery at 7 days post-surgery. Beta-diversity showed different microbial patterns between the TBI and sham surgery, but no significant difference was observed in taxonomic composition between the groups in this model. This is the first study investigating the oral microbial changes in TBI using a pig model, and the findings will provide preliminary evidence for the feasibility of using the oral microbiome in neurological diseases. Future studies are warranted with a larger sample size and longer disease duration to elucidate the role of the oral microbiome in TBI.

In summary, the findings in Chapters 3, 4, and 5 provide insights into how acquired brain injuries induce changes in intestinal and oral microbial homeostasis. Our studies provide evidence that a significant impact of ischemic stroke on gut microbial composition and diversity, and brain-targeted stroke therapy alters intestinal inflammation and membrane integrity. Furthermore, the study suggests that TBI induces potential changes in oral microbial diversity. These provide an important role of inter-organ communications in acquired brain injuries.

APPENDIX

SECONDARY INJURIES FOLLOWING THE PRIMARY INJURY OF STROKE AND TRAUMATIC BRAIN INJURY

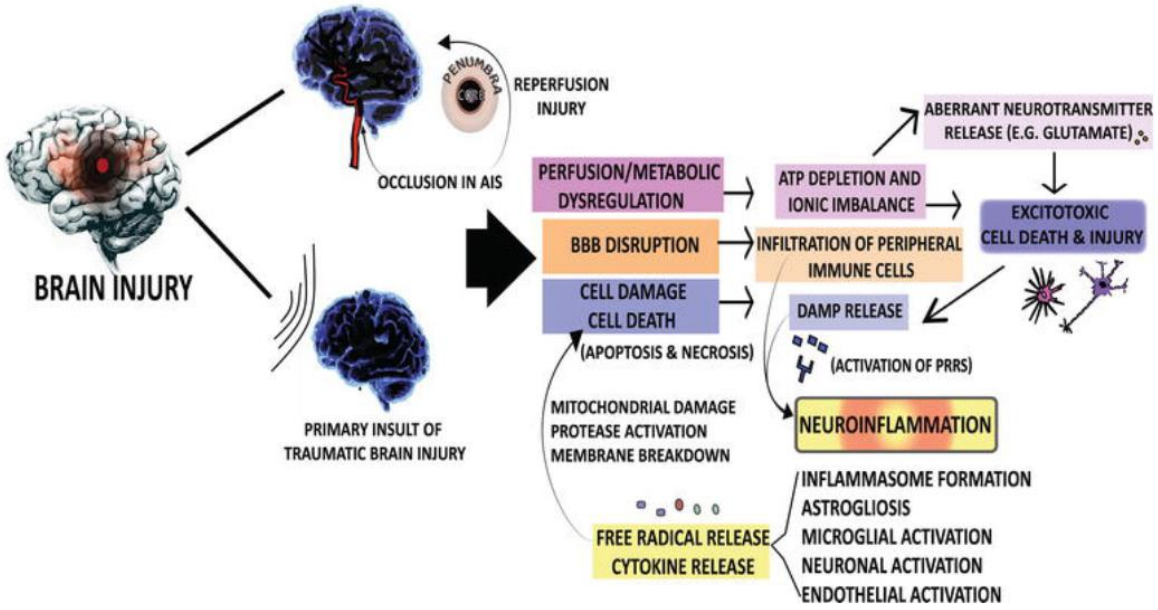

Figure 1. Secondary injuries following the primary injury of stroke and traumatic brain injury. Figure was adapted from *Mechanisms of Neuroinflammation* (p. 211), by R. Dugue et al, 2017, Intech Press.