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Preclinical Characterisation of Fingolimod as a Potential Therapeutic Agent for Stroke

Thesis presented by

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for the degree of Doctor of Philosophy

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Andrea Carolina Diaz Diaz

December 2022

Declaration of author contribution

This is to certify that this thesis was written solely by me with the input and feedback from my supervisory team, with the exception of the intracerebral haemorrhage discussion that was written in conjunction with the authors: Christian Waeber, and Kyle Malone as part of a published manuscript.

I designed the studies, performed surgeries, behaviour tests, data management, and conducted the data analysis. Jennifer Shearer performed most of the histological staining and quantification. Kyle Malone performed the flow cytometry experiments and data collection.

Andrea Carolina Diaz Diaz

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To Benjamín

The baby arriving on this world
the same day as this thesis

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Research stay

I performed a research stay in the research laboratory: Laboratorio de Investigación de Neurociencias Clínicas (LINC) at the Hospital Clínico Universitario in Santiago de Compostela, Spain with Professor Francisco Campos Pérez. The visit was completed between September and October 2016. While there I had hands-on experience learning the mouse model of in situ thromboembolic stroke, as well as reperfusion, as originally described by Orset et al. 2007

Abbreviations

BSU	Biological service unit
dH_20	Distilled water
EMA	European Medicines Agency
FDA	Food and Drug Administration
h	hour
H&E	Haematoxylin and Eosin
i.p	Intra peritoneal
i.v	Intra venous
IVC	Individually ventilated cages
ICH	Intracerebral Haemorrhage
MCA	Middle cerebral artery
MCAo	Middle cerebral artery occlusion
N_2	Nitrogen
NeuN	Neuronal Nuclei
O_2	Oxygen
PFA	Para-formaldehyde
rtPA	Recombinant tissue plasminogen activator
STAIR	Stroke Therapy Academic Industry Roundtable
tMCAo	Transient middle cerebral artery occlusion
tPA	Tissue plasminogen activator
TTC	2,3,5-Triphenyltetrazolium chloride

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Abstract

Stroke is the leading cause for death and disability worldwide, and the search for novel drug treatments has been affected by repeated clinical trial failures. One novel drug that has garnered promising results in preclinical and clinical stroke studies is fingolimod, an FDA approved drug for the treatment of multiple sclerosis. Even though there are several studies supporting the effectiveness of fingolimod for the treatment of stroke, the recommended characterisation based on the Stroke Treatment Academic Industry Roundtable (STAIR) guidelines is incomplete. Furthermore, the quality of the preclinical studies supporting fingolimod has been poor, thus rigorous studies are required to validate the effectiveness of fingolimod prior to evaluation in clinical trials. This thesis aimed to inform whether fingolimod is effective for the treatment of stroke in intracerebral haemorrhage and ischaemic stroke, and to inform whether fingolimod is a good candidate for evaluation in large randomised clinical trials. This goal was achieved by first using a model of intracerebral haemorrhage to evaluate the effect of administering fingolimod at 30 min, 24 and 48 h after stroke on lesion size and behaviour in a 14-day study on male and female mice. This was followed up by a series of studies using middle cerebral artery occlusion to cause a focal ischaemia. First an optimal dose of fingolimod was determined in a dose response study; then we evaluated the optimal drug dose in two animal model of common comorbidities associated with stroke, age and hyperlipidaemia; and the last study evaluated the effect of an extended treatment duration on stroke. For the ischaemic

stroke studies we focused on lesion and behavioural measurements as primary outcomes, and secondary data was collected from daily scores, plus additional measures where necessary. The intracerebral haemorrhage study fingolimod treatment had no measurable effect on either lesion size or behavioural outcomes irrespective of sex, the only finding was that fingolimod treatment reduced mortality in female mice. The dose response study showed no difference in lesion size or behavioural outcomes between the two fingolimod doses (0.5 and 1.0 mg/kg) and control mice, the study did show that saline treated mice had a significantly larger atrophy compared to the lower dose of fingolimod. The lower dose was selected as optimal for further studies. The study evaluating the effect of 0.5 mg/kg fingolimod on stroke in aged mice showed that fingolimod-treated mice had a significantly larger atrophy and a significant improvement in the grid score 7 d after stroke compared to saline-treated mice. The study evaluating the effect of fingolimod on stroke in hyperlipidaemic mice showed that fingolimod-treated mice had a significantly reduced lesion size, without an effect on any other outcome measures. The final study evaluated two fingolimod treatment durations compared to saline controls, the study showed no difference between any of the outcome measures, with a trend towards improved behaviour outcomes in mice receiving 10 d of fingolimod treatment. Lastly, considering the fact that the results of these studies were inconclusive, we decided to pool the data of the ischaemic studies and evaluate whether fingolimod had an effect on the primary outcome measures in a heterogenous animal population. The pooled data showed that fingolimod treatment improved behaviour 7 d after stroke without an effect on lesion size or atrophy. The results of this thesis cast a doubt on the effectiveness of fingolimod and its suitability for translation into larger clinical trials. Furthermore, they highlight the need for thorough preclinical studies for promising drugs as well as the need for studies to meet the proposed STAIR guidelines prior to translation. Confirmatory studies, like those presented here, performed with measures intended to control for internal and external biases are all good measures to be implemented for future studies of novel and highly promising drugs for stroke treatment.

Chapter 1

Introduction

Stroke is an acute event caused by blockage of a brain blood vessel or a ruptured blood vessel haemorrhaging into the brain. The interruption of the blood flow can lead to permanent cell damage depending on the length of time blood flow is interrupted (Hankey, 2017). Individuals presenting with stroke are recognised by the acute and specific neurological deficits associated with the area of the brain affected by the interrupted blood flow (Kothari et al., 1997; Yew and Cheng, 2015). The disruption of blood supply causes the cells activity to slow down, initiating a cascade of molecular events that eventually cause cell death by necrosis, apoptosis or autophagy (Dirnagl et al., 1999; Fisher, 2003).

Ischaemic and haemorrhagic are the two major types of strokes (Fig. 1.1). Ischaemic stroke represents 87% of all stroke events, and they can be further broken down into a variety of types depending on the aetiology and the duration of the blockage (Virani et al., 2020). Some strokes are transient ischaemic attacks (TIA) that can resolve quickly without requiring medical intervention. However, the majority of strokes cause some degree of neurological damage that does not resolve independently and requires medical interventions to mitigate damage (Sommer, 2017). The initial ischaemic lesion is formed within minutes of blood flow interruption, forming the necrotic core due to cellular hypoxia. The adjacent tissue forms the penumbra, an area of salvageable tissue (Hossmann, 1994).

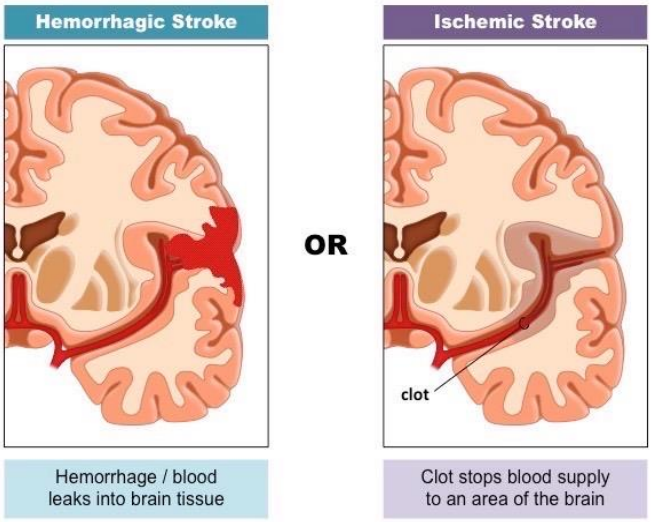

Figure 1.1: Cross-sectional diagram of a human brain with a haemorrhagic (left panel) and an ischaemic stroke (right panel). Image reproduced under permission from (Cornell, 2016).

Intracerebral haemorrhage (ICH) or haemorrhagic strokes are caused by the rupture of a blood vessel and the subsequent accumulation of blood in and around the brain parenchyma, ventricles and subarachnoid space (Sacco et al., 2013). Haemorrhagic strokes represent 10-15% of all strokes; however, they represent 50 - 60% of the morbidity and mortality associated with strokes (Virani et al., 2020; Keep, Hua and Xi, 2012; Donkor, 2018). The primary neurological deficits are caused by the haematoma and mass effect. The mass effect is a product of the accumulated blood that causes a displacement of the brain structures (Keep, Hua and Xi, 2012). The volume of blood released into the parenchyma and the increase in intracranial pressure are major determinants of the outcome post-stroke. Haematoma greater than 150 mL in volume can quickly lead to death from the compression of the brain stem (Xi, Keep and Hoff, 2006). The secondary damage is caused by brain oedema and inflammation caused in response to the injury and from the toxic effect associated with the accumulation and degradation of blood components (Wilkinson et al., 2018).

Despite stroke's major impact, there is only one pharmacological agent specific for the management of ischaemic stroke, tissue plasminogen activator (tPA). There has been extensive research over the last decades to discover and develop novel agents; however, no drug has been proven to be an effective neuroprotector. One group of drugs that has garnered a lot of interest are immunomodulators for their dynamic effects acting both as anti-inflammatory and neuroprotective drugs. There is a large body of research supporting the possibility that immunomodulators, such as fingolimod, may be effective in the management of stroke, yet more research is required (O'Collins et al., 2006; Dang et al., 2020).

The overarching goal of this thesis was to determine the effectiveness of fingolimod for the treatment of stroke in a rigorously designed preclinical study. This chapter will briefly introduce the pathophysiology of ischaemic and haemorrhagic stroke and discuss the currently available treatments for stroke. The chapter will also introduce the current state of stroke preclinical research, covering the current guidelines and model of stroke. Lastly, it will provide a background on fingolimod as a potential drug for the treatment of both haemorrhagic and ischaemic stroke and explain what makes fingolimod such a promising candidate for stroke treatment.

1.1. Stroke

1.1.1. Stroke Epidemiology

Stroke is one of the leading causes of disability worldwide, and according to The World Health Organisation, it is the second-largest cause of death (Virani et al., 2020; World Health Organization, 2018). The report from the 2016 Global Burden of Disease (GBD) Collaborators identified 80.1 million cases of stroke reported worldwide, and the mortality was 5.5 million people from all strokes, of which 2.7 million individuals died from ischaemic and 2.8 M haemorrhagic strokes (GBD 2016 Stroke Collaborators et al., 2019). The National Stroke Register of Ireland reported an incidence of 4,817 cases of stroke in 2018 (National Stroke Programme, 2019).

Men and women are both susceptible to stroke, and the risk and survival odds vary depending on age. At a younger age, men have a higher incidence of stroke compared to women and the incidence shifts after 85 years of age when women have a dramatic increase in the incidence of strokes (Manwani and McCullough, 2011). However, according to the GBD, females had a higher prevalence of stroke in 2016 (41.1 million) than males (39.0 million) (GBD 2016 Stroke Collaborators et al., 2019). The fact that women account for a higher percentage of the total deaths associated with stroke worldwide might be explained by differences in the age of the first stroke between males and females (69.2 vs 74.5 years) and the differences in life expectancy between the sexes (Virani et al., 2020).

Age is considered the strongest non-modifiable factor influencing the risk of stroke, and the risk doubles every decade past the age of 55 for people with other comorbidities and risk factors (Donkor, 2018). Yet, stroke is considered a preventable disease because it correlates with modifiable risk factors and lifestyle measures that can reduce the risk as age progresses. The modifiable risk factors associated with stroke were quantified in a recent case-control study involving 26,919 participants (10,388 ischaemic strokes, 3,059 intracerebral haemorrhage and 13,472 controls) from 32 countries; the ten most common were hypertension, physical activity, apolipoprotein ratios, diet, waist-to-hip ratio, psychosocial factors, smoking, cardiac issues, alcohol consumption and diabetes (O'Donnell et al., 2016). Furthermore, the GBD stroke collaborators attribute 91% of

strokes to similar modifiable risk factors that can vary between different regions and ethnicities (Virani et al., 2020; GBD 2016 Stroke Collaborators et al., 2019; O'Donnell et al., 2016).

The most common risk factors associated with intracerebral haemorrhage (ICH) are high blood pressure and amyloid angiopathy; less frequently ICH, is associated with brain tumours and cerebral aneurysms (Xi, Keep and Hoff, 2006). ICH has the highest mortality of all strokes, and it is among the leading causes of death and long-term disability worldwide (Benjamin et al., 2019). Low to middle-income countries also have the highest incidence and associated mortality, which represents 80% of the total ICH cases in the world (Katan and Luft, 2018).

The high incidence of stroke correlates with a high financial burden associated with the acute and chronic care and management of this condition because people that survive a stroke experience various degrees of disability. The financial burden of stroke can be measured starting from acute to long-term care and rehabilitation (Rajsic et al., 2019). In Europe, it has been estimated that the total healthcare cost of stroke was €27 billion in 2017. In the same year, in Ireland, the healthcare-related cost of strokes represented €172 million, less than 1% of the total healthcare expenditure of the country (Luengo-Fernandez et al., 2019). The burden of disability and the financial burden to families and countries makes the development and characterisation of novel drug treatments a critical area of research for preventing and managing recovery after stroke.

1.1.2. Stroke Prevention

The post-stroke treatment has many limitations and contraindications that restrict the time window of administration, as is the case with tPA administration, which limits the population for whom treatment is available. Thus, stroke prevention is the most effective intervention for the management of stroke. The American Heart Association has set forth evidence-based guidelines for the primary prevention of stroke to guide physicians on preventative treatment selection based on the modifiable and non-modifiable risk factors of each patient (Meschia et al., 2014). Age, genetics, and ethnicity are non-modifiable risk variables that cannot be addressed with behavioural changes or pharmacological therapies; however, modifiable risk factors can be pharmacologically controlled to reduce the incidence of stroke, i.e., dyslipidaemia, hypertension and diabetes. Other diseases like atrial fibrillation and coagulopathies, which increase the risk of an ischaemic stroke, can

only be treated with drugs that increase the risk of ICH (i.e., aspirin, warfarin) (Meschia et al., 2014).

After the first stroke, the incidence of stroke increases for an individual; hence, secondary stroke prevention is an important aspect of stroke treatment. Secondary prevention involves managing the risk factors that can lead to a repeat ictus with drugs and behaviour modifications similar to primary prevention. Treatments to prevent further strokes vary depending on the aetiology of the stroke (i.e., strokes caused by an embolus, a clot or a thrombus) and the risk factors of the individual. Behaviour modifications include changes in activity level and patient's diet to control comorbidities like diabetes and hypercholesterolemia. Furthermore, statins are prescribed when behaviour modifications are not enough to address lipid levels and reduce atherosclerotic related risk (Graham, 2008).

There are two classes of antithrombotic drugs effective for stroke prevention (anti-platelets and anticoagulants) that are recommended after stroke. Anti-platelet therapy reduces the 90-day risk of stroke or death. Furthermore, anticoagulant therapy for ischaemic strokes of arterial origin is just as effective at reducing the risk of repeat ictus as anti-platelet therapy (Meschia et al., 2014; Costa, Windecker and Valgimigli, 2017). High blood pressure is another risk factor for ischaemic and haemorrhagic strokes. Managing blood pressure by maintaining it below 120 mmHg reduces the risk of stroke in older individuals (Spence et al., 2020).

1.2. Stroke Pathophysiology

Stroke pathophysiology is described as a cascade of events that begins with the disruption of cerebral blood flow (CBF). Persistent low levels of perfusion cause the development of an ischaemic core in both ischaemic stroke and haemorrhagic strokes. The ischaemic core is an area that becomes necrotic and permanently damaged within minutes after blood flow interruptions. The surrounding tissue has low levels of CBF, enough to supply energy and to sustain a resting membrane potential for a short period of time but not enough energy to evoke action potentials. This area is known as the penumbra, an area of salvageable tissue that can be rescued if CBF is restored (Hossmann, 1994). The penumbra slowly disappears as cells start dying from the prolonged lack of energy, causing the expansion of the ischaemic core (Astrup, Siesjö and Symon, 1981; Hossmann, 2011).

Over the years, basic research has helped understand the pathophysiological cascade of events in ischaemic and haemorrhagic stroke. The development of neuroprotective drugs has focused on interfering with the tissue damage cascades, e.g. O_2 depletion and glutamate release, by designing drugs that counteract the events (Wilkinson et al., 2017). Animal models have helped in the understanding of the penumbra and the expansion of the ischaemic core and the contribution of cortical spreading and depolarisation mechanisms to this expansion (Fig. 1.2) (Hossmann, 1994, 2006). Advances have also been made in the understanding of inflammation, the vascular unit and immunological system and their involvement in stroke in endogenous protective mechanisms (Kleinig and Vink, 2009; Malone et al., 2019; Lambertsen, Finsen and Clausen, 2019).

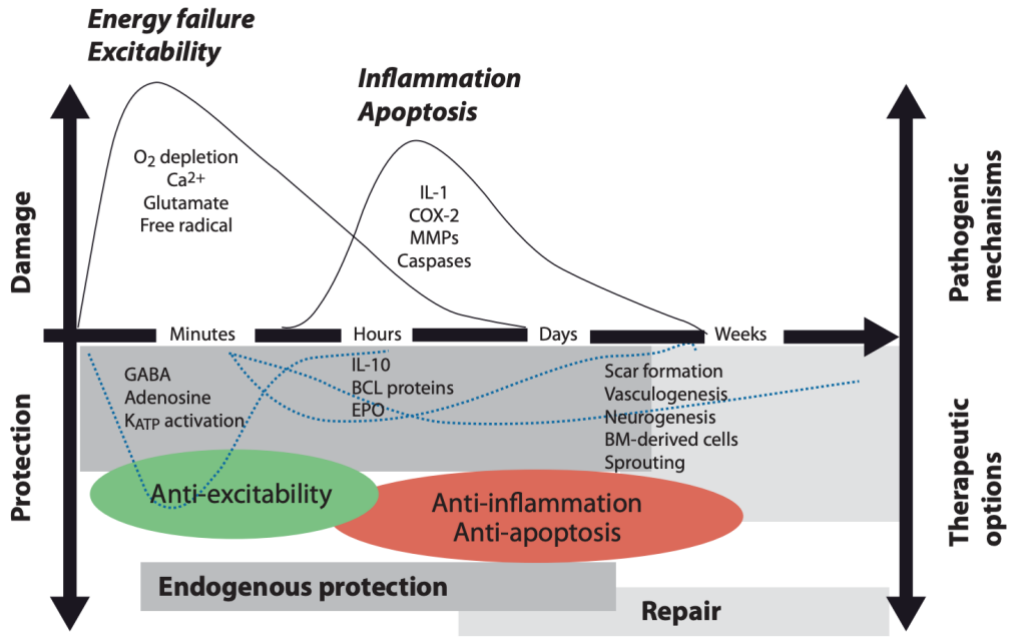

Figure 1.2: Stroke Pathophysiology progression from acute to chronic stages of the disease, damage and protection are on one side of the diagram, and pathogenic mechanism and therapeutic options are on the other end of the graph. The x axis represents time and the progression from damage to repair and energy failure to inflammation and apoptosis from minutes to weeks (Gutiérrez et al., 2009). Reproduced under copyright authorisation from Karger Intl.

The acute treatment for stroke aims to limit the ischaemic core expansion primarily by reperfusion. However, it is also important to support the recovery of the nearby tissue with neuroprotective drugs aimed at modulating the cascade of events and rescuing the cells in the penumbra and nearby tissue (Fig. 1.2). Inflammation is directly associated with stroke pathology because it has been identified as a major contributing factor for poor outcomes in stroke recovery. Inflammation can be modulated by the local and infiltrating immune cells and managing their response (Lambertsen, Finsen and Clausen, 2019; Anrather and Iadecola, 2016). Furthermore, common comorbidities are associated with low-grade inflammation that leads to a heightened inflammatory response in the brain after stroke (Przykaza, 2021). Thus, inflammation has become a research target for drug development for many years (Chen, Shao and Ma, 2021).

1.2.1. Ischaemic Stroke Pathophysiology

The expansion of the ischaemic core follows a series of events that lead to cell death by autophagy, necrosis or apoptosis. The initial events are associated with energy failure and an inability of the neurones and glia to maintain homeostasis and cell membrane

polarisation (Khoshnam et al., 2017). At the same time, there is a release of glutamate into the synaptic cleft, causing excessive calcium influx that, in turn, activates calcium dependent proteases, lipases and DNAses, leading to apoptosis. Furthermore, sodium influx is accompanied by water, causing cell swelling, oedema and reduction of extracellular space that further restricts blood flow (Lipton, 1999).

The expansion of the damaged area is also facilitated by cortical spreading depression (Hossmann, 1994), a phenomenon causing intense depolarisation of neurones and glia that propagates across neighbouring cells, furthering the loss of cellular activity and increasing swelling associated with extracellular potassium concentrations exceeding critical thresholds (Xing et al., 2012). Cell depolarisation ultimately leads to brain inflammation and oedema, a product of the abnormal ionic gradients generated by cell lysis that is exacerbated by the accumulation of blood products as the blood-brain barrier breaks down.

The cells in the ischaemic core release their contents and trigger the activation of microglia (resident immune cells) and the recruitment of peripheral immune cells (Esposito et al., 2019). The microglia activation and the infiltration of the peripheral immune cells are triggered by the recognition of damage-associated molecular patterns (DAMPs) released by dying cells. Cell death also activates the innate immune system through antigen presentation of cell proteins by antigen presenting cells such as dendritic cells (Anrather and Iadecola, 2016; Iadecola and Anrather, 2011). Locally, microglia focus on phagocytosing, clearing debris, and modulating early response by interacting with other immune cells and releasing cytokines. Early on after stroke, astrocytes also start to multiply (astrogliosis), sealing off the necrotic core scar. However, in the later stages of recovery, excessive scaring can interfere with the revascularisation of the area and limit nutrient diffusion necessary for neurogenesis (Levard et al., 2020).

The immune response after stroke involves the innate and adaptive immune systems. The response from the innate and adaptive immune system varies over time from pro-inflammatory to anti-inflammatory states. Correspondingly, these can have a negative or positive effect on recovery following stroke. Inflammation can be associated with adverse outcomes, especially in cases where there is an over-reactive immune response (Anrather and Iadecola, 2016; Harris, Rooij and Kuhl, 2019).

Neutrophils are the first responders of the innate immune system, invading the brain as early as 3 h after stroke and reaching a peak by day three (Perez-de-Puig et al., 2015). Macrophages can differentiate into M1 and M2 states and perform inflammatory or anti-inflammatory functions, respectively. The activation depends on the cytokines and

DAMPs present in the interstitial space; thus, in the early stages after stroke, there are primarily M1 (pro-inflammatory) macrophages (Kronenberg et al., 2018; Benusa, George and Dupree, 2020). Later on, the macrophages start to shift to an M2 (anti-inflammatory), helping with the recovery process after stroke.

The adaptive immune system also responds after stroke insult and the release of inflammatory cytokines. The response involves T cells that infiltrate and differentiate into cytotoxic T cells or regulatory T cells (Tregs). The activated Tregs have been associated with balancing between pro- and anti-inflammatory responses (Fu et al., 2015; Yoshimura and Ito, 2020). Furthermore, Tregs have been reported in preclinical studies to promote an anti-inflammatory environment by inhibiting the secretion of pro-inflammatory cytokines, and they are associated with better outcomes and improved recovery after stroke (Wang et al., 2021).

Furthermore, several studies have found that B cells also have an effect on stroke recovery as they become activated and recognise self-antigens of the brain cells as part of the adaptive immune response to stroke (Hum et al., 2007; Ren et al., 2011; Ortega et al., 2015, 2020). The use of immune deficient mice (SCID) lacking both B and T cells showed a reduced lesion, implicating both cells in the inflammatory process after stroke (Hum et al., 2007). Additional studies have shown positive effects associated with the presence of B cells in the injury, and this is supported by the fact that mice lacking B cells had larger lesion sizes and higher mortality than wild type mice (Ren et al., 2011). These results reflect the complexities of the recovery following stroke and the dynamic changes involved in the pathology and recovery, as well as the involvement of different immune cell types.

The possibility of modulating the immune response has further developed an interest in immunomodulatory drugs that can also interfere with the response of macrophages and astrocytes, as well as interfere with inflammatory cytokines (IL-1β and TNFα) (Malone et al., 2018). Immunomodulation has become a major area of research focused on understanding the pathways involved in recovery and the inflammatory response after stroke (Malone et al., 2018), and it is an active area of research for drug development (Malone et al., 2019; Lambertsen, Finsen and Clausen, 2019; Ren et al., 2020). Neuroprotective drug research has focused on the period in which there is salvageable tissue (penumbra) by limiting the expansion of the necrotic core and reducing apoptosis. Clinical results with these drugs have been disappointing, possibly because they target events occurring during a short time window. Yet, there is still a chance to limit the damage by treating with immunomodulators that can target inflammation, a more

protracted phenomenon, and prevent an overreactive immune response after stroke (Fig. 1.2).

1.2.2. Haemorrhagic Stroke Pathophysiology

Specifically in haemorrhagic stroke, the pathophysiological cascade is triggered by the accumulation of blood within the parenchyma of the brain. This can be divided into the physical and molecular aspects associated with blood accumulation and haematoma. The physical aspect relates directly to the volume and location of the blood and is associated with its accumulation in the parenchyma, causing intraventricular distention (Asch et al., 2010; Zhu et al., 2019). Bleeding stops for most patients after the initial ictus, but some continue to bleed and suffer from haematoma expansion and mass effect. The haematoma expansion limits blood flow to the nearby tissue, and it negatively correlates with the chances of survival. The increase in intracranial pressure and mass effect cause further damage, necrosis and death (Ye et al., 2020).

The molecular aspects of the injury are associated with the blood, clotting cascade, cell lysis, and the accumulation of haemoglobin in the parenchyma of the brain. Haemoglobin, iron and clotting cascade components have each been independently identified to negatively impact the lesion following stroke causing inflammation and apoptosis (Xi, Keep and Hoff, 2006; Wilkinson et al., 2018). The cascade of events leads to oedema and inflammation following the initial injury, that can further increase intracranial pressure and disrupt blood flow.

Furthermore, the infiltrating macrophages and local microglia are activated and contribute to the inflammatory response by the secretion of cytokines and phagocytosis of haemoglobin and cellular debris (Zhu et al., 2019). In contrast to the inflammatory of ischaemic stroke that operates over days or even weeks, the inflammatory response after ICH is much faster. The inflammatory response is triggered by the extravasated blood that quickly activates both the innate and adaptive immune system in response to exposure to antigens previously unavailable to the immune system (Keep, Hua and Xi, 2012; Tschoe et al., 2020). Being that inflammation is a shared factor between ischaemic and haemorrhagic strokes, various anti-inflammatory agents have been tested for both types of stroke (Kleinig and Vink, 2009).

1.3. Clinical Management Of Stroke Patients

Stroke recovery is highly dependent on the type of stroke and the access to medical treatment. The goal of stroke treatment and management is to minimise brain tissue damage, disability and risk of death. The availability of adequate medical care shortly after people first experience stroke symptoms is critical because early intervention highly correlates with recovery and survivability (Hossmann, 2006; Powers et al., 2019).

Recanalisation is the only available specific treatment for ischaemic stroke. While it can be spontaneous, as in transient ischaemic attacks, most cases require medical interventions. Recanalisation can be achieved by thrombolysis with tissue plasminogen activator (tPA; Alteplase), or by mechanical removal of the clot or embolus. However, the implementation of these treatment modalities are restricted to patients arriving at the hospital within a narrow time window from the onset of symptoms, limiting their use (Meschia et al., 2014). On the other hand, haemorrhagic stroke management is more dependent on the arrival of the patient to a hospital with a capable team for the clinical management of ICH because there are no specific pharmacological treatments for intracerebral haemorrhage.

1.3.1. Stroke Units

The most effective care for patients with stroke is achieved in highly specialised stroke units. The stroke units have been developed on the basis of many clinical trials aimed at refining and improving the standards of care. There are specialised stroke units that focus on ischaemic stroke and haemorrhagic stroke care. They are composed of a multi-disciplinary team that can diagnose, treat and stabilise the patient in the shortest amount of time after arriving at the hospital (Ringelstein et al., 2013; Hostettler, Seiffge and Werring, 2019). The highly specialised stroke units offer a comprehensive team of neurologists, neurosurgeons, radiologists, intensivists, emergency and internal medicine specialists that can increase the odds of survival for the most deadly types of stroke

(Hostettler, Seiffge and Werring, 2019). These specialised facilities also include rehabilitation, physical and occupational therapy as part of the treatments to aid the long-term recovery of stroke patients (Gonzales and Grotta, 2016).

The treatments are defined by pre-established algorithms that allow the medical team to quickly determine individualised care based on the specific characteristics of each patient. The algorithms were developed to increase the number of patients that receive the best care possible and to expand the usage of thrombolysis (tPA) by standardising the selection of ischaemic stroke patients (Hankey, 2017). Patients will also be treated to prevent deterioration and secondary strokes by controlling blood pressure, blood glucose levels and other common comorbidities that patients have at the time of stroke (Powers et al., 2019; Specogna et al., 2014).

1.3.2. Ischaemic Stroke Treatment

1.3.2.1. Tissue Plasminogen Activator

Tissue plasminogen activator (tPA) is the only drug approved by regulatory agencies for the reperfusion and recanalisation of brain arteries. The drug proved effective for reducing stroke injuries in two pilot studies evaluating safety in 0-90 min and 91-180 min time windows from symptom onset (Brott et al., 1992; Haley et al., 1992). The follow-up double-blinded randomised trial carried out by the National Institute of Neurological Disorders and Stroke (NINDS) confirmed the safety of administering tPA within 3 h of symptom onset. It showed that reduced neurological deficits and improved clinical outcomes three months after stroke outweighed the risk of haemorrhagic transformation and provided the first guidelines for the use of tPA (National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995).

In Europe, tPA was approved for clinical use by the European Medicine Agency after the completion of The Safe Implementation of thrombolysis in Stroke-Monitoring Study (SITS-MOST). The study evaluated the safety and best therapeutic window and confirmed that a 3 h treatment window was adequate, with very low rates of haemorrhagic transformation at 24 h (1.7%) (Wahlgren et al., 2007). In an effort to expand the treatment window, two clinical trials evaluated a treatment window of up to 6 h from the onset of symptoms, The European Cooperative Acute Stroke Study (ECASS) and ECASS II

(Hacke, 1995; Hacke et al., 1998). However, these studies failed to show an overall positive effect of a 6 h treatment window.

A systematic review and meta-analysis of the ECASS and ECASS II clinical trials determined that a treatment window extension of up to 4.5 h after symptom onset provided the best outcomes. The updated treatment window (3-4.5h) was confirmed by the ECASS III clinical trial, proving that treatment with alteplase in a subset of eligible patients improved clinical outcomes even though there was an increased frequency of haemorrhagic transformation associated with tPA (Hacke et al., 2008). Based on these and other studies, an extensive list of criteria that a patient must meet was developed to determine eligibility for tPA treatment, summarised in table 1.1 (Powers et al., 2019).

Even though thrombolysis with tPA is a lifesaving treatment, there is still a significant number of patients that do not receive thrombolysis even when they might benefit from treatment. In Ireland, only 11.2% of patients presenting with ischaemic stroke received thrombolysis in 2018, and the remainder of the patients were not treated because of contraindications (61%) or non-specified reasons (27.4%) (National Stroke Programme, 2019). This shows that there is a substantial number of patients that would benefit from novel treatment options that can protect the brain and limit disability, especially for patients that do not and cannot receive recanalisation treatment. Furthermore, a novel treatment could expand the use of tPA by limiting the side-effects associated with delayed tPA treatment. One example of such a novel drug that can expand the treatment window is fingolimod because it reduced haemorrhagic transformation in a mouse model of ischaemic stroke that was treated with tPA and improved outcomes in the case of non-recanalisation (Campos et al., 2013). These preclinical findings were later evaluated in a small proof of concept clinical trial wherein fingolimod reduced reperfusion injury and improved outcome measures (Zhu et al., 2015).

Table 1.1: Up-to-date summary of the most common inclusion and exclusion criteria for intravenous thrombolysis

Inclusion criteria:
< 3 h from symptom onset
• Age ≥ 18 years • Acute stroke with clear symptomatology (mild to severe and disabling symptoms)
3 - 4.5 h
• Age ≤ 80 years • No history of diabetes or prior stroke • NIHSS score ≤ 25 • Not on oral anticoagulants • Imaging reports that the injury is not greater than one-third of the MCA territory
Exclusion criteria
• Intracranial haemorrhage in CT or MRI • Brain CT with clear hypo-attenuation • Prior ischaemia within 3 months • Intracranial or spinal surgery 3 months prior • History of intracranial haemorrhage • Symptomatology suggesting subarachnoid haemorrhage • Gastrointestinal bleeding event reported within the last 21 d • Platelets < 100.000 mm3 • Treatment with low molecular weight heparin within the last 24 h • Warfarin and use of factor Xa inhibitors • Suspicion of infective endocarditis • Known or suspected aortic dissection • Intra-axial intracranial neoplasm • Unruptured or unsecured aneurysm • If MRI is available: high burden of cerebral micro-bleeds

Adapted from (Powers et al., 2019)

1.3.2.2. Mechanical Thrombectomy

Mechanical thrombectomy is an endovascular technique adapted from cardiovascular procedures that has been successfully used in the removal of an embolus from brain arteries. This is a novel procedure based on stent and clot retrievers commonly used in the cardiovascular field (Meyers et al., 2011; Smith and Furlan, 2018). The procedure has expanded the treatment options available for recanalisation, especially for those patients with large brain vessel occlusions. The American Heart Association recently presented guidelines intended to expand the use of endovascular procedures in stroke patients (Powers et al., 2019).

A meta-analysis of multi-centre clinical trials found that endovascular procedures

improved neurological outcomes and 90-day survivability after stroke without increasing the risk of haemorrhagic transformation when compared to tPA or standard of care (Badhiwala et al., 2015). Furthermore, recent clinical trials have evaluated the potential use of mechanical revascularisation in combination with tPA and other neuroprotectants with the aims of improving life expectancy, degree of disability and functional independence (Badhiwala et al., 2015; Fischer et al., 2017; Yang, Yang and Chen, 2019). The meta-analysis of the small proof of concept trial shows no difference between the tPA alone and tPA in combination with mechanical thrombectomy, and yet a larger randomised control clinical trial might be necessary (Phan et al., 2017). Neuroprotectants, like K3A-APC (a recombinant variant of human activated protein C), have been found promising for the reduction of haemorrhagic transformation (Lyden et al., 2019), and others, like fingolimod, have been proposed for clinical trial evaluation in combination with mechanical thrombectomy (NCT04718064 - Revascularization Pretreated With Fingolimod in Acute Stroke - REPAIR FAST).

1.3.2.3. Other Available Treatments

There are additional treatment options for patients that can be used in conjunction or independently for recanalisation. Prolonged treatment with anti-platelets, i.e., aspirin, is recommended for all patients within 24-48 h after onset of symptoms, but not as a replacement for tPA in eligible patients. For patients that receive tPA, aspirin should be initiated 24 h after infusion. Anticoagulant usage is not a well-established treatment following an ischaemic stroke, and more research is necessary to establish the safety and use of thrombin inhibitors at the acute and subacute treatment phases (Powers et al., 2019). Lastly, acute and chronic blood pressure management after stroke is necessary for the prevention of repeat strokes.

1.2.3. Haemorrhagic Stroke Treatment

Unlike ischaemic stroke, haemorrhagic stroke does not have a specific pharmacological treatment that has been proven effective. Clinical trials are underway evaluating anti-inflammatory drugs and drugs promoting haematoma and iron clearance

(Wilkinson et al., 2017). The cell surface marker CD47, a “do not eat me” marker, can be blocked with an antibody and in both mice, and aged rats, it has led to effective and accelerated haematoma clearance (Jing et al., 2019; Tao et al., 2019). Other work has focused on targeting the immune system with the development of treatments targeting Sphingosine-1-Phosphate receptors, Toll like receptor 4 and Cyclooxygenase 2 to reduce inflammation and manage cell infiltration (Shao et al., 2019).

High blood pressure and anticoagulant prescriptions are two of the major causes of haemorrhagic stroke; thus, the focus of the current standard of care is to control their effects after the stroke. The treatment course involves the medical management of blood pressure and coagulopathies to control the volume and limit the expansion of the haematoma. It is recommended that systolic blood pressure values be lowered to values equal to or below 130-140 mmHg within an hour of admission to prevent adverse events (Hostettler, Seiffge and Werring, 2019). Traditional methods for counteracting the anticoagulant therapies with platelet transfusions have shown negative effects in various clinical trials, and newer therapeutic agents are being evaluated to reverse the effects of both direct oral anticoagulants and vitamin K antagonists (for review, see (Kuramatsu, Sembill and Huttner, 2019)).

International Surgical Trial in Intracerebral Haemorrhage (STICH, STICH II) evaluated open skull surgery to remove the haematoma in two clinical trials that failed to show benefits when compared to conservative (non-surgical) treatments (Wilkinson et al., 2017). Recommended surgical or endovascular evacuation of the haematoma for some patients depends on the size and location of the haematoma (Keep, Hua and Xi, 2012; Luzzi et al., 2019). Patients with a moderate to large haematoma undergoing surgical evacuation show a reduction in mortality compared to non-surgical controls (Hegde, Menon and Kumar, 2019). However, more conservative stroke management recommends surgery only for patients with a haemorrhage smaller than 30 mL in volume, and there are still debates on what is the best timing for any surgical procedure (Luzzi et al., 2019). Less invasive catheter based procedures to reduce the size of the haematoma have been clinically evaluated in several trials. However, the recent report from the Minimally Invasive Surgery Plus Rt-PA for ICH Evacuation Phase III (MISTIE-III) clinical trial did not show improved functional outcomes though there were trends for reduced mortality at 365 d in the experimental arm compared to standard of care (Hanley et al., 2019).

1.4. Preclinical Stroke Research

Basic stroke research has been evaluating the cellular and molecular mechanisms of stroke for decades; preclinical research has focused on identifying potential drugs for clinical use. Basic stroke research has elucidated the cascade of events following the reduction in CBF in ischaemic models ranging from global to focal ischaemia. The knowledge gained about the mechanisms involved in stroke pathology has allowed for the development of drug targets that modify different injury pathways (Dirnagl and Endres, 2014). Neuro- and cytoprotection have been targeted with anti-inflammatory drugs, antioxidants, thrombolytics, stimulants, calcium and excitotoxicity modulating drugs (O'Collins et al., 2006). However, all of the time and money invested in preclinical and clinical studies have failed to produce clinically effective drugs; there have been no new pharmaceuticals specifically approved for the acute treatment of stroke since tPA was approved in 1995 (Hankey, 2017; National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995).

Many drugs with different modes of action, have been tested in preclinical studies and clinical trials. Glucocorticoids, mineralocorticoids, and cyclooxygenase (COX) inhibitors have been evaluated for their anti-inflammatory effects and potential benefits on stroke, and even though some produced promising effects in pilot studies (preclinical and clinical), they have all failed in large randomised controlled clinical trials (O'Collins et al., 2006). The interest in anti-inflammatory drugs remains; however, it is now focused on immunomodulatory drugs that affect lymphocytes populations and immunoreactivity after stroke. Immunomodulators as anti-inflammatory drugs have shown promise in preclinical research, and some are in the early stages of clinical trials for the treatment of stroke, including fingolimod, because they limit the immune cell infiltration and reactivity after cell death in stroke (Malone et al., 2019).

Clinical trials have been impacted by repeated failures to translate potential drugs from the laboratory into the clinic, leading to a translational gap that consequently exposed a reproducibility crisis. O'Collins and colleagues evaluated the drugs selected for clinical trials and examined which drugs were taken forward to clinical trials based on 1,026 drugs tested in animals (O'Collins et al., 2006). Their findings showed that the drugs chosen for clinical testing (114) did not show more robust efficacy in animal studies than the drugs that were not selected for clinical studies (912). This observation led the

authors to question whether the best drugs are being selected for clinical trials (O'Collins et al., 2006; Dirnagl, 2006; Ioannidis, 2017).

The effect of publication bias and small sample sizes are other aspects related to the quality and reproducibility of research that have been identified through a meta-analysis of preclinical studies of drugs that failed in clinical trials. The publication bias favours positive findings, and this is supported by the near absence of published studies showing no-effect or negative findings, which leads to the overestimation of drug efficacy (Sena et al., 2010). Furthermore, the small sample sizes frequently used in basic and preclinical studies reduce the likelihood that a statistically significant finding represents the true effect (Button et al., 2013; Tsilidis et al., 2013). All of these factors highlight the possibility that a proportion of published work might be false positives and that drugs selected for clinical trials are bound to fail (Ioannidis, 2005).

Even though much continues to be said about the reproducibility crisis, only a few researchers have adopted methodologies that reduce the effect of internal and external bias in their work (Tsilidis et al., 2013). A study performed to evaluate the quality and extent of methodological rigour improvement in cardiovascular studies over the past decade revealed that only 1 in 5 articles reported randomisation, a third blinding and about 2% performed sample size estimations (Ramirez et al., 2017).

The external validity and reproducibility of the studies also rely on the selection of the animal models, first by modelling the disease with different methods of causing stroke and modelling underlying comorbidities along with stroke to closely mimic the human population that suffer from strokes (Sommer, 2017). However, comorbidities commonly associated with stroke patients are rarely used when characterising promising drugs, a factor that could improve the translatability of the experimental results (Kleinschnitz, Fluri and Schuhmann, 2015; Cho and Yang, 2018).

As part of the effort to bridge the translational gap, several sets of guidelines have been proposed by expert groups to promote well-designed rigorous research. The ultimate goal of the guidelines is to overcome the reproducibility crisis and identify the best drugs for clinical evaluation in a more robust manner. Conducting research studies with rigour involves following expert guidelines as well as adhering to general principles of preclinical study design, and differentiating between exploratory and confirmatory studies (Macleod and Mohan, 2019; Dirnagl, 2019). One part of the recommendation involves the pre-registration of the study and the methods of analysis intending to reduce p-hacking and data manipulations to achieve significant finding that is driven by the publication bias in favour of positive findings (Ioannidis et al., 2014; Nosek et al., 2018).

1.4.1. Stroke Research Guidelines

Several guidelines have been introduced that are specific to the field of neuroscience that are aimed at improving the reproducibility crisis (Baker, 2016; Voelkl and Würbel, 2016). Improving reproducibility is part of efforts to bridge the translational gap in stroke research. These guidelines provide a checklist for preclinical researchers to ensure that the work performed is going to support clinical trials and help meet the needs of the affected population.

1.4.1.1. STAIR Guidelines

The Stroke Therapy Academic Industry Roundtable (STAIR) gathered experts with academic and industry backgrounds to propose the first set of guidelines for the preclinical characterisation of drugs intended for stroke treatment. The guidelines aimed to improve the quality of the preclinical research in order for clinical trials to be based on results from rigorously conducted studies (Table 1.2) (Stroke Therapy Academic Industry Roundtable (STAIR), 1999). The goal was to reduce internal bias by encouraging researchers to perform blinded and randomised studies and to characterise an optimal dose and treatment window. These guidelines gave researchers a benchmark for designing studies for the characterisation of new drugs for clinical use.

Table 1.2: STAIR preclinical guidelines 1999

1. Adequate dose-response curve
2. Define the time window in a well-characterised model
3. Blinded, physiologically controlled reproducible studies
4. Histological and functional outcomes assessed acutely and long-term
5. Initial rodent studies, then consider gyrencephalic species
6. Permanent occlusion, then transient in most cases

(Stroke Therapy Academic Industry Roundtable (STAIR), 1999)

However, this first set of guidelines did not appear to have solved the translation gap. NXY-059, a free radical spin trap agent with neuroprotective properties, had been evaluated in preclinical studies and scored highly in the STAIR checklist defined from the 1999 guidelines in table 1.2 (O'Collins et al., 2006; Macleod et al., 2008; Sutherland et al., 2012). However, the Stroke–Acute Ischaemic NXY Treatment II (SAINT II), a

larger follow-up study to the SAINT I clinical trial, found NXY-059 to be ineffective for the treatment of ischaemic stroke (Lees et al., 2006; Shuaib et al., 2007). The failure of NXY-059 came as a surprise because it had been evaluated in several preclinical studies, and it was considered a promising drug based. After the failure of the SAINT II trial, a refined set of guidelines for preclinical research were proposed in the 2009 STAIR meeting (Shuaib et al., 2007; Fisher et al., 2009).

The updated guidelines aimed to address several shortcomings identified through systematic reviews and meta-analyses of several preclinically evaluated drugs that, like NXY-059, had failed in clinical trials in the decade since the original recommendations (Fisher et al., 2009). The guidelines expanded on the first set by being more specific on the characterisation requirements for drugs in preclinical studies prior to advancing to them into clinical trials (Table 1.3), and there was an additional set of recommendations for good scientific inquiry (Table 1.4) to aid in the designing of the experiments and standardisation across research laboratories.

In 2017, the most recent STAIR meeting shifted the focus of stroke research towards cytoprotective therapies, i.e. therapies treating all cells and not only neurones; and towards preclinical studies mimicking endovascular recanalisation. The shift involves recommendations for the use of the filament stroke model because it achieves reperfusion with similar complications to those experienced in endovascular procedures and away from permanent occlusions (Savitz et al., 2019). Furthermore, there is an increased interest in using larger animal models that could undergo endovascular procedures in combination with thrombolytics and other novel therapeutics.

Table 1.3: Updated STAIR preclinical guidelines 2009

1. Adequate dose-response curve: the minimum and maximum tolerated dose should be defined, as well as the optimal tissue concentration target.
2. Define the time window in a well-characterised model: optimal treatment window to salvage penumbral tissue
3. Relevant outcome measures: evaluation of multiple endpoints, including both histological and behavioural outcomes, as well as delayed survival measures (2 - 3 weeks after stroke)
4. Basic physiological monitoring: monitoring of cerebral blood flow in the occlusion area, as well as blood pressure, gasses, glucose and body temperature.
5. Efficacy evaluated in multiple species: rodent and rabbit studies followed by cats or primates as a second species
6. Reproducibility evaluation: positive results from one laboratory need to be replicated in at least one independent laboratory.
7. Follow the Recommendation for good scientific enquiry (Table 1.4)

(Fisher et al., 2009)

Table 1.4: Recommendations for ensuring good scientific inquiry

Sample size calculation	Describe how the size of the experiment was planned. If a sample size calculation was performed, including the expected difference between groups, the expected variance, the planned analysis method, the desired statistical power, and the sample size thus calculated.
Inclusion and exclusion criteria	If a pre-specified lesion size is required for inclusion, then this should be detailed, as well as the corresponding exclusion criteria.
Randomisation	Describe the method by which animals were allocated to experimental groups.
Allocation concealment	Concealed allocation if the investigator responsible for the induction of ischaemia and care of experimental animals has no knowledge of the experimental group to which an animal belongs.
Reporting of animals excluded from analysis	All randomised animals (both overall and by treatment group) should be accounted for in the data presented.
Blinded assessment of outcome	The assessment of outcome is blinded if the investigator responsible for measuring infarct volume, scoring neurobehavioural outcome or determining any other outcome measures has no knowledge of the experimental group to which an animal belongs.
Reporting potential conflicts of interest and study funding	Any relationship that could be perceived to introduce a potential conflict of interest, or the absence of such a relationship, should be disclosed in an acknowledgements section, along with information on study funding and, for instance, supply of drugs or equipment.

(Fisher et al., 2009)

Similar to the STAIR roundtable, clinicians and researchers working on haemorrhagic stroke gathered to form the Haemorrhagic Stroke Academia and Industry (HEADS) roundtable in efforts to address the translational gap in haemorrhagic stroke research. This new organisation is focusing on the unmet needs and challenges that are specific to haemorrhagic stroke, and in their first meeting, produced guidelines for basic, translational, and clinical research, thus establishing a guided path for novel treatments being developed for ICH (The Haemorrhagic Stroke Academia Industry (HEADS) Roundtable Participants et al., 2018a; b).

Following the neutral results of several ICH clinical trials published after the first meeting, the HEADS met again to refine the recommendation with a focus on quality and rigour in study design (The Haemorrhagic Stroke Academia Industry (HEADS) Roundtable Participants et al., 2020). The updated HEADS guidelines recommend that drugs be evaluated for efficacy and safety, and that the results be replicated. The guidelines also recommend the use of multiple models of ICH and the inclusion of animals of different ages, sexes, strains and species (The Haemorrhagic Stroke Academia Industry (HEADS) Roundtable Participants et al., 2018a).

1.4.1.2. Other Guidelines

As mentioned previously, the reproducibility crisis is not unique to stroke and neuroscience research; it is a problem of animal research in general. Because the problem is not limited to the rigour of the studies, but they are also poorly reported, additional guidelines have been proposed to increase both the quality of the reporting and the quality of the research performed.

The ARRIVE guidelines (Animals in Research: Reporting In Vivo Experiments) were proposed to improve the quality of all research reporting of bioscience and preclinical research (Kilkenny et al., 2010). The guidelines aim to improve the information shared in manuscripts in order to communicate the quality and reliability of the work and provide others with information that would allow for study replication. Even though the guidelines have been widely endorsed, the quality of the reporting and level has not been enough (Leung et al., 2018). The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) revised the original guidelines and divided them into “the essential 10” and “the recommended set” (Sert et al., 2020). The renewed effort has the goal of facilitating and increasing adherence to the guidelines because setbacks and failures in animal research have been blamed on poor experimental design and lack of reproducibility. Thus, these guidelines are intended to be used for experimental design and manuscript preparation.

The IMPROVE Guidelines (Ischaemia Models: Procedural Refinements of In-Vivo Experiments) were also proposed by the NC3Rs in conjunction with stroke research experts from academic and industry backgrounds. These guidelines provide incremental fine-tuning of all aspects of care and experimental procedure for stroke modelling (Sert et al., 2017). Their goal is to foster the translational success of pharmacological agents to treat stroke. The guidelines are useful for both established and novice researchers working with rodent stroke models as they help standardise procedures between laboratories and institutions. The use of standard techniques will enhance reproducibility and the possibility of translating the best treatments into the clinic (Ferreira et al., 2020; Eggel and Würbel, 2020).

1.4.1.4. Preclinical Trials

The increased focus on improving the quality of preclinical research led to the proposal and development of the concept of preclinical trials (Bath, Macleod and Green, 2009; Dirnagl and Fisher, 2012). Preclinical trials are designed based on clinical trial methodology to determine the efficacy of a drug in animal models, and they can include a multicentre approach as part of the drug development process (Mergenthaler and Meisel, 2012). The preclinical studies would follow clinical trials parameters to increase the complexity and diversity of animal populations evaluated with the ultimate goal of informing the design of a randomised clinical trial (Fig. 1.3) (Macleod et al., 2008; Sena et al., 2007)

The preclinical trials focus on evaluating drug toxicity, safety, and efficacy and include different animal populations (age, sex, strain and comorbidities). Preclinical studies can inform the predictive value of clinical trials based on extensive and high quality research (Boltze et al., 2016). This study design is also intended to solve the reproducibility crisis because it can address internal and external validities of experimental studies. Internal validity refers to internal bias and whether they are controlled for, i.e., randomisation, blinding, and allocation concealment. The external validity of an experiment refers to how applicable the findings are to the real world, addressed by modelling stroke in aged animals, animals with comorbidities, and evaluation of drug interactions (Dirnagl, 2019; Eggel and Würbel, 2020)

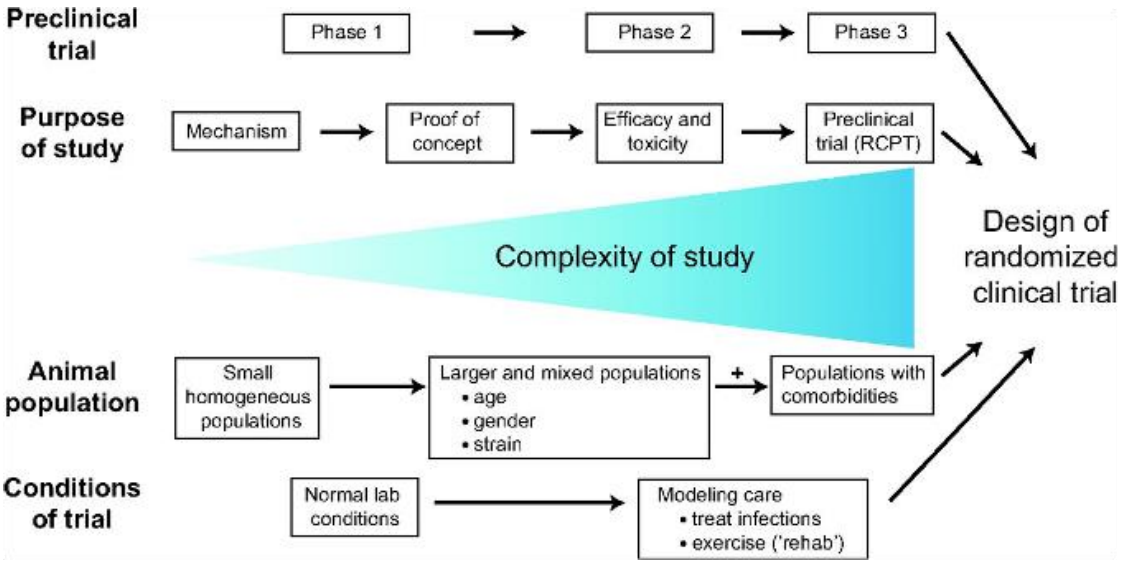

Figure 1.3: "Preclinical trial phases of translational stroke" Increasing complexity of studies and drug characterisation leading to the design and execution of preclinical studies with mixed populations of animals encompassing age, sex and comorbidities with the ultimate goal of designing clinical trials. Reproduced from (Mergenthaler and Meisel, 2012) under Creative Commons Licence 3.0.

Preclinical trials have been carried out for interleukin-1 receptor antagonist (IL-1 Ra) and Anti-CD49d, two drugs identified through systematic reviews and meta-analyses to be candidates for further preclinical characterisation and clinical studies. Interleukin-1 receptor antagonist was evaluated and found successful for the treatment of stroke in a cross-laboratory preclinical study (Maysami et al., 2015b). These results were followed by a randomised controlled phase II clinical trial, SCIL-STROKE (Subcutaneous Interleukin-1 Receptor Antagonist in Ischaemic Stroke); however, it only identified a reduction in plasma inflammatory markers without an effect on the modified Rankin scale, a measure of the degree of disability or dependence in day-to-day activities (Smith et al., 2018).

The multicentre preclinical randomised controlled trial (pRCT) for anti-CD49d (Natalizumab) was performed after the positive outcomes of the ACTION Phase II clinical trial (Elkins et al., 2017). However, the pRCT observed conflicting results in the lesion size measurements, with a lesion size reduction in the permanent ischaemic model and no effect in the transient ischaemic model (filament) (Llovera et al., 2015). Notwithstanding, two clinical trials were performed after these conflicting and inconsistent preclinical results were published, the first and smaller trial showed some efficacy, and the second larger trial failed (Elkins et al., 2017; Elkind et al., 2020). A follow-up preclinical study using a thromboembolic stroke model found no lasting improvements in lesion size or grip strength in mice treated with anti-CD49d, matching

the clinical trial results (Drieu et al., 2019).

Preclinical trials and rigorously designed animal experiments are fundamental to the advancement of any drug into the clinic. The examples presented here evaluated different animal strains and sexes, as well as different stroke models, producing results that put in doubt the efficacy of the drugs, and yet they were tested in clinical trials that eventually failed. Lastly, the animal studies lacked animals with comorbidities, thus limiting the external validity and predictive value of these studies because most individuals in the clinical trials had one or more comorbidities.

1.4.2. Animal Models of Stroke

Animal models of stroke have been developed in rodents, dogs, pigs, and non-human primates, to name a few species. The use of stroke models has helped in the understanding of major pathways involved in the pathological cascade of events following stroke (Howells et al., 2010). Knowledge of the pathophysiological cascade has allowed the evaluation of targeted drugs for disrupting the cascade of events in an effort to identify novel drug treatments (Dirnagl and Endres, 2014).

Each method for modelling stroke has its advantages and disadvantages when it comes to mimicking human strokes, which makes the selection of the model a critical step in the study design (Macrae, 2011). There are several methods of inducing different types of cerebral ischaemia. Strokes can be global, with a critical reduction of cerebral blood flow to the whole brain, or focal, with a reduction of blood flow to a specific area of the brain (Traystman, 2003). There are also models of haemorrhagic strokes in which blood vessels are disrupted with collagenase, or blood is injected directly into the brain (MacLellan et al., 2010). The model selected for a study affects the translation potential of the findings into humans (Braeuninger and Kleinschnitz, 2009). An ischaemic and a haemorrhagic stroke model were selected for characterising the effect of fingolimod.

1.4.2.1. Ischaemic Stroke Models

The two common surgical approaches to model ischaemic stroke involve accessing the middle cerebral artery (MCA) through the internal carotid artery (proximal occlusion)

or directly occluding the MCA either through the skull or by craniotomy (distal occlusion) (Fig. 1.4). Inducing a stroke through the internal carotid artery can be achieved with a filament or by injection of preformed clots. The filament model was first developed in rats; it produces large strokes (37% of the brain) that vary in size depending on the duration of the occlusion or whether the occlusion is transient or permanent (Longa et al., 1989).

Larger infarcts are easier to measure. This facilitates the detection of changes in lesion size associated with treatment. However, the filament model has a high mortality rate that correlates with the duration of the occlusion. The vessel walls can be damaged when introducing and withdrawing the filament, and the permanent ligation of the carotid artery causes an overall reduction of blood flow to the brain, limiting the usage of the model in long-term studies (Hossmann, 2011; Howells et al., 2010). The injection of preformed clots was developed in dogs and then adapted to rats (Kudo et al., 1982). The clots can occlude MCA and other arterial and capillary branches, causing large diffuse strokes. However, one limitation is that this model has high variability in lesion size between animals, hindering reproducibility (Howells et al., 2010).

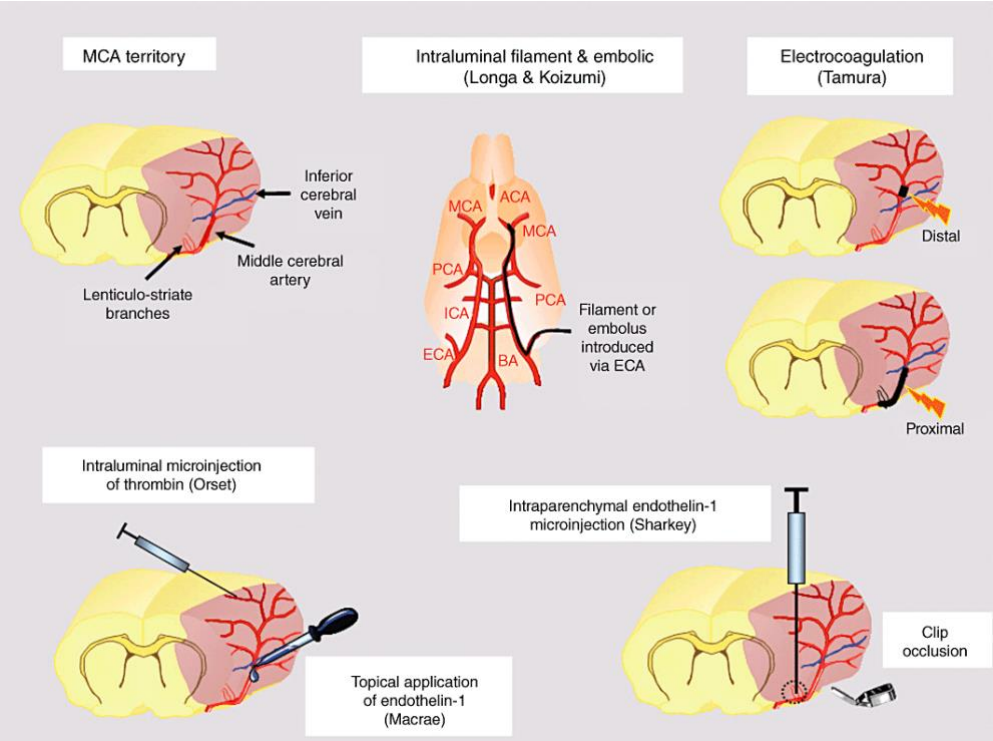

Figure 1.4: Diagrams depicting different methods for inducing stroke in rodents by occluding the middle cerebral artery (MCA). The top left image shows the brain with the MCA territory in pink. The other images show the location of the MCA occlusion and the different methods of occlusion. Reproduced from (Macrae, 2011) under permission from John Wiley & Sons, Inc.

Distal focal ischaemias are lesions that are mostly limited to the brain cortex, and they

are caused by the occlusion of the distal parts of the MCA (Fig. 1.4). There are several physical methods for inducing a permanent or transient focal ischaemia using sutures or clips to cause the occlusion. Other methods to cause focal ischaemia use electrocoagulation, endothelin injection or photochemicals (i.e., rose bengal) to only cause permanent occlusions (Howells et al., 2010; Macrae, 2011; Bacigaluppi, Comi and Hermann, 2010). These methods produce cortical injuries with comparable size and location that are all highly reproducible with a low mortality rate. However, the disadvantages of these methods are in part associated with the fact that these methods do not resemble humans because the occlusion does not respond to recanalisation treatment. However, the impact of this shortcoming is limited by the fact that a large proportion of the population does not reperfuse or does not receive tPA treatment (Sommer, 2017). Another limitation specific to the photo-thrombotic model is that this method does not produce a salvageable penumbra.

A novel procedure originally described by Orset et al. in 2007 produces a thromboembolic MCA occlusion by injecting thrombin into a distal branch of the MCA to create a clot in situ. The model mimics the human ischaemic stroke caused by a clot located in the middle cerebral artery (MCA) that can be treated with tPA to produce reperfusion. The thromboembolic MCA occlusion (tMCAo) model has been characterised and used by others after the original publication (Campos et al., 2013; Ansar et al., 2014; Correa-Paz, 2019) and a meta-analysis of its usage found that early (<3 h) tPA administration produced favourable results, as opposed to late (>3 h) administration that produces haemorrhagic transformation, an effect that can be counteracted with fingolimod treatment (Campos et al., 2013; Salas-Perdomo et al., 2019). Based on these reports, we initially selected this model to evaluate the effects of fingolimod in the presence or absence of recanalisation following tPA treatment, adding to the external validity and translatability of the results.

1.4.2.2. Intracerebral Haemorrhage models

There are two commonly used methods for modelling haemorrhagic stroke: a collagenase-based method in which the blood vessels are disrupted by an enzymatic weakening of the vessel wall and a blood injection, wherein a volume of blood is injected into the brain parenchyma. The limitation of blood injection is that it requires blood collected from a donor mouse or from the femoral artery of the same mouse. Autologous

blood requires the ligation of the femoral artery, a step that has been reported to affect behavioural test results (Ma et al., 2011). The injection of blood can also lead to increased intracranial pressure causing mass effect and a high chance of reflux up the needle tract (Manaenko et al., 2011). On the other hand, the collagenase model simply disrupts blood vessels with the limitation that the enzyme can have unintended effects (Keep, Hua and Xi, 2012). The collagenase method was selected for the studies herein because it provides a consistent injury, and it can mimic the haematoma expansion that humans experience from continuous bleeding.

1.4.2.3. Animal Models of Comorbidities

Comorbidities are modifiable and non-modifiable conditions affecting many patients presenting with a stroke. Most patients that present with stroke range between 50-80 years old and have modifiable comorbidities; the most commonly associated with stroke are dyslipidaemia, hyperglycaemia, and high blood pressure. Modifiable comorbidities can be managed with drugs and behaviour modifications, such as exercise, diet and drugs to reduce the risk of stroke, and other cardiovascular diseases. On the other hand, age is an example of a non-modifiable comorbidity.

As part of the effort to improve the quality of preclinical stroke research, increasing emphasis is being placed on the use of animals with comorbidities in studies evaluating the effect of a drug on stroke (Sommer, 2017; Dirnagl, 2006; Ergul et al., 2016; Dirnagl et al., 2018). Testing in animals with comorbidities becomes critical as the positive evidence for a drug accumulates, and the drug is being considered for translation into the clinic (Yuan et al., 2012; Buga, Napoli and Popa-Wagner, 2013). Failure to do so may underlie the reproducibility crisis and the low predictive value of preclinical studies for the success of clinical trials (O'Collins et al., 2006; McCann and Lawrence, 2020).

The presence of comorbidities tends to exacerbate stroke pathology both in rodents and humans (Sommer, 2017; Cho and Yang, 2018; Macleod et al., 2008), and the fact that most failed clinical trials used drugs that were not evaluated in preclinical studies with comorbid models is reported as a finding in a recent meta-analysis evaluating possible factors contributing to clinical trial failure. This study found that preclinical studies involving comorbidities show neutral results compared to studies with healthy animals (Schmidt-Pogoda et al., 2020). Thus, the evaluation of drugs in animals with comorbidities prior to translation might limit unnecessary risk to patients, and the time

and money invested in drugs that are bound to fail.

Comorbidities are associated with a specific inflammatory profile unique to each condition that could be the factors that exacerbate stroke pathophysiology (Przykaza, 2021). By using comorbid mice, we can evaluate how a drug behaves in these pathological conditions and inform the feasibility and likelihood of translation of a drug. At the time of the designing of these studies, fingolimod had not been evaluated in any comorbid model, and since then, only one study has done so. A study evaluating the intersection of diabetes, stroke and fingolimod treatment found that fingolimod exacerbated oedema while reducing inflammation (Li et al., 2020)

The use of aged mice would mimic the age of stroke onset in humans would add to the understanding of the pathophysiology and the pharmacological responses after stroke (Buga, Napoli and Popa-Wagner, 2013; Flurkey, Currer and Harrison, 2007; Dutta and Sengupta, 2016). Additionally, modelling hyperlipidaemia as a modifiable comorbidity by feeding a high-fat diet to ApoE-/-mice combines the genetic background and diet that accelerates the development of hypercholesterolemia and the accumulation of the atherosclerotic plaques in the aorta (Nakashima et al., 1993; Maganto-Garcia, Tarrio and Lichtman, 2012). Both of these models have an underlying inflammatory state that could also be modulated by fingolimod treatment.

1.5. Fingolimod

Fingolimod (FTY720; Gilenya ®) is an immunosuppressive drug derived from the naturally occurring molecule myriocin and approved for the treatment of multiple sclerosis (Adachi et al., 1995). Fingolimod is rapidly phosphorylated by sphingosine kinase 2, one of the two sphingosine kinases (SPHK1 & SPHK2), to become the structural analogue of the sphingosine-1-phosphate (S1P) (Blondeau et al., 2007; Wacker, Park and Gidday, 2009; Chun and Hartung, 2010). The phosphorylated form of fingolimod binds to sphingosine receptor 1 (S1P1), S1P3, S1P4 and S1P5 but not S1P2 (Kim et al., 2015). S1P receptors are a family of G-protein coupled receptors that are expressed throughout the body and have effects that are specific to the tissue in which it is expressed (O'Sullivan and Dev, 2017).

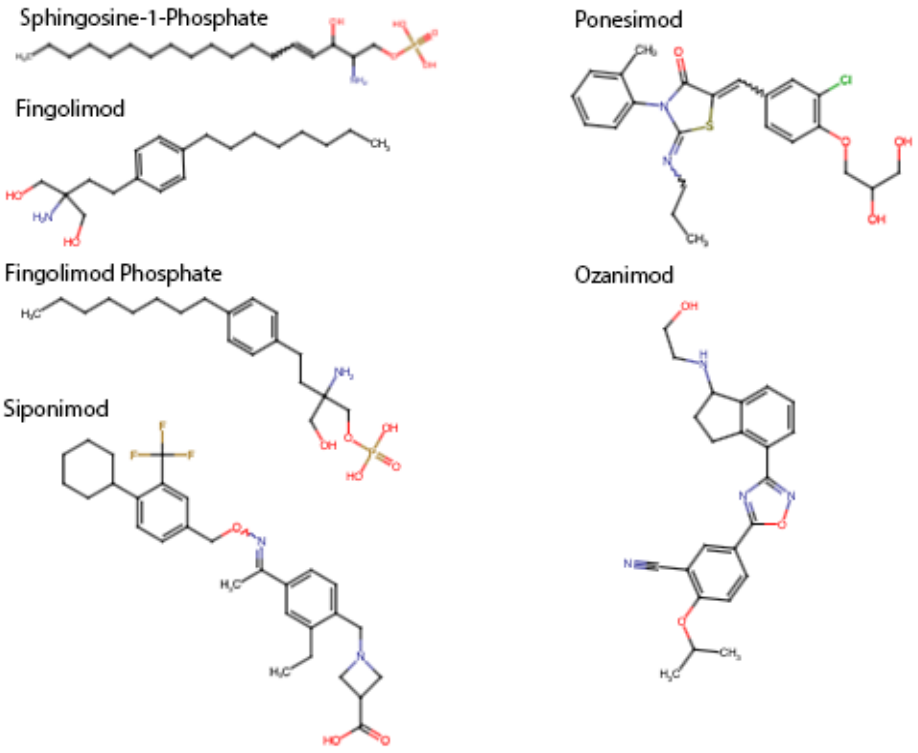

Figure 1.5: Chemical structure of Sphingosine-1-Phosphate and structural analogues that bind selectively to S1P receptors.

Orally administered fingolimod reaches peak concentrations of 1.1 ng/mL 12 h after administration, conversely the peak concentrations of the active fingolimod-phosphate were 1.6 to 2.8 ng/mL at 6 to 8 h after dosing (Kovarik et al., 2007). The drug is rapidly

absorbed and distributed mostly to the lymphoid tissue where it accumulates, resulting in a ten times higher concentration in lymphoid tissues than in blood (Sensken, Bode and Gräler, 2008). The large pool of fingolimod present in the lymphoid tissue is phosphorylated locally by SPHK2, where it then exerts its function of blocking the egress of lymphocytes from the lymph nodes by functioning as a functional antagonist of the S1P1 receptor (Sensken, Bode and Gräler, 2008). Shortly after the first dose, some patients experience a drop in blood pressure and heart rate, being the most common side effect associated with fingolimod treatment. Fingolimod has a half-life in humans of 6-9 d, independent of the number of doses, and after one to two months, lymphocyte levels return to normal (David, Kovarik and Schmouder, 2012). The elimination of fingolimod-phosphate happens via dephosphorylation back to fingolimod, fingolimod is eliminated by oxidative processes and excreted via urine, and a small fraction is excreted in faeces (Meno-Tetang et al., 2006; Zollinger et al., 2011; FDA, n.d.).

Even though fingolimod was the first drug approved for multiple sclerosis treatment, more recently, a second generation of S1P modulators has been approved to treat other types of multiple sclerosis (Park and Im, 2017). The newer drugs were developed to have a shorter half-life, higher specificity to S1P1 receptor binding and not bind to the S1P3 receptor, the receptor commonly expressed in the cardiovascular system and thought to be the cause of the bradycardia observed after the first dose of fingolimod. Siponimod (BAF312) binds to S1P1 and S1P5 receptors, and it does not require phosphorylation; however, the lack of binding to the S1P3 receptor did not eliminate bradycardia as a side effect (O'Sullivan et al., 2016; Cohan et al., 2022). Siponimod. was approved for the treatment of secondary progressive multiple sclerosis (Kipp, 2020).

Ozanimod (RPC1063) is another derivative of fingolimod that binds to S1P1 and S1P5 without the need for phosphorylation in order to exert its effects, and it has been approved for the treatment of relapsing forms of MS and moderate to severe ulcerative colitis (Scott et al., 2016). Ozanimod has a wide distribution with slow absorption, and it has a relatively short half-life of 19 h. These characteristics have been found to decrease the cardiovascular effects associated with the first dose of S1P1 modulators (Scott et al., 2016; Sandborn et al., 2016). Ponesimod (ACT-128800) is a selective S1P1 receptors agonist that has rapid absorption and a short half-life (30 h) that, with rapid elimination, allows for the lymphocyte counts to return to baseline one week after stopping treatment (Dash, Rais and Srinivas, 2018; Ruggieri, Quartuccio and Prosperini, 2022). Lastly, amiselimod (MT1303) is a selective S1P1 modulator that lacks the cardiovascular side effects associated with other S1P1 modulators, but it is no longer being evaluated for MS,

yet there is continued research in the inflammatory bowel disease and colitis fields (Kappos et al., 2016; Shimano et al., 2019).

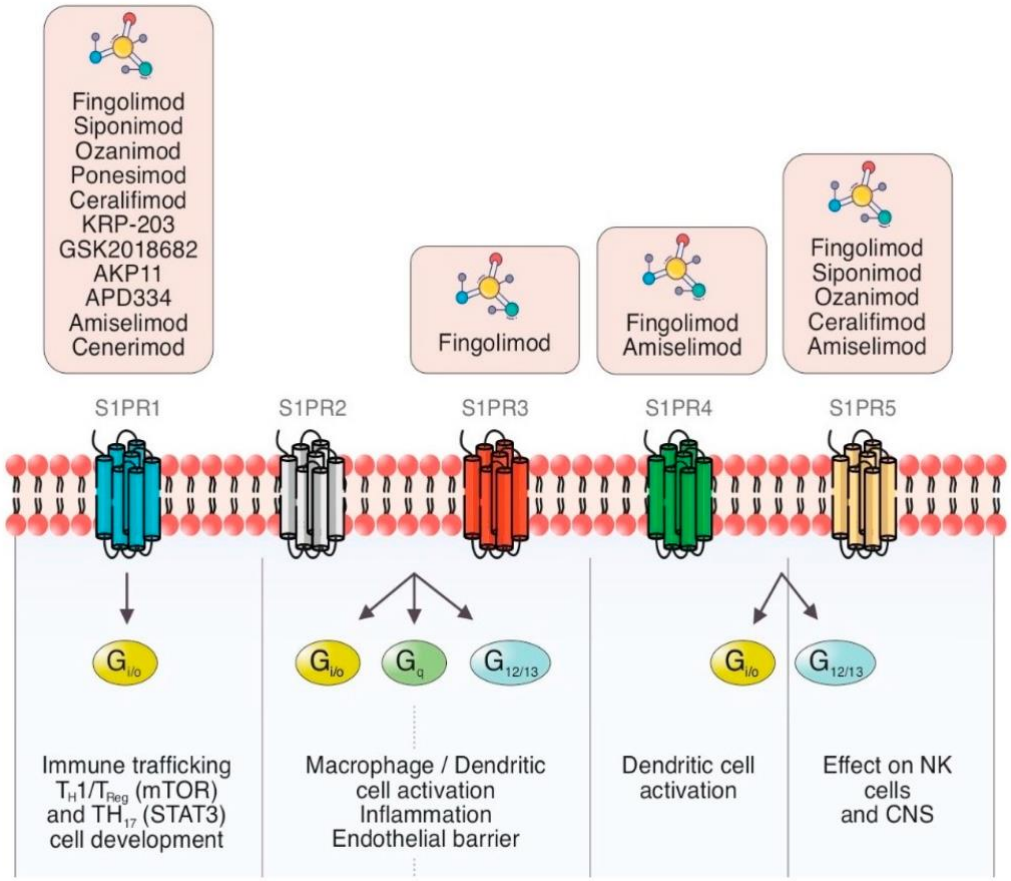

Figure 1.6: Schematic summary of S1P receptor signalling pathways through different G proteins and the therapeutics that target each receptor. Reproduced from (Bravo et al., 2022) under Creative Commons Licence 4.0.

The S1P receptors are five G protein-coupled receptors expressed throughout the body, most notably in the cardiovascular, immune and nervous systems, with unique functions in each tissue (Fig. 1.6) (Brinkmann, 2007; Obinata and Hla, 2011). The use of global S1P1 knockout mice has demonstrated that S1P1 receptors are involved in the maturation of blood vessels and neurogenesis, and S1P2 and S1P3 receptors have redundant or supportive roles in the development of the blood vessels (Obinata and Hla, 2011; Brinkmann et al., 2010). S1P3 is involved in the regulation of myocardial perfusion and vasodilation, and it is considered pro-inflammatory. Lastly, S1P4 and S1P5 mediate the immunosuppressive effects of S1P in T cells, where they are mostly expressed (Obinata and Hla, 2011).

Fingolimod acts as a full agonist of S1P1, S1P4 and S1P5, and it is a partial agonist for S1P3 receptors (Fig. 1.6). Furthermore, fingolimod acts as a functional antagonist at S1P1 receptors expressed on T cells. Fingolimod causes the internalisation of the S1P1

receptor leading to the sequestration of lymphocytes in lymph nodes, the mechanism that makes fingolimod an immunomodulator and anti-inflammatory agent (Matloubian et al., 2004). The binding and activation of the S1P1 receptor is highly potent (Kd=0.3 nM) and with higher efficacy than S1P. Fingolimod phosphate is a functional antagonist of the S1P1 receptor on lymphocytes causing the internalisation and degradation of the receptor due to overactivation (Matloubian et al., 2004; Arnon et al., 2011).

Although the effects on S1P1 have been more extensively investigated, the effects on S1P3, S1P4 and S1P5 also have the potential to influence recovery after stroke. In the central nervous system, sphingosine receptors (S1P1, S1P3, and S1P5) are expressed on astrocytes, neurones, microglia and oligodendrocytes, suggesting that brain cells can be targeted for modulation with fingolimod, where it can exert additional effects in stroke recovery (Fig. 1.7) (O'Sullivan and Dev, 2017). Fingolimod also acts directly in the brain by reducing reactive astrogliosis and scar formation (Brunkhorst et al., 2013). Furthermore, the expression of S1P receptors on lymphocytes varies depending on the cell type and maturation stage, and the effect of S1P receptor activation produces pro or anti-inflammatory effects depending on the cells (Fig. 1.6) (Bravo et al., 2022).

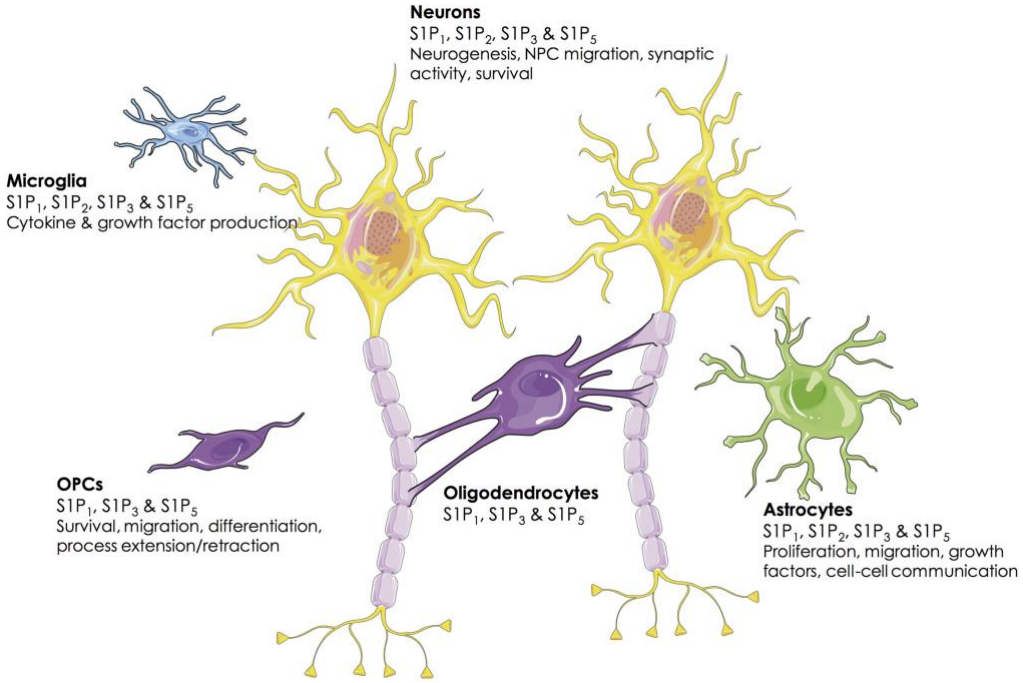

Figure 1.7: S1P receptor family expression in central nervous system cells. All cells have S1P1, and the others are not consistently expressed in the cells. Copyright (O'Sullivan and Dev, 2017); reproduced under license authorisation.

The reduction of circulating lymphocytes limits the number of lymphocytes that can infiltrate into to CNS (Liesz et al., 2011; Rolland et al., 2013). This is facilitated by the inability of lymphocytes to egress from the lymph nodes after stimulation with fingolimod (Matloubian et al., 2004). Lymphocyte egress is normally mediated by S1P1 receptor

recognition and an S1P gradient (Brinkmann, 2007), and in the presence of S1P1 receptor modulators, the S1P1 receptor is internalised, and the lymphocytes can no longer exit the lymph node (Brinkmann, 2007). The ensuing lack of S1P receptors on the T cell surface from fingolimod treatment prevents lymphocytes from egressing the lymph nodes into the general circulation. The reduction in circulatory T cells prevents the recirculation of inflammatory T cell subtypes that have been associated with detrimental effects on multiple sclerosis and stroke (Weaver et al., 2006). The effect of fingolimod is measurable five days after the last dose by a lymphocyte count reduction (Malone et al., 2021).

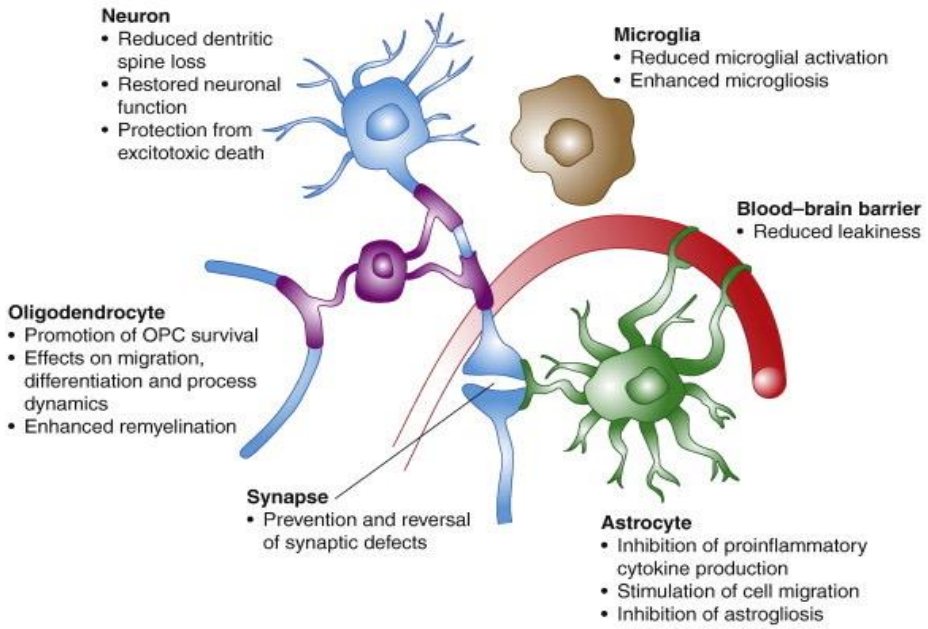

Figure 1.8: The effect of fingolimod on brain cells by interaction with the S1P receptors. Reproduced under license authorisation. Copyright to (Groves, Kihara and Chun, 2013).

The reduction of circulating lymphocytes associated with the treatment with fingolimod led to the characterisation and study of its effects in the model of experimental autoimmune encephalitis (EAE) (Webb et al., 2004). EAE is a model of CNS inflammation that leads to demyelination and neurodegeneration similar to that observed in multiple sclerosis. Fingolimod treatment resulted in a sustained improvement of the clinical signs in a mouse model of EAE, thus prompting the clinical evaluation of fingolimod (Webb et al., 2004). Long-term treatment with fingolimod in mice and humans has led to remyelination of neuronal axons (Groves, Kihara and Chun, 2013). Other treatment effects associated with fingolimod have been identified with the use of other disease models, i.e., LPS-mediated lung injury, in which fingolimod reduced microvascular permeability and inflammation that is consistent with the observation that fingolimod prevents blood brain barrier breakdown after stroke (Fig. 1.8) (Campos et al.,

2013; Peng et al., 2004).

In stroke, one of the mechanisms of action associated with fingolimod is the modulation of infiltrating immune cells into the brain, which has been shown to reduce ischaemic lesions and behavioural outcomes. Furthermore, fingolimod has been found to protect the vascular unit and prevent the degradation of the tight junctions of the blood brain barrier (Foster et al., 2008; Pinheiro et al., 2016). Being that fingolimod is neuroprotective by protecting against excitotoxic cell death (Menna et al., 2013); and is neuroregenerative by increasing brain-derived neurotrophic factor levels in models of Rest syndrome and Alzheimer's disease (Fukumoto et al., 2014), it is a promising agent for the treatment of stroke (Groves, Kihara and Chun, 2013; Hunter, Bowen and Reder, 2016).

1.5.1. Approved Clinical Use

Fingolimod was approved by the Federal Drug Administration in September 2010 for the treatment of relapsing forms of multiple sclerosis (Brinkmann et al., 2010; Sharma et al., 2011), and the European Medicines Agency approved it the following year. Currently, the drug is approved for use in patients older than 10 years of age since the effectiveness in individuals 10-18 of age was recently confirmed in a phase 3 clinical trial comparing fingolimod with interferon beta-1a, another approved treatment for MS (Chitnis et al., 2018).

The first study that led to the approval of fingolimod for MS was a double-blinded proof of concept clinical trial where fingolimod treatment reduced the number of gadolinium-enhanced lesions and frequency of relapses in patients with multiple sclerosis compared to placebo controls (Kappos et al., 2006). These results were followed up by two phase III trials: the TRANSFORMS (Trial Assessing Injectable Interferon vs FTY720 Oral in RRMS) evaluated the effectiveness of 0.5 mg and 1.25 mg doses of fingolimod vs the standard of care (interferon beta-1a) (Cohen et al., 2010), and the FREEDOMS trial (FTY720 Research Evaluating Effects of Daily Oral therapy in Multiple Sclerosis) evaluated two fingolimod doses compared to placebo (Kappos et al., 2010). Both studies found fingolimod to be effective and significantly better than standard of care (interferon beta-1a) or placebo.

1.5.2. Fingolimod in Stroke Research

The commonalities between the pathophysiological processes behind MS and stroke are the reasons supporting attempts at using MS drugs for the treatment of stroke. This makes fingolimod a good candidate drug for the treatment of ischaemic and haemorrhagic stroke. Some of the early studies on ischaemic stroke found that S1P receptor modulation with fingolimod reduced stroke lesion size and improved neurological scores (Wacker, Park and Gidday, 2009; Shichita et al., 2009; Czech et al., 2009). These and other findings were later evaluated in two meta-analyses and systematic reviews to assess the effect size and possible benefit of fingolimod in a larger population (Dang et al., 2020; Liu et al., 2012).

The first meta-analysis included 9 reports with a median quality score of 6, with the lowest score of 2; the scores were based on an 11 item quality assessment using the STAIR guidelines (Liu et al., 2012). Even though the overall finding is that fingolimod reduces lesion size and behavioural deficits, the low scores suggest that more studies of higher quality studies are necessary in order to support fingolimod for evaluation in large clinical trials. The lower overall quality score correlates with the fact that only two studies reported sample size calculations (Pfeilschifter et al., 2011b; a), and four studies reported some degree of randomisation (Czech et al., 2009; Pfeilschifter et al., 2011a; Wei, Zhiquiang and Li, 2011; Hasegawa et al., 2010). There were 194 animals assessed in the review, and none of them modelled a comorbid state like age, diabetes, hypertension or dyslipidaemia or included female mice.

The second meta-analysis published in 2020 included seventeen studies, nine publications previously summarised and eight newer articles published between 2009-2019 (Table 1.5) (Dang et al., 2020). The overall study quality remained low (median [IQR] 6 [4-8]). The median score did not change because the newer studies did not meet all of the CAMARADES checklist parameters, and only two new studies reported doing power calculations (4 total) (Brunkhorst et al., 2013; Cai et al., 2013). Ten publications reported a randomised study design, six reported blinded allocation, and nine reported blinded outcome assessment, but no study evaluated fingolimod in animals with comorbidities.

Table 1.5: Quality characteristics of included studies (CAMARADES checklist) (Dang et al., 2020)

Author (year)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	Total
Czech (2009)	√	√	√	√	√	√			√		7
Shichita (2009)	√	√				√					3
Wacker (2009)	√	√				√			√		4
Hasegawa (2010)	√	√	√		√	√			√		6
Liesz (2011)	√	√				√			√		4
Pfeilschifter (2011A)	√	√	√	√	√	√		√	√	√	9
Pfeilschifter (2011B)	√	√	√	√	√	√		√	√		8
Ying (2011)	√	√				√			√		4
Wei (2011)	√	√	√								3
Brunkhorst (2013)	√				√	√		√	√		5
Cai (2013)	√		√	√	√	√		√	√	√	8
Campos (2013)	√	√				√			√		4
Hasegawa (2013)	√	√	√		√	√			√	√	7
Kraft (2013)	√	√	√	√	√	√			√	√	8
Xu (2014)						√					1
Nazari (2016)	√	√	√			√			√	√	6
Schuhmann (2016)	√	√	√	√	√	√			√	√	8

Studies fulfilling the following criteria: (1) published in a peer-reviewed journal; (2) stated that body temperature was controlled; (3) allocated to groups by randomisation; (4) experimenters were blinded to allocation; (5) estimation of outcome was blinded; (6) anaesthetic used during the study did not have intrinsic neuroprotective activity; (7) use of comorbid subjects (aged, diabetic or hypertensive); (8) reported that sample size was determined by power calculation; (9) complied with internationally recognised animal welfare regulations; (10) statement of potential conflicts of interest.

The overall effect sizes for stroke lesion size and neurological function in the second meta-analyses were similar to the first, with an overall reduction in lesion size and an improvement in neurological function. So far, fingolimod research has only partially met the recommended STAIR guidelines, and more research is necessary to fully satisfy these recommendations (Table 1.6). In particular, the studies fail to use male and female mice, evaluate the effect of comorbidities on treatment response and use a small number of mice that are not the product of a power calculation and do not consistently control for internal bias by blinding and randomisation. The aim of our studies was to fill this gap.

Table 1.6: STAIR checklist for fingolimod stroke research.

STAIR recommendation	Description	Criteria met?
Dose Response	Two or more doses have been studied in five publications	Partial
Therapeutic Window	A few different time points of initiation of therapy have been evaluated	Partial
Outcome measures	Multiple endpoints, with histological and behavioural outcomes, have been investigated. Most studies evaluated 1-3 d short term effects, and a few evaluated 7 d or more.	Partial
Physiological monitoring	Intra-operative blood pressure, temperature, blood glucose, and blood gases were measured.	Partial
Multiple species	Treatment has been evaluated in rodents (mice and rats)	Partial
Reproducibility	Two meta-analyses show positive results of FTY720 that have been replicated in independent laboratories.	Yes
Permanent occlusion	Only two used permanent occlusion models	Partial
Randomisation, inclusion and exclusion criteria	Randomisation and defined exclusion criteria were reported in some studies.	Partial
Power calculations	Sample size calculation not commonly reported	Partial
Disclosure of potential conflict of interest	The information on whether a potential conflict of interest exists was given in some studies.	Partial
Animals with comorbidities	One study evaluated diabetes as a comorbidity. Age, hypertension, and hypercholesterolemia have not been evaluated.	Partial
Measurements of relevant biomarker	Diffusion/perfusion MRI or serum markers of tissue injury were not reported.	No
Drug interaction studies	The interaction of fingolimod with thrombolysis (tPA) and other drugs has not been investigated thus far.	Partial

Table adapted from (Liu et al., 2012) and (Dang et al., 2020).

Even though there are many studies evaluating the effect of fingolimod, there might be an overestimation of the benefits associated with fingolimod treatment. This can be inferred by the low number of neutral studies, the small sample sizes, and the lack of control affecting variables, thus, increasing the odds that the results are false (Button et al., 2013; Ioannidis, 2005, 2014). Furthermore, there is a preponderance of short-term studies that use the filament model, causing large strokes, and only a handful of studies that evaluate small distal ischaemic strokes that are limited to the brain cortex (Dang et al., 2020; Liu et al., 2012). The filament stroke model was typically used in short-term studies because this model has a high mortality, although many of these studies do not report mortality rates that could confound the data and lead to an overestimation of the treatment effect (Sommer, 2017; Ström et al., 2013).

The evaluation of long-term outcomes using stroke models that are compatible with

long-term survival (i.e., focal ischaemia), along with different treatment durations, dictates the use of a distal model, as well as the evaluation of fingolimod treatment in stroke models with comorbidities (age, diabetes and hyperlipidaemia). In this context, it is worth mentioning that a recent study reported that fingolimod exacerbated oedema formation in the acute phase of stroke in diabetic mice (Li et al., 2020); yet its effect might be different in other comorbidities. Studies evaluating other drugs have observed different treatment effects between comorbidities (Przykaza, 2021; Popa-Wagner et al., 2020; Grisotto et al., 2021). For example, one study showed that mice fed a HFD for 2 months had significant metabolic alterations that caused increased infarct volume and haemorrhagic complications (Grisotto et al., 2021).

When it comes to ICH, as mentioned above, fingolimod has also been evaluated as a potential treatment for intracerebral haemorrhage in a few preclinical studies and one open label clinical study (Fu et al., 2014a). There are three preclinical studies evaluating fingolimod that showed both behavioural and histological improvements in short and long-term studies (Rolland et al., 2013, 2011; Lu et al., 2014). There are two studies evaluating siponimod, a more specific S1P1 modulator that was also shown to be beneficial in haemorrhagic stroke by also acting as an immunomodulator (Bobinger et al., 2019, 2020). The use of fingolimod and siponimod are all motivated by the need to reduce inflammation and modulate the immune response and infiltration into the brain after intracerebral haemorrhage. However, these studies are not sufficient to validate fingolimod for clinical trials, and more research is necessary.

More studies using models of ICH are required to evaluate different animal populations, including both male and female mice and animals with comorbidities, i.e., aged mice. There is also a need to evaluate a variety of treatment regimens in order to meet characterisation standards prior to clinical translation of fingolimod. As far as ICH studies, there is a lack of variety of animals strains, they predominantly have small group sizes, and the studies focus on short-term outcomes (Table 1.7), all of the shortcomings that are also common in ischaemic stroke studies.

Table 1.7: Comparison between the published research evaluating S1P1 modulation for ICH treatment.

	Roland et al, 2011	**Rolland et al, 2013**		**Lu et al, 2014**	**Bobinger et al, 2019**
Drug, dose, route	Fingolimod, 1mg/kg, i.p.	Fingolimod, 1mg/kg, i.p.		Fingolimod, 0.5mg/kg, i.p.	□ Siponimod, 0.3mg/kg, i.p. □ Siponimod, 3mg/kg, i.p.
Treatment regimen	1h after surgery	□ 1h after surgery □ 1h after surgery and once daily on the following two days		30 min after surgery and once daily on the following two days	□ 30 min after surgery □ 30 min after surgery and once daily on the following two days
Sex, strain, species	CD-1 mice (sex not reported)	Male CD-1 mice	Male Sprague-Dawley rats	Male CD1 mice	Male C57BL/6 mice
Model	Collagenase	□ Collagenase □ Autologous blood	Collagenase	Collagenase	Collagenase
Group sizes	5 or 10	7		9-10	10
Histological outcome					
Edema	Wet-dry: p<0.05 (D1 and D3)	Wet-dry: p<0.05 (D1 and D3)		Wet-dry: p<0.05, D3 (n=5)	□ MRI: p=0.02 at D3 (multiple dosage) □ Wet-dry: p=0.022, p=0.0013, p=0.04 for single and multiple 0.3, and multiple 3mg/kg doses
Atrophy/Tissue loss			p<0.05 at 10 weeks	p<0.01, D14	
Apoptotic cells				p<0.05, D3	
Behavioural outcome					
Composite neuroscore	p<0.05 D1 and D3	p<0.05 D1 and D3		p<0.05, D3	No effect at D1 p=0.03 at D3
Wire hanging	p<0.05 (D1) n.s. (D3)	p<0.05 (D1)* n.s. (D3)*		p<0.05, D3 and D14	
Beam balance	p<0.05 (D1) n.s. (D3)	p<0.05 (D1)* n.s. (D3)*			
Forelimb use asymmetry		n.s. at D1 and D3*			
Corner test		p<0.05 D1 and D3			
Paw placement		p<0.05 D1 and D3	p<0.05 D1 and D2 No effect at D3 and 10 weeks		
Weight loss				P<0.01 (D1), p<0.05 (D3)	p=0.036 (multiple 0.3mg/kg)

(Diaz Diaz et al., 2020)

1.5.3. Stroke Clinical Trials of Fingolimod

Based on the preclinical work published on fingolimod, a few open label clinical trials have been performed in both intracerebral haemorrhage (Fu et al., 2014a) and ischaemic stroke (Zhu et al., 2015; Fu et al., 2014b; Tian et al., 2018; Liantao et al., 2019). Eleven participants were treated with a 3 d course of 0.5 mg fingolimod in a 2-arm proof-of-concept study involving 23 participants with a medium sized haemorrhage; clinical fingolimod treatment reduced perihaematomal oedema at 7 and 14 d after stroke compared to matched the controls (Fu et al., 2014a). In another randomised study evaluating a 3 d treatment with fingolimod or standard of care on ischaemic stroke in 22 patients presenting with an acute ischaemic stroke beyond the tPA treatment window were found that individuals in the fingolimod treatment group had improved neurological rehabilitation and a significant reduction of the secondary lesion compared to controls up to 90 d after stroke (Fu et al., 2014b).

These two proof of concept studies and the fact that alteplase remains the most effective treatment were the basis for clinical trials evaluating the co-administration of fingolimod and alteplase. A small 22 patient study in which individuals were randomised to control or the co-administration of alteplase with 3 d of 0.5 mg fingolimod found that fingolimod treatment reduced secondary lesion expansion and limited the haemorrhagic transformation events compared to the control group (Zhu et al., 2015). A larger follow-up randomised study involving 46 patients evaluated the efficacy of alteplase with fingolimod and reported improved outcome measures, improved anterograde perfusion and retrograde collateral flow in non-recanalised alteplase treated patients (Tian et al., 2018).

These results are all promising for the clinical effectiveness of fingolimod, and several follow-up clinical trial studies have been proposed. One such study is the Fingolimod with Alteplase bridging with Mechanical Thrombectomy in Acute Ischaemic Stroke (FAMTAIS) that was planned to involve 98 participants receiving alteplase along with mechanical thrombectomy randomised into receiving fingolimod or placebo (Zhang et al., 2017); however, this study was withdrawn in March 2020. Lastly, a double-blinded placebo-controlled pilot trial of Fingolimod as a Treatment of Cerebral Oedema After Intracerebral Haemorrhage (FITCH) has started recruiting patients, and the treatment plan involved the administration of a single dose of fingolimod (0.5 mg) and evaluate outcomes up until 365 d after stroke (NCT04088630, 2021).

Siponimod, a more selective S1P receptor binding agent that only binds to S1P1 and S1P5, has also been evaluated for ICH in phase 2 randomised controlled clinical trial (NCT03338998), where it was found to be ineffective at reducing perihaematomal oedemas as opposed to the results of a preclinical study (Bobinger et al., 2019). The failed trial for ICH could also mean that in a larger study that includes a more varied population of patients that fingolimod might also have neutral results, again emphasising the need for thorough evaluation in preclinical studies prior to the performance of large randomised controlled trials.

1.6. Hypothesis and Objectives

In summary, based on the background showing the potential benefits associated with fingolimod, we designed a series of preclinical studies with the goal of fulfilling the STAIR guidelines.

This would be achieved by designing and executing robust studies that address internal and external biases, thus filling the gap in fingolimod research for the use of comorbid mice.

Overall objective:

To evaluate and characterise the effects of fingolimod in preclinical stroke studies using mouse models of both ischaemic and haemorrhagic stroke.

Intracerebral haemorrhage study:

The aim of this study was to evaluate the effect of fingolimod in a model of intracerebral haemorrhagic stroke in male and female mice.

Hypothesis: Male and female mice treated for 3 d starting 2 h after intracerebral haemorrhagic stroke with 0.5 mg/kg of fingolimod will have a better outcome than those treated with vehicle (saline) 14 d after intracerebral haemorrhage.

Dose response study:

The aim of this study was to determine the optimal dose of fingolimod for the treatment of a stroke in mice by performing a dose response test.

Hypothesis: Mice treated for 3 d starting 2 h after ischaemic stroke with a low dose (0.5mg/kg) or a higher dose (1.0 mg/kg) of fingolimod will have improved outcomes than mice treated with vehicle (saline) in a dose dependent manner 7 d after stroke. A dose-response relationship should be observed.

Aged mice study:

The aim of this study was to evaluate the effectiveness of an optimal dose of fingolimod for the treatment of ischaemic stroke in aged mice.

Hypothesis: Aged mice treated for 3 d starting 2 h after ischaemic stroke with an optimal dose of fingolimod will have better outcomes than mice treated with vehicle (saline) 7 d after stroke.

Hyperlipidaemic mice study:

The aim of this study was to evaluate the effectiveness of fingolimod for the treatment of ischaemic stroke in a mouse model of hyperlipidaemia.

Hypothesis: Mice with hyperlipidaemia treated for 3 d starting 2 h after ischaemic stroke with an optimal dose of fingolimod will have better outcomes than mice treated with vehicle (saline) 7 d after stroke.

Treatment duration study:

The aim of this study was to evaluate the effect of different treatment durations for the treatment of an ischaemic stroke in mice.

Hypothesis: Mice treated with an optimal dose of fingolimod for 10 d will have better outcomes than mice treated with fingolimod for 5 d and saline-treated controls after ischaemic stroke.

Chapter 2

Methods

The overall aim of this project was to systematically evaluate the efficacy of fingolimod in a series of rigorously designed studies to inform whether this drug is suitable for further testing in clinical trials.

Our studies were designed to meet the currently accepted quality standards in preclinical research with power calculation, randomisation, and blinding to reduce internal bias. To control for external biases, we used aged and hyperlipidaemic mice (Fisher et al., 2009). The outcome measures focused on histological lesion size and behavioural measurements with experimental endpoints of seven or more days. The goal of assessing these long-term outcomes was to expand on the literature because most previously published studies have focused on fingolimod's short-term effects (24-72 h post-stroke) (Wacker, Park and Gidday, 2009; Liesz et al., 2011; Hasegawa et al., 2010, 2013).

The methods section is divided into four subsections: animal models, experimental outcome measures, experimental design, and experimental studies.

2.1. Animals

All animal studies were performed with the approval and authorisation of: (1) the Irish Health Products Regulatory Authority (HPRA, project authorisation AE19130/P042) and (2) the University College Cork Animal Experimentation Ethics Committee (AEEC; authorisation 2016-004). Furthermore, the studies were performed according to the National Institute of Health Guide for Care and Use of Laboratory Animals (National Research Council, 2011).

2.1.1. Experimental Mice

Young adult mice:

C57BL/6J mice from Envigo (C57BL/6JOlaHsd) were received from the UK colony and housed in groups of 3-4. Male and female mice were ordered at 6-8 weeks. Female mice were used only in the intracerebral haemorrhage study along with male mice and all other studies used exclusively male mice.

Male mice were used at 8-16 weeks of age. Conversely, female mice were used when they weighed ≥ 20 grams or were 10-16 weeks of age. This was because female mice were observed to have difficulty recovering after surgery when they weighed ≤ 20 grams.

Aged mice:

Thirty-three male mice (C57BL/6NCrl) were received at 58 weeks of age from Charles River (UK). The delivery included two singly housed mice, and a third mouse was single-housed after its cage-mate died during the acclimatisation period. Mice were aged in-house for an additional 16 weeks until 74 weeks, approximately 18 months.

ApoE knockout mice:

Forty male ApoE/J (Apoetm1Unc) mice were ordered from Charles River (Italy); 20 were four weeks of age, and 20 were five weeks old. Atherosclerosis was induced in ApoE -/- mice at eight weeks by feeding them an adjusted calorie diet for 12 weeks. The high-fat diet (HFD) contained 43% calories from fat and 1.5 g/kg of cholesterol (0.2% total cholesterol), and it was sourced from Envigo (TD.88137).

Mice on HFD are known to develop skin lesions due to higher cholesterol levels (Zhang et al., 2015). Thus, mice were monitored daily for their health status and to ensure proper levels of food. Mice were housed in groups of 4, but ten mice were singly housed at the end of the study because of skin lesions and signs of fighting. Mice started to develop skin lesions at about 16 weeks of age (8 weeks after starting the high-fat diet), and the lesions were treated with Calamine solution once a day.

2.1.2. Animal Husbandry

The animal husbandry varied locations during the study because the university was transitioning between two Biological Services Unit (BSU) facilities. The first study evaluating the effect of fingolimod on intracerebral haemorrhage was completed in the older facility, Doughcloyne-BSU. Western Gateway Building BSU (WGB-BSU), the new facility, opened in 2018 and was the location for all other experimental studies. Mice were housed maintaining the groups in which they arrived, and they were allowed to acclimate for at least seven days before procedures were performed.

Doughcloyne facility

Mice were housed in conventional open-top cages in groups of 3-4 unless otherwise noted. Cages were lined with aspen wood-chip bedding, shredded paper, and additional enrichment (cardboard cylinder and chewing blocks). They had a 12 h light and dark cycle, with food and water provided ad libitum. Mice were fed a regular high-protein diet (Envigo TD2018, Teklad global 18% protein).

The facility was positive for numerous FELASA (Federation of European Laboratory Animal Science Associations) exclusion pathogens, such as MNV (murine norovirus) and MPV (mouse parvovirus). Even though some exclusion pathogens were under control, others were endemic. The room temperature and humidity were monitored, yet they fluctuated throughout the year and were challenging to control.

Western Gateway facility

The facility is a Specific-pathogen-free (SPF) unit free from the FELASA exclusion pathogens. Mice were housed in groups of 3-4 in individually ventilated cages (IVCs) connected to an air handling unit that controls temperature and humidity (Tecniplast). The cages were lined with aspen wood-chip bedding, and the mice were provided with

shredded paper and cardboard tunnels as enrichment. Mice had free access to food and water in a 12 h dark and light cycle. Animals were fed an irradiated high-protein diet (Envigo TD2918, Teklad global 18% protein, Irradiated). Alternatively, mice in the atherosclerotic diet (ApoE-/-) were housed in groups of 4 in IVC cages with paper-based bedding used to wick excess fat from food (Alpha-dri bedding; Shepherd Specialty Papers, UK).

Individual housing

Mice were individually housed only in cases where the welfare of the mice required it. Individually housed mice were provided extra enrichment. Single housing was necessary for two reasons: fighting and the development of skin lesions. The aggressor mouse was separated from the group and singly housed when the fighting was evident.

Handling

Mice were handled by researchers and BSU staff with a tunnel. Tunnel handling ensures reduced anxiety and stress levels even after brief tail handling necessary for intraperitoneal injections (Hurst and West, 2010; Gouveia and Hurst, 2017; Clarkson et al., 2018).

Pre-operative stroke care

Mice were given a hydration gel (Hydrogel, ClearH2O, USA) with a wet diet to facilitate eating and maintain hydration. The gel was provided two days before surgery to allow mice to get used to the novel object and ensure that they consumed it after surgery.

Post-operative care and monitoring

After the stroke induction, mice were monitored daily with a scoring sheet that measured and scored body weight, appearance, behaviour and neurologic aspects starting one day after surgery (Appendix 1). The scores were collected and analysed as an outcome measure (see Scoresheet measurements).

2.1.3. Focal Ischaemic Stroke Models

2.1.3.1. Thromboembolic Stroke

Thromboembolic middle cerebral artery occlusion (tMCAo) was induced on C57BL/6 mice anaesthetised with 2% isoflurane in 100% oxygen or 30%/70% oxygen/nitrogen mixture. Mice were placed in an induction box, and the isoflurane was set to 2% for induction of anaesthesia; once the mouse fell over and their breathing slowed down, the pedal reflex was tested for depth of anaesthesia. Anaesthetised mice were transferred to the heated thermo-regulated surgical mat that uses a rectal probe to monitor the temperature and provide feedback to maintain the mouse body temperature around 37°C (NeosBiotec, Spain). The mouse was placed in a stereotaxic frame by placing the head onto a bite bar within the anaesthetic nose cone and stabilising it with the ear bars and tape to a 20° to 30° (Fig. 2.1a). The lower body of the mouse was covered with a surgical drape to prevent heat loss.

Next, LacriLube was applied to the eyes to prevent drying or ulceration. A local anaesthetic (0.05 mL 0.25% bupivacaine) was injected into the surgical site between the left eye and left ear. The area was shaved with a cream hair remover and sterilised by alternating between 70% alcohol and Povidone-iodine solution three times. Once the surgical area was sterile and dry, a 1 cm incision was opened vertically between the left eye and ear, the skin was retracted, and the parietal muscle was dissected and retracted from the insertion point to the parietal bone using a suture. Then, the middle cerebral artery (MCA) and its branches were quickly identified through the bone.

A Laser Doppler device (Moor VMS-LDF, Moor Instruments, United Kingdom) was used to measure the blood flow and confirm occlusions by attaching the tip of the laser Doppler probe above the territory perfused by the MCA. The attachment of the optic fibre was performed by first determining the area of interest, followed by lightly thinning the skull with a micro-drill to facilitate adhesion and flow detection. The fibre optic probe was glued perpendicularly to the skull with cyanoacrylate glue and an accelerator to set the glue quickly. A blood flow greater than 90 perfusion units (PU) was expected for baseline, and if the flow was lower than 90 PU, the probe was removed and reattached until a higher flow was detected.

The next step was to create a window centred over the MCA bifurcation with the

micro-drill. The edge of the circumference was thinned down, and a thicker centre was maintained. Once the outer area of the circle was thin enough, the skull flap was removed using 5SF or 5/45 forceps (Fine Science Tools). The meninges were removed by puncturing with a 30 G needle bent 90° and delicately pulling them back with the needle or with forceps and a small cotton tip, thus completely exposing the MCA. Once the meninges were removed, the area was maintained clean and hydrated with saline and the aid of a small metal shaft cotton tip.

Figure 2.1: Representative images of the thromboembolic surgery setup for a right MCA occlusion. (a) Top view of a mouse in the stereotaxic instrument with a Doppler flow probe attached to the skull and microneedle in place for injection (b) Closeup of the mouse head positioning in the stereotaxic instrument. (c) The middle cerebral artery with an in situ clot visualised through the microscope eyepiece.

Thrombin was injected into the bifurcation of the MCA to form an intraluminal clot using a microneedle (a needle made from a glass micropipette, as described in a separate section). A 50 mL syringe with a catheter tip and PE50 tubing was attached to the glass pipette to pneumatically load the microneedle with thrombin (2 µL; 2U/µL); once loaded, the microneedle was attached to the probe holder on the stereotaxic instrument. The microneedle was advanced towards the MCA bifurcation, once in the vicinity of the injection site, the tubing was reattached to the 50 mL syringe, and positive pressure was maintained on the syringe to prevent blood absorption by capillary action that would block the microneedle.

The microneedle was pushed into the MCA lumen, and the thrombin was injected. During the injection, white streams of clotted blood were observed, and the blood flow

was monitored by laser Doppler flowmetry until a blood flow reduction of 80-90% and a stable clot had formed in the bifurcation. After a clot was established, the microneedle was left in place for 10 min and then slowly retracted, preventing disturbance of the clot. Blood flow was monitored for 20 minutes before the bone defect was covered with bone wax. The Doppler probe was removed, the muscle returned to its place, and the incision was closed with a 5-0 prolene suture. The mice were placed in a recovery chamber for 30 min at 32°C before being returned to their home cage.

2.1.3.1.1. Glass Micropipettes

The glass micropipettes used for the thrombin injection were 1-2-3-4-5 µL capacity disposable micropipettes with ring marks (No. 40555003; Hecht Glaswarenfabrik GmbH & Co, Germany). The microneedles were prepared with a P-1000 Pipette Puller (Sutter Instruments, USA), and the optimal pulling protocol was established with the Pipette Cookbook 2018.

The first step was to determine the melting temperature of the glass micropipette using the ramp test function on the P-1000 machine. Test pulls were carried out to establish the optimal protocol to achieve the long tapered microneedle required with the known ramp value. The following tables present the Pipette Puller settings used for each test, followed by a brief description of the microneedles produced.

Test no. 1: Standard thick wall pipet pulling protocol from the Sutter Instruments Pipette Cookbook

	Heat	Pull	Velocity	Time	Pressure
Settings	504	90	70	80	200

Product: the microneedles were too long and curved once the glass had cooled.

Test no. 2: Increase time delay by 10 units (1 unit is 0.5 milliseconds)

	Heat	Pull	Velocity	Time	Pressure
Settings	504	90	70	90	200

Product: the microneedles had a good taper. One end was curved and brittle.

Test no. 3: Reduce ramp heat by five units

	Heat	Pull	Velocity	Time	Pressure

Settings	499	90	70	90	200

Product: both ends were sharp yet curved

Test no. 4: Revert to standard thick wall pipet pulling protocol and decrease velocity

	Heat	Pull	Velocity	Time	Pressure
Settings	504	90	60	80	200

Product: the resulting microneedle was too long and curved.

Test no. 5: Reduce pull intensity

	Heat	Pull	Velocity	Time	Pressure
Settings	504	85	60	80	200

Product: the resulting microneedle was too long and brittle.

Test no. 6: Increase pull delay

	Heat	Pull	Velocity	Time	Pressure
Settings	504	85	60	90	200

Product: the microneedle had no curling, had a good length-to-taper ratio and was selected as the optimal pull program for future preparation of micropipette needles.

Microneedle cutting and bevelling

The recommended microneedle tip size for thrombin injection ranges from 20 to 40 µm (Orset et al., 2007). Microneedles were cut back and measured with an Olympus BX43 microscope with Stream Essential software (Olympus; V2.3.3.) to verify the microneedle diameter. The next step was to test the microneedles for being able to breach the vessel wall to inject the lumen of the MCA: one set of microneedles were cutback with iris spring scissors and bevelled with a BV-10 Beveler (Sutter Instruments, USA) to 25°, and 45°; and the other set of microneedles were cut back with the spring scissors at a 45° angle. The bevelled microneedles, though clean-cut, could not breach the vessel to inject the thrombin (Fig. 2.2.a & b); however, the slightly jagged edge of the scissor-cut microneedles was able to breach the vessel wall (Fig. 2.2.c).

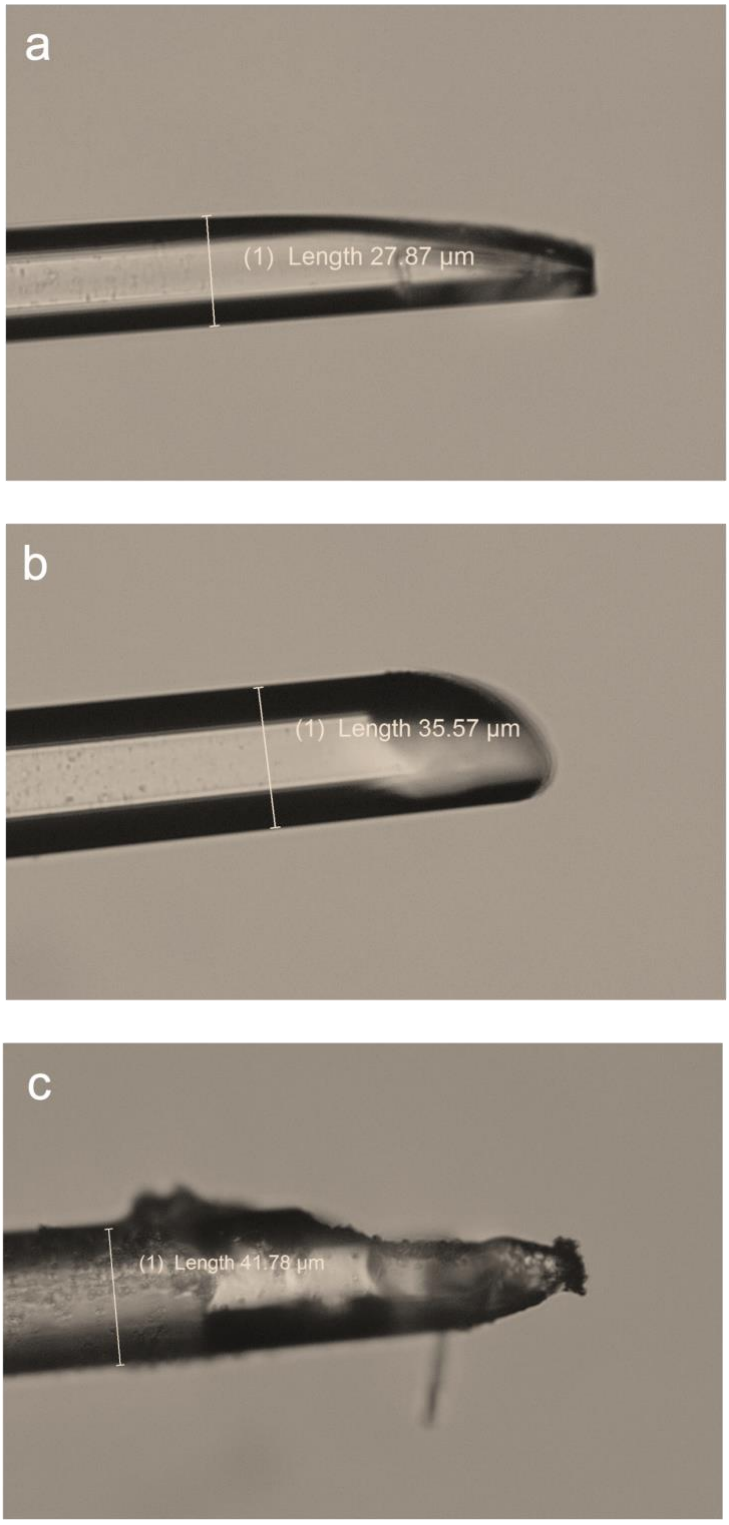

Figure 2.2: Representative images of the bevelled microneedles; (a) 25° and (b) 45° bevel microneedle that were instrument bevelled; and (c) microneedle bevelled with a scissor that was successfully used for thrombin injection into the middle cerebral artery. The diameter was measured with a calibrated microscope and is represented as a length in the images.

2.1.3.1.2. Thrombin Dilution and Characterisation

Human Alpha-Thrombin (HTC-0020; Haematologic Technologies Inc, USA) was used to induce the formation of a clot in the bifurcation of the MCA. The thrombin product was delivered as 50% glycerol/H_20 (vol/vol) solution at 0.1mg/mL concentration. Each batch of thrombin had a unique activity, and it was diluted to a final concentration of 2 U/µL with 18% glycerol by adding saline and glycerol. Fifteen microlitre aliquots were stored at –20°C.

Thrombin injection and clot formation

During the setup stages of the tMCAo surgical model, 1-2 µL of 2U/µL thrombin was injected into the MCA lumen. The clots produced were unstable, and early reperfusion was observed shortly after the thrombin injection. As a part of the setup of this model, we decided to evaluate a few controlled scenarios (Table 2.1) to determine whether the concentration of thrombin or isoflurane levels affects clot stability.

Cerebral blood flow (CBF) positively correlates with isoflurane concentrations, with higher concentrations leading to higher CBF, which could affect the stability of the clot (Munting et al., 2019). In addition, lesion size may depend on thrombin concentration. A study using C57Bl/6 mice observed a concentration-dependent lesion with 1.5 and 3.0 UI of thrombin (Ansar et al., 2014); however, a meta-analysis of published and unpublished data found that concentrations greater than 2 UI led to clots that were resistant to thrombolysis with tPA (Orset et al., 2016). Hence, we limited the trial to comparing 1.5 and 2 UI of thrombin and two isoflurane concentrations to evaluate their effect on clot formation and stability.

Table 2.1: Thrombin optimisation trial

Number of mice	Isoflurane concentration	Buffer	U/µL
2	2 - 2.5%	PBS	1.5
2	2 - 2.5%	Saline	2
2	1.5 - 2%	PBS	2
2	1.5 - 2%	Saline	1.5
Total mice= 8			

2.1.3.1.3. Thrombolysis

The human recombinant tissue plasminogen activator (tPA, Alteplase) (Actilyse ®, Boehringer Ingelheim Limited) was used to dissolve the clot formed by thrombin injection. We used the recommended dose for rodents (10 mg/kg), which is ten times higher than the clinically used dose of 0.9 mg/kg because rodent clots are less susceptible to lysis than human clots (Korninger and Collen, 1981). The Alteplase was prepared from 10 mg powder into a 2 g/mL solution using sterile saline as solvent. For a 25 g mouse, 188 µL of the 2mg/mL solution were mixed with 113 µL of saline for a final volume of 300 µL, of which 200 µL were infused.

For tPA infusion, mice were anaesthetised with an injectable anaesthetic cocktail containing ketamine (75 mg/kg) and xylazine (1 mg/kg). Mice were maintained warm and placed on a heating pad for the infusion of tPA (10 mg/kg; 200 µL), administered with a syringe pump into the tail vein with a 10% bolus, and the remainder infused over 40 min. After the infusion, mice were injected with 100 µL of Revertor (Atipamezole hydrochloride, 1 mg/kg) to wake them up following the anaesthesia. Mice were monitored until they became recumbent, at which point they were returned to their home cage.

2.1.3.1.4. Cerebral Blood Flow Measurement

Laser Doppler flow (LDF) measurements were performed for the thromboembolic model to measure the percentage reduction of CBF in the MCA perfusion area. A reduction of >90% that was stable for 20 minutes after removing the microneedle was considered successful. The LDF also allowed us to exclude any animals that spontaneously reperfused or had an insufficient level of reduction of flow during the observation period (Fig. 2.3). The Laser Doppler Monitor system was calibrated as per manufacturer instructions before use, and the fibre-optic filament was cut back to remove any remaining cyanoacrylate glue from the previous usage. The filament was then attached to the skull, and the baseline CBF was determined. Once the MCA was ready for injection, the recording was started. After the injection, a CBF drop was observed (Fig. 2.3), and the clot stability was monitored until the probe was removed 20 min after injection.

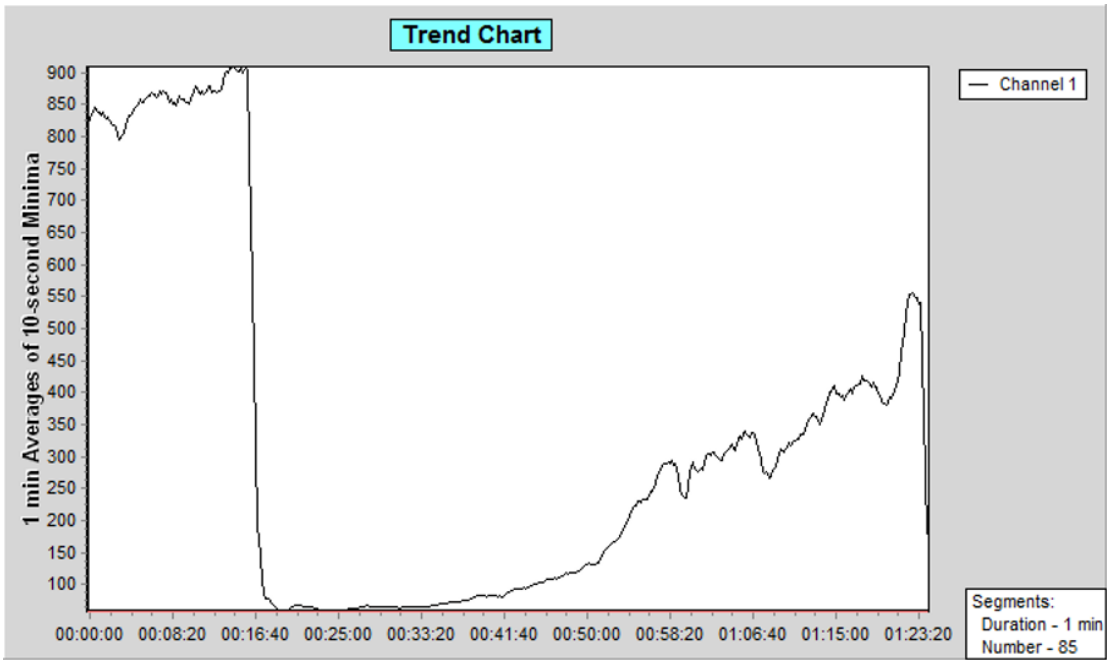

Figure 2.3: Graph representing the cerebral blood flow of a mouse that received thrombin into the MCA. The mouse was observed for one hour after the thrombin injection. After 25 min, there was an increase in perfusion due to clot instability.

2.1.3.1.5. Experimental Setup of Thromboembolic Stroke Model

The thromboembolic stroke procedure was learned on a research stay at the Laboratorio de Investigación de Neurociencias Clínicas (LINC) at the Hospital Clínico Universitario in Santiago de Compostela, Spain, with Professor Francisco Campos Pérez. In Spain, the occlusion was performed on the right MCA of BalbC mice, as previously described by Orset et al. 2007. The procedure was implemented in Cork and set up on the left MCA mice due to the equipment availability (Stereotaxic instrument orientation). We used C57Bl/Bl6 mice because the overall study aimed to use a variety of models that use C57Bl/B6 as genetic background, and aged mice are more commonly available in that strain. During the effort to establish this stroke model, we used 59 mice (Fig. 2.4).

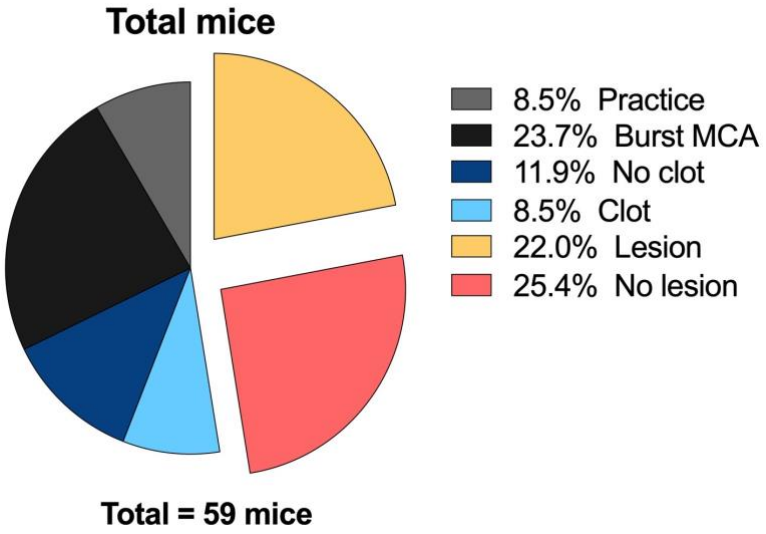

Figure 2.4: Pie chart representing the total number of mice used to set up the thromboembolic stroke model. Grey: Practice animals that were not recovered. Black and dark blue: euthanised mice that suffered a burst MCA or no clot formation. Light blue: non-recovery mice with a stable clot. Yellow represent the recovered mice with a lesion, and pink represents the mice that did not have a lesion.

The procedure was initially performed under non-recovery conditions to practice and become familiar with the steps of the overall procedure (5 animals; 8.5%). Once familiar with the procedure and consistent access to the MCA without rupturing it, the thrombin

injection practice stage was started observing for clot formation and stability (Fig. 2.4). There was a considerable failure rate of 23.7% (14 animals), mainly due to rupturing of the MCA causing uncontrollable bleeds and a lack of clot formation in the MCA bifurcation (7 animals; 11.9%).

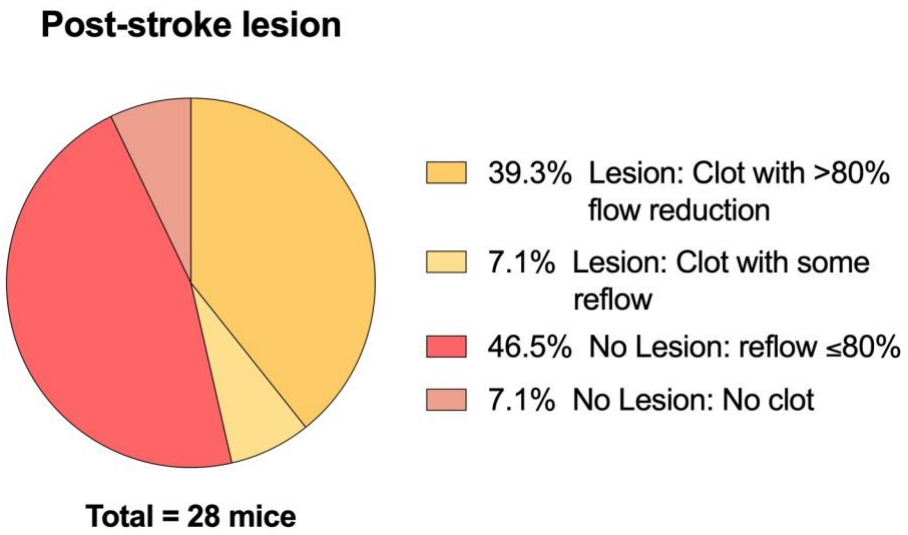

Figure 2.5: Evaluation of the success of the clot and the development of a lesion size following the thromboembolic stroke surgery. Dark yellow represents mice with a lesion with a stable clot (a clot was formed with a greater than 80% reduction in blood flow) and mice with a lesion with a clot with significant reflow (light yellow). Dark pink represents mice without a lesion that had a clot with significant reflow, and pale pink represents mice that did not have a clot and no lesion.

Once the number of successful clots observed during practice procedures was consistent, mice were allowed to recover. A stable clot was determined by an 80% reduction of cerebral blood flow (CBF) or greater that remained in place for 10 minutes. In cases where a clot was unstable, and there was reflow, we recorded the information and compared it to the lesion size measurements after 24 to 72 h. The brains were evaluated with TCC for the presence of a lesion (Fig. 2.5).

As expected, mice with no clots had no lesion (2 animals; 7.1%), and most mice with reflow did not have a lesion (13 animals; 46.5%); however, some mice with considerable reflow did have a measurable lesion. Lastly, mice with stable clots and greater than 80% reduction of CBF always led to a lesion (11 animals; 39.3%).

To identify the cause of the variability in the success rate of this procedure, we compared two different anaesthesia concentrations along with two different thrombin concentrations, as they both could affect clot formation (Munting et al., 2019; Orset et al., 2016). Our results suggest that a higher concentration of isoflurane with a lower concentration of thrombin is more successful than the other combinations (Fig. 2.6).

Overall, 2.5% of isoflurane had a higher frequency of success for clot formation (3:1) vs 1.5% with only one lesion observed. On the other hand, thrombin seems to have had a higher success rate when using the lower 1.5UI (3:1) than 2UI.

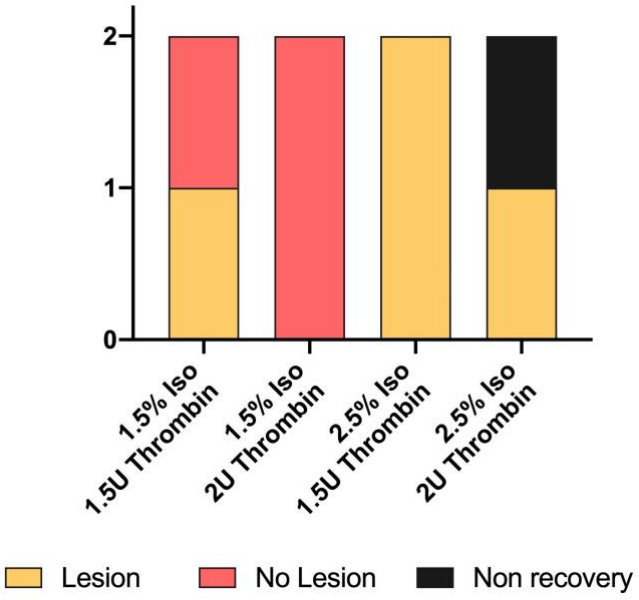

Figure 2.6: Results of the pilot study comparing two concentrations of isoflurane with two concentrations of thrombin. Four groups were studied by the number the outcomes. The observed outcomes were lesion (yellow), No-lesion (pink) and Non-recovery (black).

These results highlight the difficulties experienced while attempting to establish this model. While considering the risk that proceeding with this model might require additional mice to achieve the intended number of mice per group, we decided to use another stroke model that produces a similarly sized stroke lesion (Macrae, 2011). The electrocautery stroke model was selected, and all subsequent studies were performed with the new method because it has a high success rate, is highly reproducible, and has low mortality.

2.1.3.3. Electrocautery Stroke

Mice were anaesthetised with 2% isoflurane using 100% O_2 or 30% O_2:70% N_2 gas mixture as the carrier gas. After induction, the anaesthesia was maintained at 1.5-2% isoflurane using the face mask on the stereotaxic frame. The head was placed in the nose cone, angled to 20-30° and stabilised with the ear bars for a better view and access to the left side of the head (Fig. 2.7). Once the animal was in place, the rectal probe was inserted as part of the homeothermic blanket system (NeosBiotec) used to maintain the animal's

temperature at 37°C. LacriLube was applied to avoid cornea ulceration during the procedure. The surgical site was infiltrated with 0.25% w/v bupivacaine (0.05 ml). The fur between the eye and the ear was removed with depilatory cream, and the area was cleaned with three cycles of iodine solution/70% isopropyl alcohol.

An incision was made vertically on the skin midway between the left eye and the ear. The parietal muscle was cut at the insertion point, and the muscle was retracted using a suture to expose the parietal bone. The left middle cerebral artery was identified through the skull in the area posterior to the zygomatic arch. The skull was thinned in a circular pattern around the bifurcation of the MCA using a micro-drill. Once the edge of the circle was thin enough to break with forceps, the skull flap was lifted and removed. The dura was then pierced and pulled away from the artery, allowing access to the MCA.

The MCA was occluded by bipolar electrocoagulation with a Bovie Bantam Pro electrosurgical generator (A952) with the aid of McPherson 3 1/2" straight forceps (A842) (Symmetry Surgical Inc, USA). The voltage ranged from 0.7V on ApoE mice up to 1.5V used on older mice, with younger mice falling in the middle at 1.0V. The MCA bifurcation and accessory branches were occluded by electrocoagulation (Fig. 2.7). Occlusion was visually confirmed once the blood turned white and the clot was stable for a few minutes. The absence of bleeding after nicking the MCA distally to the coagulation site confirmed the occlusion. The bone defect was covered with bone wax to prevent adhesions, and the muscle was released and returned to its position. The incision was closed with 5-0 prolene sutures, anaesthesia was discontinued, and the mouse was allowed to recover in a heated chamber at 32°C for 30 minutes before being returned to its home cage.

Figure 2.7: Electrocautery surgical setup: Top image shows the surgical microscope, electrocautery machine, dental drill, heated surgical pad and stereotaxic instrument. The bottom picture shows an anaesthetised mouse with the head in the nose cone at a 30° angle; the mouse also has the rectal temperature probe in place for the feedback control of the heated surgical pad.

2.1.4. Haemorrhagic Stroke

Experimental intracerebral haemorrhage was induced by injecting collagenase into the right striatum with some modifications to a previously described method (Lu et al., 2014). Briefly, mice were anaesthetised with 2% isoflurane in 100% O_2. Once deeply

anaesthetised, mice were placed in the stereotaxic instrument: the head was held in place with the ear bars, which were covered with a topical anaesthetic (EMLA Cream 5% lidocaine/prilocaine). The skull was levelled by adjusting the bite bar that also served as a nose cone (see Fig. 2.8). LacriLube (Allergan Ltd) was used to protect the eyes from drying and ulceration. Body temperature was monitored and maintained through feedback thermoregulated surgical mat (NeosBiotec, Spain).

The surgical area was infiltrated with 0.05 mL of Bupivacaine (0.25% w/v solution), and the fur was removed with cream hair remover (Veet, Reckitt Benckiser). The surgical area was sterilised by alternating 70% alcohol and Povidone-iodine solution three times. Once the area was sterilised, a 1 cm incision was made on the scalp, and the skin was drawn back with a Colibri retractor (Fine Science Tools, Germany) to expose the skull.

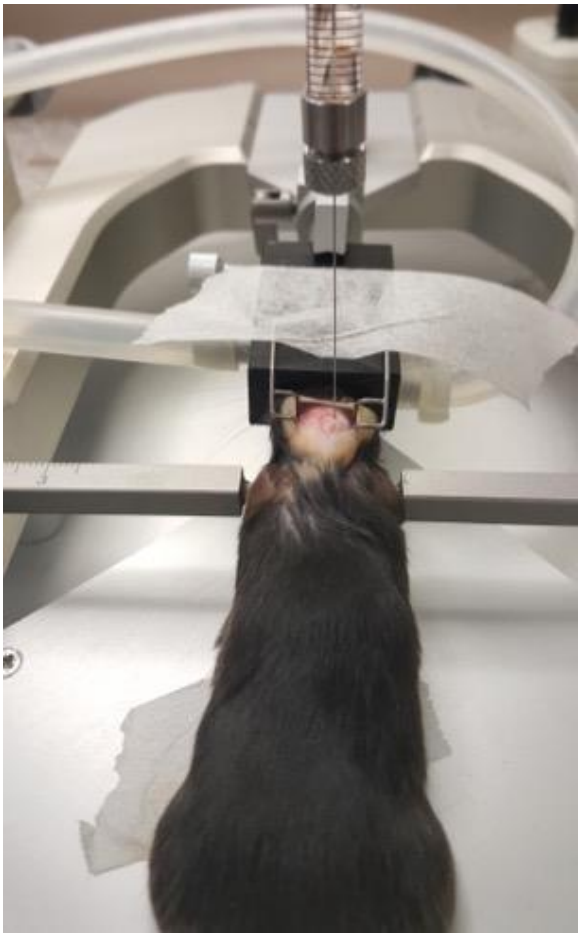

Figure 2.8: Intracerebral haemorrhage surgery. Mouse with a needle in the right hemisphere following the injection of collagenase for the induction of haemorrhagic stroke

A 2.5 µL Hamilton syringe (RN701, needle 30G, blunt, 4 inches long) was loaded with 0.075 U of collagenase (0.5 µL, Bacterial collagenase VII-S, Sigma Aldrich, UK) and placed in the syringe holder. The needle was aligned to bregma and then moved 2 mm right, 0.2 mm anteriorly; when in place, the skull was marked. The needle was lowered through a 1 mm burr hole created with a micro-drill using a 0.9 mm burr. The needle went 3.5 mm into the brain, and the distance was counted from the point where

the needle bevel was level with the skull. The collagenase injection was performed over 15 seconds; the needle was left in place for 10 min to allow the collagenase to act and prevent reflux through the needle track. Lastly, the needle was slowly retracted, and the burr hole was covered with bone wax. The scalp was closed with 5-0 sutures, and the mice were allowed to recover in a heated chamber at 32°C for 30 min before being returned to their home cage.

2.2. Experimental Outcome Measures

The primary outcome measures evaluated are the stroke lesion size and performance in behaviour tests. The secondary outcomes comprise the weights and daily scores after surgery, as well as, when relevant, other supplemental data such as cholesterol levels and lymphocyte counts. The lesion size was measured from histological slides, and the behaviour was assessed by cylinder test, foot fault and wire-hanging test.

2.2.1. Histology

2.2.1.1. Tissue Collection

Mice were euthanised by anaesthetic overdose (20-30 µL intraperitoneal injection of pentobarbital, Euthatal 200 mg/ml). The depth of anaesthesia was verified by testing pain reflexes with a tail or toe pinch. The ribcage was opened by cutting it from the sides and lifting it towards the head. A small incision was made into the left atrium, and 15 mL of cold phosphate-buffered saline (PBS) was slowly injected into the left ventricle until the perfusate coming out through the left atrium ran clear.

An incision was made down the middle of the scalp and retracted to the sides to dissect the brain. The skull was cut along the lambdoid suture and sagittal sutures with sharp scissors. The base of the skull was pulled down with forceps to expose the cerebellum, followed by the retraction of the parietal bones to expose the brain completely. Lastly, the brain was removed and stored on ice until further processing.

2.2.2.2. Tissue Staining

The following sections describe the different methods for visualising the stroke lesion once the brain has been isolated. Fresh tissue can be stained with 2,3,5-triphenyl tetrazolium chloride (TTC) to visualise lesions 1-3 d after stroke (Zille et al., 2011). However, this technique cannot be used beyond three days after stroke because

infiltrating immune cells might produce uneven staining (Liszczak et al., 1984). TTC was implemented to verify the injury in both thromboembolic and electrocautery models. Histological sections from cryopreserved brains were stained with haematoxylin and eosin and immunohistochemistry.

2.2.2.2.1. 2,3,5-Triphenyl Tetrazolium Chloride Staining

Fresh ischaemic stroke tissue was characterised 24-72 h after stroke with TTC staining. Brains were collected from euthanised mice, perfused with PBS and stored on ice in 15 mL tubes. The brains were transferred onto an ice-cold brain matrix (RBMS-200C, World Precession Instruments, UK) and cut into 1 mm thick coronal sections with cold razor blades.

Figure 2.9: Mouse brain sections (1 mm) 24 h after an electrocautery-induced stroke stained with TTC. Left hemisphere stroke lesion in white, and healthy tissue in red.

The sections were transferred to a 5 cm Petri dish with 5 mL of 2% TTC in PBS. The plate was wrapped with foil and placed in an incubator at 37°C for 30 min. The ischaemic regions remained white in successfully stained tissue, and the healthy tissue stained red (Fig. 2.9). Sections were then imaged next to a calibration ruler with a camera or scanner, and the lesion was quantified with ImageJ (v1.51f, NIH).

2.2.2.2.2. Tissue Freezing and Sectioning

Brains were frozen in isopentane (2-methyl butane) that was chilled on dry ice and maintained at –40°C. The freshly isolated brains were submerged in the isopentane and then removed, lightly covered with embedding matrix (M-1, Thermo Fisher Scientific) and chilled again before being stored. The frozen brains were stored at –80°C.

For sectioning, brains were transferred to a –20°C freezer overnight to stabilise the tissue to the sectioning temperature. The brains were sectioned with a Leica CM 1900 cryostat at –20°C. For sectioning, the brains were mounted perpendicularly onto the cryostat chuck inside the cryostat at –20°C by adding fresh embedding matrix and attaching the brain perpendicularly to the chuck. Once the embedding matrix was set, the brain and the base were thinly coated with more embedding matrix to create one block. Once the embedding matrix sets, the brain could be sectioned.

The sectioning was performed by first aligning the chuck perpendicularly to the blade and beginning to slice the embedding matrix and the anterior part of the brain. The alignment was adjusted to ensure that the sections were even. The sections were collected onto a single slide (SuperFrost Plus, Thermo Fisher Scientific) based on brain anatomy. The sections were collected at 500 µm intervals from 1.54 mm anterior-to-bregma to 2.92 mm posterior-to-bregma throughout the cortex. Any deviations in the interval distance were recorded and accounted for in volume calculations. The 20 µm thick section were collected onto ten slides per brain in serial coronal sections. The completed slides were air-dried and stored at –20°C until staining was performed. Before staining, all slides were air-dried for 1 h and scanned to allow accurate hemispheric volume measurements in case of damage or lifting of sections during staining.

2.2.2.2.4. Haematoxylin and Eosin Staining

Air-dried slides were fixed in 4% formalin for 5 min and then rehydrated in a series of graded alcohols (100%, 95% and 70%) for 2 min each, followed by 2 min in distilled water. Slides were placed in Mayer’s haematoxylin solution (Sigma Aldrich, UK) for 4 min, followed by rinsing in tap water until the water ran clear. Slides were then dipped into 0.25% Eosin Y (Sigma Aldrich, UK) solution for 1 min. Lastly, slides were washed in 70% alcohol and then dehydrated through a series of alcohols (70%, 90% and 100%) for 2 min each, cleared in Histochoice (xylene alternative; Sigma Aldrich, UK) and coverslipped with Permount mounting medium (Fisher Scientific).

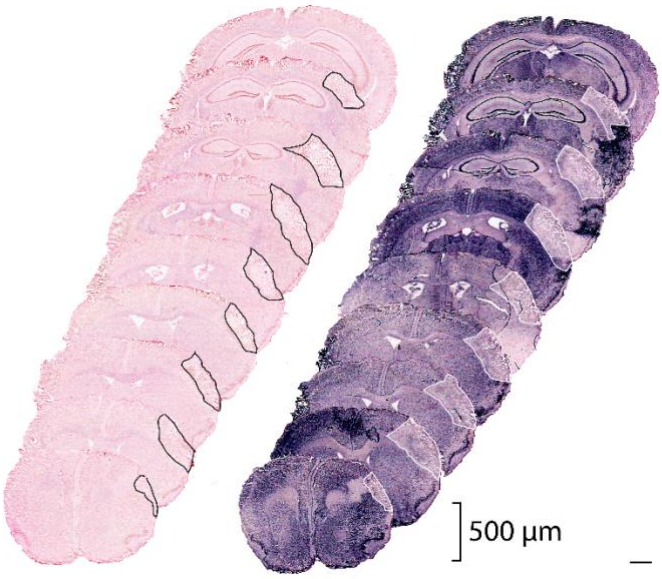

Figure 2.10: Brain sections from mice subject to ischaemic stroke. The sections are 20 µm thick and 500 µm apart. The sections to the left are stained with Haematoxylin and Eosin, and the sections to the right are stained by immunohistochemistry detection of NeuN. The brain sections have the stroke injury delineated in a contrasting colour (black for H&E and white for NeuN). These sample images belong to the same mouse and compare the detectable ischaemic area under each staining condition. Scale bar 1 mm

2.2.2.2.3. Cresyl violet

The goal was to increase the clarity and definition of the injury. Llovera et al. described the possibility of having clear boundaries of the lesion, in contrast to the results observed in H&E staining. The Cresyl violet staining was performed as previously described (Llovera et al., 2014). For establishing the method, a range of concentrations of Cresyl violet (0.1, 0.2 and 0.5%) were dissolved in dH_2O, acid dH_2O or acetate buffer. The different parameters that were evaluated resulted in different intensities of staining; however, the best staining would have paler staining in the ischaemic area as opposed to what we were able to observe in figure 2.11.

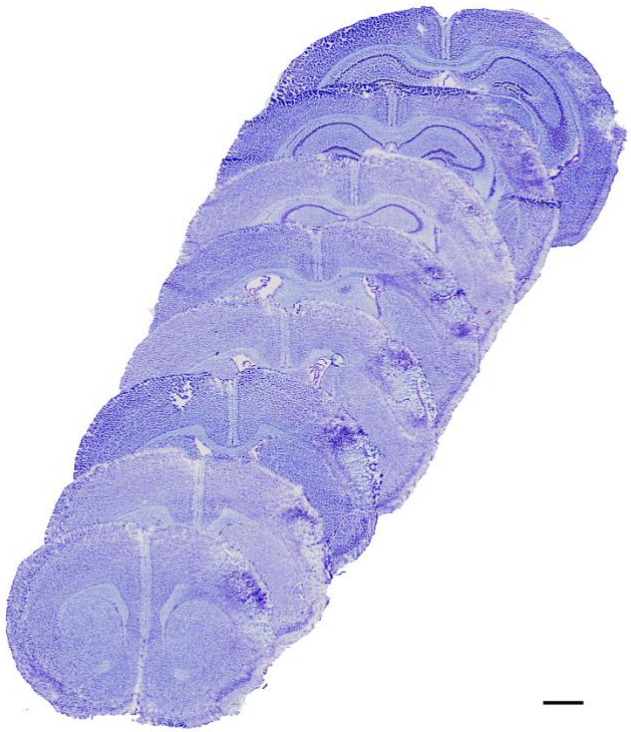

Figure 2.11: Representative image of brain sections from a mouse with a stroke. The sections are 20 µm thick section 500 µm apart. Scale bar 1 mm

The staining protocol was performed on tissue dried overnight and fixed for 5 minutes in 4% paraformaldehyde (PFA). The tissue was rehydrated for 2 minutes in a series of alcohols (100%, 95%, and 70% ethanol) and washed in distilled water for 2 minutes. The slides were stained for 5 minutes in a 0.2% solution of Cresyl violet and then washed in distilled water until the excess stain was removed. The slides were dehydrated in a series of alcohols, cleared with histolene, and coverslipped. The 0.2% Cresyl violet stain solution was prepared in H_2O acidified to pH 4.0 with glacial acetic acid. The solution was heated to 60°C to dissolve the Cresyl violet powder and then filtered. Even though the stain was achieved, it was challenging to differentiate the injured tissue from the healthy tissue, primarily due to unclear borders.

2.2.2.2.5. Immunohistochemistry (NeuN) Staining

NeuN is a protein expressed in the neuronal nucleus (Badan et al., 2003; Hughes et al., 2009). Immunohistochemistry was used to determine the injury size by quantifying the area negative for NeuN antibody staining, as previously published (Katchanov et al., 2006). The rabbit monoclonal Anti-NeuN antibody (ab177487, Abcam) was selected over

the commonly used mouse monoclonal anti-NeuN antibody (MAB377, Chemicon) because the mouse monoclonal would have required extra blocking steps to eliminate background binding on mouse brain tissue. After staining with the secondary antibody, biotinylated Goat Anti-Rabbit IgG (ab207995, Abcam) and conjugated to avidin-biotin horseradish peroxidase complex solution (ABC Kit pk-4000, Vector Laboratories), the signal was visualised with 3,3'-Diaminobenzidine (DAB Kit SK-4100, Vector Laboratories).

Slides with brain sections were air-dried overnight, followed by 20 minutes of methanol (Sigma Aldrich, UK) fixation at –20°C. Slides were incubated in cold 10% hydrogen peroxide methanol solution for 10 min, followed by blocking in 5% normal goat serum (S-1000, Vector Laboratories) in 0.3% TritonX TBS. Anti-NeuN antibody (1:1500) was incubated for 1 h at RT, followed by secondary antibody Goat Anti-Rabbit biotinylated IgG (1:250) for 1 h at RT. Slides were washed three times with TBS, incubated in avidin-biotin horseradish peroxidase complex solution (ABC Kit PK-4000, Vector Laboratories) for 30 min, and the reaction product was visualised with diaminobenzidine (DAB Kit SK-4100, Vector Laboratories). Lastly, the sections were dehydrated in a series of alcohols, cleared with histochoice and coverslipped with Permount mounting medium (Fisher Scientific).

2.2.1.3. Stroke Injury Quantification

The measurements associated with injury quantification were performed by scanning the slides before and after staining with a high-resolution scanner (Epson V600) to 3200 dpi (dots per inch) resolution. The measurements were obtained with ImageJ (Version 1.51.f, NIH); for this, the file was opened with ImageJ, and the calibration was set to 3200 dpi (125.98 pixels per mm). So all measurements were obtained in mm^2. Brain hemispheres and the lesioned area were measured, and the volumes were calculated by multiplying the area in each section by the known distance (500 µm) between sections. The total volume was taken as the sum of these individual volumes (Sommer, 2016). Oedema expressed as [(Ipsilateral hemisphere – contralateral hemisphere) / contralateral hemisphere * 100].

The intracerebral haemorrhage regions of interest included measurement of the hemispheric volumes, ventricles and infarct sizes associated with haemorrhage cavitation and ventricular dilation (Fig. 2.10). The injured tissue was observed under a light

microscope to determine the boundary of the lesion, and that was transcribed onto the scanned image for measurement of the injured area. Injured tissue was identified as brain tissue with abnormal morphology. Depending on the brain, this could include bleeding, cavitation and areas of tissue necrosis with visible pallor and abnormal nuclei.

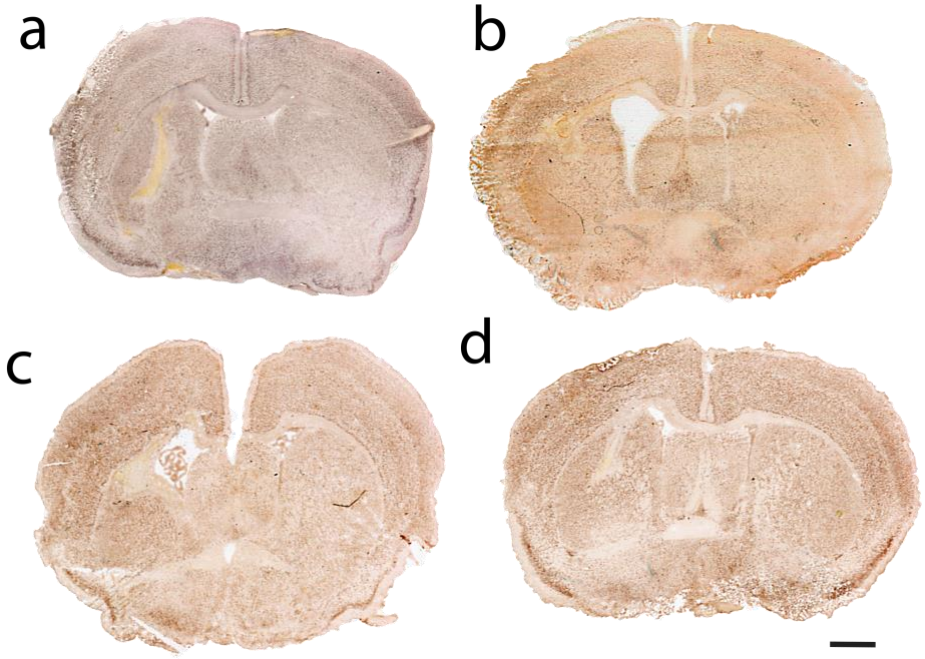

Figure 2.12: Intracerebral haemorrhage NeuN stained representative images of a) Saline and b) fingolimod-treated male mice; c) Saline and d) fingolimod-treated female mice. Scale bar 1 mm.

For the ischaemic stroke model, the regions of interest (hemispheric volumes, ventricles and infarct) were measured in H&E and NeuN stained tissue (Fig. 2.10). Injured tissue was observed, and the boundary was determined under a light microscope. Infarct tissue was identified area with abnormal morphology. Depending on the extent of damage, included areas of necrosis, haemorrhage or cavitation with immune cell infiltration and, in some cases, glial scar formation at the boundary. The infarct boundary was identified where the abnormal tissue transitioned to a normal morphology. The edge was verified under the microscope, especially in the case of H&E stained samples, wherein the border was diffuse, and the boundary between the infarct and healthy tissue had to be closely identified.

2.2.1.4. Ventricle Measurements

The enlarged ventricles were measured similarly to all other regions of interest. They were identified in each section and outlined. Furthermore, the samples were compared with the unstained tissue to ensure none of it was lost during the staining procedure. The volume was calculated by multiplying the area of the ventricle by the thickness of the section (20 µm) and by the separation between the sections (500 µm)

2.2.2. Behaviour

Behaviour evaluation was utilised to characterise the effect of stroke and fingolimod treatment on functional recovery. Animals were tested with cylinder and foot fault tests to evaluate the asymmetry associated with unilateral stroke damage. The wire-hanging test evaluates grip strength, balance, and endurance (Balkaya et al., 2012). The tests were performed at baseline and, depending on the study, at 7 and 14 d; 3 and 7 d; or 2, 5 and 10 d after surgery. A Canon Legria HF R706 camera was used to record all the tests for offline analysis.

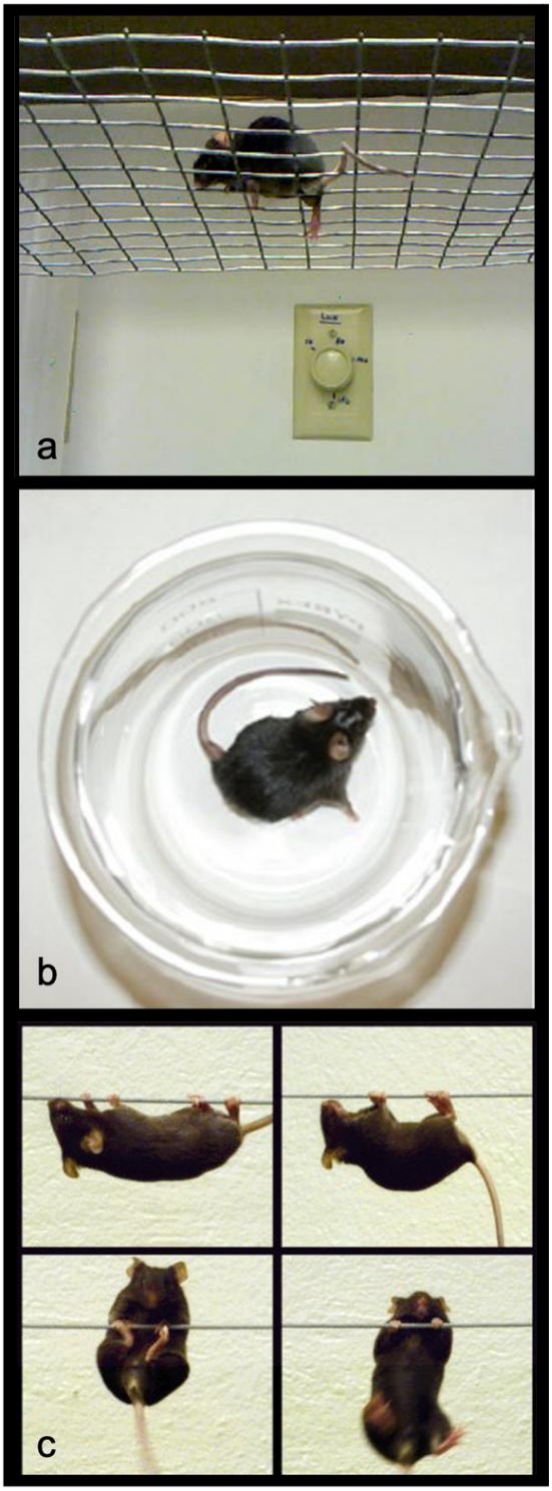

Figure 2.13: Behaviour test views matching video recording style used for each test; a) Foot fault test: mouse on the grid viewed at an angle from below; b) Cylinder test: mouse in the cylinder viewed from above; c) Wire-hanging test: frontal view of a mouse hanging off the wire.

2.2.2.1. Cylinder Test

The cylinder was selected because it is a widely used behaviour test that can be compared to other studies (Balkaya et al., 2012). The test was performed in a large glass beaker of 12.5 cm in diameter by 23.5 cm in height. The recording was done from above

to allow visualisation of the times the mice reared and touched the cylinder wall (Fig. 2.13b). A mouse was placed in the cylinder for the test and allowed to explore until it performed 20 rears and wall touches.

Recordings were analysed by an investigator blinded to the treatment groups at 0.5X speed and frame by frame when necessary to determine the paw usage. The numbers of independent touches with ipsilateral (I), contralateral (C) or both (B) forepaws were used to calculate a score for each mouse and time points with the equation (C–I)/(C+I+B).

2.2.2.2. Foot Fault Test

The test evaluated the level of laterality a mouse experienced after a stroke by counting the number of times the mouse missed a step (Schaar, Brenneman and Savitz, 2010). For this test, a metal grid (25 x 35 cm) with 1 cm^2 openings was placed over a pair of mouse cages (Fig. 2.13a). The test was performed by placing a mouse on the grid and inducing it to walk across from one end to the other using a cardboard cylinder and the smell of the home cage.

The videos were recorded at an angle from below the grid to visualise the missed steps (Fig. 2.14). Videos were viewed frame by frame by a researcher blinded to the treatment groups. The score was calculated by the number of missed ipsilateral steps over the contralateral ones out of 100 steps.

Figure 2.14: Recording setup for the grid walking test: The grid is set on top of two cages, and the video is shot from below.

2.5.2.3. Wire-Hanging Test

The wire-hanging test was performed using a 45 cm long metal wire placed between two wooden poles 35 cm above a padded base. Mice were allowed to grab onto the middle of the wire with their forepaws for the test (Fig. 2.13 c). The scores ranged from 0 to 5, based on the ability of the mice to hang onto the wire and escape.

0 - unable to remain on the wire for more than 30s

1 - failed to hold onto the wire with both forepaws and hind paws

2 - held on to the wire with both forepaws and hind paws but not the tail

3 - held on to the wire using its tail along with all four paws

4 - moved along the wire with all four paws plus the tail

5 - scored four and ambulated down the post supporting the wire

Three attempts were recorded and scored by a researcher blinded to the treatment and measurement time-point. The final score was the median of the attempts.

2.2.3. Post-operative recovery scores

The post-operative recovery was assessed with a scoresheet (Appendix 1). The data collected served as a measure and a sign of the recovery after stroke, informing whether there were differences in the recovery-associated treatment. Body weight was measured at baseline to determine the daily weight loss percentage. Based on the percentage of weight loss, a score was assigned as follows: 0 = <5%, 1 = 5-10%, 2=11-15%, 3=16-20%, and mice that has lost 20% of their original weight required humane endpoint.

Appearance scores were determined based on the grooming behaviour of the mice and the degree of piloerection they were experiencing. The scores were 1= coat slightly unkempt, 2= slight piloerection and 3= marked piloerection. Behaviour scores were based on the ability of mice to move around the cage and their response to handling. The scores were 1= slightly abnormal gait, 2= markedly abnormal gait, 3= significant mobility problems, and mice that experienced prolonged immobility (>24hrs) required a humane endpoint. Additionally, mice tense and nervous on handling scored a 2; when markedly distressed, they scored a three.

Neurological scores were determined using the Bederson scale to assess the recovery of the mice after stroke (Pinkernell, Becker and Lindauer, 2016). The grading scale of 0-3 is used, with 0= normal and 3= the highest level of disability; 0= no observable deficit, 1= forelimb flexion, 2 = decreased resistance to lateral push (and forelimb flexion) without circling, 3 = same behaviour as grade 2, with circling

Lastly, a composite score was calculated by adding the individual scores from weight, appearance and behaviour. The score was adjusted by adding one more point every time there was a score of 3. The composite score defined additional post-operative care, such that mice scoring 4-5 were given supplementary care, scoring 6-9 were reviewed by BSU staff, and mice scoring ≥10 points were humanely euthanised by anaesthetic overdose or cervical dislocation.

2.2.4. Blood Sampling

Blood samples were collected from mice in the atherosclerotic study by submandibular facial vein puncture. The procedure was performed with modifications to a previously described method (Golde, Gollobin and Rodriguez, 2005). A 16G needle was used to puncture the vein, and 2-3 drops of blood were collected (about 100 µL) in a 500 µL Eppendorf tube.

Blood was collected from all mice after euthanasia and prior to cardiac perfusion. For this procedure, the abdomen was cut open, and the viscera were gently moved aside to expose the abdominal aorta. A 26G needle attached to a 2 mL syringe was used to collect 0.5-1 mL of blood.

2.5.3.1. Flow Cytometry Analysis

For the analysis of the lymphocyte counts, approximately 500 µL of blood was collected from the abdominal aorta of euthanised mice. The blood was transferred into EDTA tubes until further processing. The blood was mixed with 5 ml of 1X Red Blood Cell Lysis Buffer (eBioscience, #430054) and incubated for 5 minutes to remove red blood cells. The lysis reaction was stopped by adding 20 ml of 1X PBS. The cell suspension was then washed twice by spinning at 300 x g at 2-8°C and re-suspending in the appropriate volume of 1X PBS. The washed and re-suspended cells were counted with trypan blue to determine the total cell concentration and viability.

For the confirming of the delivery and subsequent reduction in lymphocytes counts in the intracerebral haemorrhage studies, the cell samples were incubated with the following fluorophore-conjugated antibodies at 4°C for 30 minutes: anti-mouse CD3ε PE-Cy-7 (Invitrogen, Clone 145-2C11), anti-mouse CD4 FITC (Invitrogen, Clone RM4-5) and anti-mouse CD8a PerCp-Cy5.5 (Invitrogen, Clone 53-6.7). All antibodies were used at concentrations estimated by titration experiments. Fixable Viability Dye eFluor 780 (eBioscience) was added as a live/dead cell stain. The total cell counts were estimated using “CountBright absolute counting beads” (Invitrogen). An LSR II Flow Cytometer instrument (Becton Dickinson) was used for the flow cytometric analysis, and the data were analysed using FlowJo software (v8.6.3) as previously described (Diaz Diaz et al.,

2020).

Similarly, the ischaemic stroke study samples were incubated for 5 min with 50 µL of anti-mouse CD16/CD32 (Clone 93, 1:100; eBioscience). The cell suspensions were then stained for anti-mouse CD45 (PerCP-CY5.5) (30-F11, 1:100), CD3(PE-Cy7) (145-2C11, 1:100), CD4 (FITC) (RM4-5, 1:800), CD8(Pacific Blue) (5H10, 1:100), and CD25 (APC) (PC61.5, 1:100) (eBioscience). A live/dead stain (1:10,000 solution) was also added to each sample (Fixable Viability Dye eFluor 780, eBioscience). The samples were incubated in the dark for 30 min at 2–8°C, washed, fixed, and re-suspended in PBS. The Flow cytometric analysis was performed with an LSRII flow cytometer (Becton Dickinson). The compensation control was set using BD CompBead Anti-Rat/Anti-Hamster Particles. Data were analysed using FlowJo (v10). Gates were set according to unstained samples and fluorescent minus one (FMO) controls. Absolute cell counts for all tissues were calculated based on the instructions provided with the CountBright Absolute Counting Beads (Molecular Probes) (Malone et al., 2021; Diaz et al., 2021, 2022).

2.2.3.2. Serum Analysis

The mice in the atherosclerosis comorbidity study (ApoE-/-) naturally present with higher levels of cholesterol (412 mg/dL) and higher glycemia (127 mg/dL) than C57BL/6 mice fed regular chow (Yin et al., 2012). Thus, serum samples were used to evaluate glucose and lipid levels in the atherosclerotic study.

Blood samples were allowed to clot at room temperature for 30 min. The serum was prepared by centrifugation at 2000 x g for 15 min at 4°C. The supernatant (serum) was collected, transferred into a fresh tube, and stored at –80°C. The serum was collected twice, two days before stroke and at the experimental endpoint.

2.2.3.2.1. Total Cholesterol

Cholesterol levels were determined with a Cholesterol Fluorometric Assay kit (No. 10007640, Cayman Chemical) following the manufacturer's instructions. Cholesterol levels in the experimental serum samples were expected to fall between 10-90 mM (500-

3500 mg/dL), beyond the standard curve for the assay (0-20 µM); thus, the samples were diluted to fall within the range of the assay with a dilution buffer provided in the kit. The assay was run in duplicates. The plate was covered to protect the reaction from light and incubated at 37°C for 30 min. Lastly, the fluorescence was read using 530nm excitation and 590nm emission wavelength using a SpectraMax M3 microplate reader (Molecular Devices, USA).

2.2.5. Atherosclerotic Lesions Quantification

Aorta isolation

The whole aorta was dissected from transcardially perfused mice, starting from its bifurcation into the iliac arteries. The abdominal organs were pulled aside to expose the aorta and its branches that were cut off. The diaphragm was cut away to access the base of the thoracic aorta; once in the thorax, the lungs were removed to access the aortic arch. Once the aorta was removed from the body, further dissection was completed through the length of the aorta to remove excess connective tissue. The aortas were fixed in 5 mL of 10% neutral paraformaldehyde overnight on a shaker at 4°C.

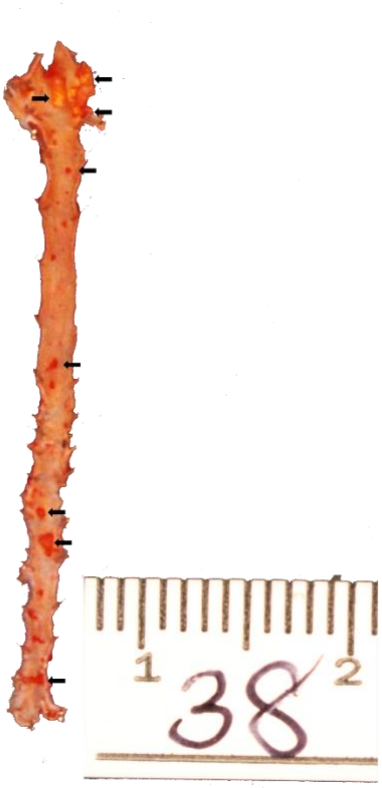

Figure 2.15: Oil Red O staining of a whole mounted aorta. At the top of the image, the aortic arch has several atherosclerotic plaques (arrows), and the lower section of the aorta also has atherosclerotic plaques. A ruler with the animal number is included at the bottom part of the image

Oil red O staining

The procedure for staining and the solution preparation was carried out as previously described (Maganto-Garcia, Tarrio and Lichtman, 2012). Briefly, for a 0.5% Oil Red O solution, 3 grams of Oil red O (ORO) were saturated in 10 mL of isopropanol for three

days, the solution was centrifuged for 15 min at 900 x G, and the supernatant was diluted in a 6:4 ratio with dH_20 and filtered through 0.45 mm filter.

The aortas were washed overnight in PBS at 4°C and dehydrated for 2 min in propylene glycol. The aortas were incubated for 2 h at room temperature in 5 mL of 0.5% ORO solution on a tube roller. The excess staining was removed in four washes with 85% propylene glycol for 1 minute each, thus differentiating the lesions from the aortic wall. Aortas were washed in 5 mL PBS overnight in PBS on a tube roller at 4°C.

Lastly, the aortas were cut open to expose their inner face, and atherosclerotic plaques were exposed and pinned onto silicon-elastomers plates using stainless steel minutien pins. Pictures were acquired, and the number of plaques on the inside surface of the aorta were counted (Fig. 2.15).

2.3. Experimental Design

Based on the preclinical studies published up to date and the promising results of pilot clinical trials, it is worth further exploring the effect of fingolimod and expanding the quality of the preclinical research because there is still a need for better quality research. The two meta-analyses have demonstrated that the overall quality of the preclinical studies evaluating fingolimod is low, and these studies often lack controls for factors that increase internal or external bias (Dang et al., 2020; Liu et al., 2012). To address these shortcomings in the literature, many have recommended using power calculations, randomisations and blinding to address the internal biases. Other recommendations address the external biases by adding diversity models, either by comorbidities or evaluating both sexes in one study (Mergenthaler and Meisel, 2012). This first section of the methods will focus on the measures adopted to reduce internal bias in these studies.

2.3.1. Sample Size Calculation

The sample size was calculated a priori based on an estimated effect size from a meta-analysis that evaluated the efficacy of fingolimod in different animal models of stroke from research publications between 2009 and 2011 (Liu et al., 2012). The sample size calculation was performed for an unpaired t-test comparing the stroke lesion size between saline-treated (control) and fingolimod-treated groups. G*Power (Faul et al., 2009) was used to perform the *a priori* power calculation. The data variability was chosen to be equal between the groups, assuming alpha=0.05, power=0.80, and allocation ratio 1:1, resulting in 15 mice/group required for each study.

2.3.2. Exclusion Criteria

Exclusion criteria were predetermined for all mice undergoing experimental procedures; mice that did not meet the criteria were euthanised.

1. Mice that were not in good health prior to initiation of experimentation were excluded (good health: animals without fight wounds, signs of infection, wasting or

malocclusion);

2. During surgery, mice that did not have an MCA bifurcation or had fine branches of the MCA (affecting lesion size), were excluded;
3. During surgery, mice that had an uncontrollable haemorrhage following the MCA occlusion (by electrocautery or thrombin injection) or had damage to the cortex, were excluded;
4. For the thromboembolic stroke, mice that did not have a reduction in cerebral blood flow $\leq 90\%$; and mice that did not have a stable clot for up to 10 minutes following the needle retraction were excluded.

Mice that passed the first set of exclusion criteria could then be excluded from the data analysis under the following circumstances:

1. Mice that did not wake up after the surgical procedure, from either ischaemic or haemorrhage surgeries, or that did not survive to the last experimental endpoint;
2. Mice that did not have a measurable lesion in histological slides;
3. Mice from which histological brain sections were damaged during processing;
4. Mice that had incomplete histological or behaviour datasets.

2.3.3. Randomisation and Blinding

Randomisation:

All mice allocations were randomly allocated to treatment groups prior to experimentation. The randomisation was done with a pseudorandom number generator (randomizer.org) that can generate various sequences based on how many numbers are required, the number of sets, and the range of numbers in a set (Fig. 2.16).

Mice were randomised into sets of two or three depending on the number of treatment groups. The number of sets were determined by the number of mice per group (Table 2.2). The allocation was revealed when mice had recovered from surgery, and the first dose was prepared. Mice euthanised prior to surgical recovery were excluded from randomisation assignment to prevent disparity in the total of animals assigned to each group.

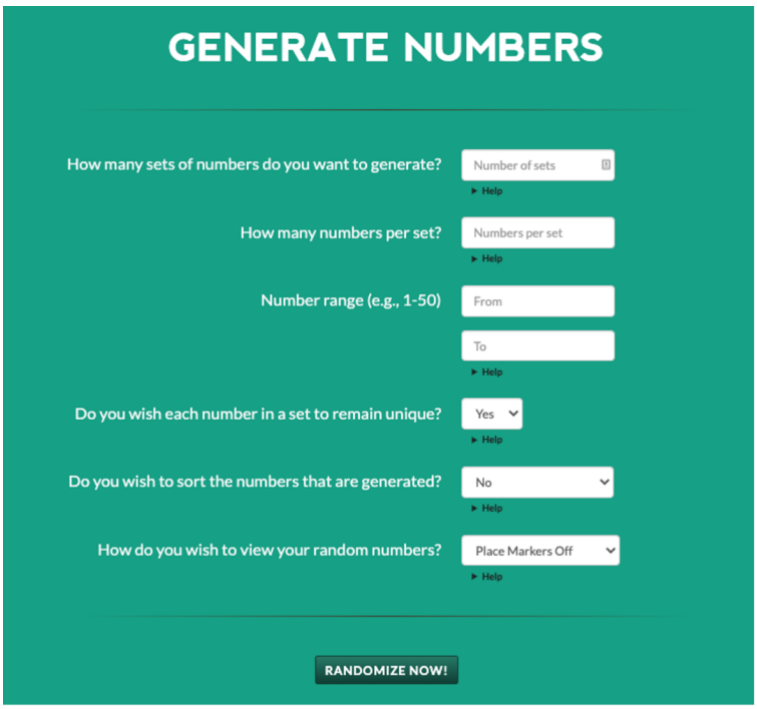

Figure 2.16: Screen capture from the Randomizer website with options for generating random number sets.

Table 2.2: Example of randomisation (where 1 and 2 are the treatment groups and the group size is 15).

15 sets of 2 unique numbers per set						
Range: from 1 to 2						
Set 1	Set 2	Set 3	Set 4	Set 5	Set 6	…
1	2	1	1	2	1	…
2	1	2	2	1	2	…

Blinding:

Blinding and coding were performed by a researcher not involved with the study. The researcher carrying out the surgeries and dosing remained blinded. Furthermore, the analyses were blinded, as the researcher doing the analyses was unaware of the animal's allocation, and the blinding was decoded after the completion of the analysis.

2.3.4. Data Analysis and Statistical Tests

Our studies collected various data types (i.e., ordinal and continuous). The primary outcomes were lesion size and behavioural scores. Secondary outcome measures were the scoresheet data that included weights, appearance, behaviour and neuro-scores.

The Shapiro-Wilk test was used to evaluate the data distribution, and the ROUT method (Q=1%) was used to screen for outliers. The appropriate statistical test was selected to assess differences between groups based on the normal distribution or lack thereof.

Kaplan-Meier visualised the time to death (survival) after stroke (ischaemic and haemorrhagic), and statistical differences between groups were assessed using a Gehan-Breslow test. One-way and two-way ANOVA were used to compare multiple variables, and repeated measures ANOVA to evaluate variables over time. All p values are reported, and a $p<0.05$ was accepted as significant. Analyses and graphing were done with Prism 9 (GraphPad Software v9.3.1). Once the data were analysed, researchers were unblinded to the treatment allocation.

2.3.5. Drug Preparation and Dosing

Fingolimod hydrochloride (Novartis) solutions were prepared in saline and titrated to pH 7.0. The concentration was based on the weight of the mice and a maximum target volume of 250 µL for intraperitoneal injection in mice. The average weight of the mice varied depending on the study; thus, the concentration of the solution varied between studies — in the case of the aged mice, a solution of 1 mg/mL concentration was prepared to account for their higher average weight.

The drug solutions were prepared in advance, and their identity was concealed by a researcher not involved in the project. The tubes were delivered to the investigator performing the surgery and administering the treatment at the appropriate time points. Dosage was calculated for every mouse based on their body weight.

2.4. Experimental Studies

Fingolimod was evaluated for stroke treatment in a series of studies. Briefly, the first study used a model of intracerebral haemorrhage to characterise the effect of 0.5 mg/kg of fingolimod in a long-term recovery study.

The second set of studies were performed using the electrocautery ischaemic stroke model. We conducted a dose-response study to establish the best dosage for ischaemic stroke treatment with this model. The dose-response was followed up with the characterisation of the effect of fingolimod on stroke in two comorbid states, age and hyperlipidaemia. Lastly, a study was performed to evaluate the effects of longer treatment durations on stroke lesion size and recovery. This section describes each study design and the timeline of the studies.

2.4.1. Intracerebral Haemorrhage Study

This study aimed to evaluate the effect of fingolimod in a model of haemorrhagic stroke. This study used male and female C57Bl/6J mice (7-8 weeks of age) to evaluate the effect of a 3-day treatment with fingolimod on a 14-day recovery study following ICH.

Male and female mice were operated on the same day to reduce the effect of day-to-day variability. Treatment with saline and 0.5 mg/kg of fingolimod was administered 30 min post-stroke induction, followed by doses at 24 and 48 h. The dose was selected because it was previously reported to be effective in a long-term study (Lu et al., 2014).

Table 2.3: ICH experimental timeline

Time-Point	Task
-10 d	Minimum acclimatisation period;
-3 d	Baseline behaviour (cylinder, foot fault, wire-hanging);
0 d	Surgical induction of a haemorrhagic stroke;
t = 0-30 min	Mice were allowed to recover for 30 min in an incubator prior to being returned to their home cage;
t = 0.5 h	Drug treatment: intraperitoneal injection;
24 h	Drug treatment: intraperitoneal injection;
48 h	Drug treatment: intraperitoneal injection;
6 d	Behaviour: cylinder test;
7 d	Behaviour: foot fault and wire-hanging tests;
13 d	Behaviour: cylinder test;
14 d	Behaviour: foot fault and wire-hanging tests; Euthanasia and tissue collection.

2.4.2. Dose-Response Study in Ischaemic Stroke

The overall aim of this study was to determine the optimal dose of fingolimod for the treatment of ischaemic stroke in mice. Mice received one of 3 treatments; vehicle (saline), 0.5mg/kg fingolimod or 1mg/kg fingolimod administered intraperitoneally. These fingolimod concentrations were selected based on studies showing an effect in ischaemic stroke models (Liu et al., 2012). Treatment was administered 2, 24, and 48 h post-MCAo.

The dose-response study was performed twice. The first was performed using 100% oxygen, and the second instance was performed using 30% oxygen mixed with 70% nitrogen. The second dose-response study excluded the hanging wire test from the primary outcomes and included blood collection for FACS analyses as an additional secondary outcome.

Table 2.4: Dose-response experimental timeline

Time-Point	**Task**
-10	Minimum acclimatisation period;
-3 d	Baseline behaviour (cylinder, foot fault, wire-hanging);
0 d	pMCAO surgery (electrocoagulation);
t = 30 min	Mice were allowed to recover in an incubator for 30 minutes and returned to their home cage;
t = 2 h	Drug treatment: Intraperitoneal injection;
24 h	Drug treatment: Intraperitoneal injection;
48 h	Drug treatment: Intraperitoneal injection;
3 d	Behaviour (cylinder, foot fault, wire-hanging);
7 d	Behaviour (cylinder, foot fault, wire-hanging); Euthanasia and tissue collection.

2.4.3. Effect of Anaesthetic Carrier Gas

This small study aimed to evaluate the effect of oxygen concentrations on ischaemic stroke injury in mice. This study was performed as a follow-up to the first dose-response study results because there might have been a floor effect caused by the protective effects of 100% O_2 that masked the effect of the fingolimod treatment.

This study involved mice operated under isoflurane anaesthetic evaporated with either 100% O_2 or a mixture with 30% O_2 and 70% N_2**.** Male C57Bl/6 mice from Envigo were used at 14 weeks of age; n=6/per group.

Table 2.5: Carrier gas experimental timeline

Time-Point	**Task**
- 10 d	Minimum acclimatisation period;
- 3 d	Baseline behaviour (cylinder and grid walking);
0 d	pMCAO surgery (Electrocautery);
30 min	Mice were allowed to recover in an incubator for 30 minutes and returned to their home cage;
3 d	Behaviour (cylinder, grid walking);
5 d	Behaviour (cylinder, grid walking); Euthanasia and tissue collection.

2.4.4. Treatment Duration Study

This study aimed to evaluate the effect of extended treatment duration with fingolimod (0.5mg/kg) in a mouse model of ischaemic stroke. This study had three experimental groups that received a daily intraperitoneal injection for ten days. The mice were randomised to one of three treatment regimens; a) 10 d of saline, b) 5 d of fingolimod followed by 5 d of saline or c) 10 d of fingolimod. The behaviour evaluation was performed with the foot fault test, as it had the most sensitivity in our hands for observing deficits and improvements.

Table 2.6: Treatment duration experimental timeline

Time-Point	**Task**
-10 d	Minimum acclimatisation period;
- 3 d	Baseline behaviour (foot fault);
0 d	pMCAO surgery (electrocoagulation);
30 min	Mice were allowed to recover in an incubator for 30 minutes and returned to their home cage;
2 h	Drug treatment: intraperitoneal injection;
24 h	Drug treatment: intraperitoneal injection;
48 h	Drug treatment: intraperitoneal injection;
3 d	Drug treatment: intraperitoneal injection; Behaviour (foot fault);
4 d	Drug treatment: intraperitoneal injection;
5 d	Drug treatment: intraperitoneal injection; Behaviour (foot fault);
6 d	Drug treatment: intraperitoneal injection;
7 d	Drug treatment: intraperitoneal injection;
8 d	Drug treatment: intraperitoneal injection;
9 d	Drug treatment: intraperitoneal injection;
10 d	Behaviour (foot fault); Euthanasia and tissue collection.

2.4.5. Effect of Comorbidities

2.4.5.1. Age Study

This study aimed to evaluate the long-term effect of fingolimod treatment on ischaemic stroke in aged C57Bl6/J mice. Mice were kept in the animal facility for 13 weeks to reach the target age of 71-73 weeks at the time of surgery. Vehicle (saline) or fingolimod (0.5 mg/kg) were delivered intraperitoneal 2, 24 and 48 h after stroke induction. Behavioural assessment was completed at baseline, 3 and 7 d post-op (cylinder and foot fault).

Table 2.7: Aged study experimental timeline

Time-Point	**Task**
-10 d	Minimum acclimatisation period;
- 3 d	Baseline behaviour (cylinder, grid walking);
0 d	pMCAO surgery (electrocoagulation model);
30 min	Mice were allowed to recover in an incubator for 30 minutes and returned to their home cage;
t = 2 h	Drug treatment: intraperitoneal injection;
24 h	Drug treatment: intraperitoneal injection;
48 h	Drug treatment: intraperitoneal injection;
3 d	Behaviour (cylinder and grid walking);
7 d	Behaviour (cylinder and grid walking); Euthanasia and tissue collection.

2.4.5.2. Hyperlipidaemia Study

This study aimed to evaluate the effect of the optimal dose of fingolimod in a model of ischaemic stroke induced in ApoE-/- mice fed a high-fat diet for 12 weeks to induce hyperlipidaemia. Mice received one of 2 treatments, intraperitoneal vehicle (saline) or fingolimod (0.5 mg/kg), at 2, 24, and 48 h post ischaemic stroke.

Table 2.8: Hyperlipidaemia experimental timeline

Time-Point	**Task**
-14 to 13 weeks	Minimum acclimatisation period;
-12 weeks	Initiation of the high-fat diet;
- 3 d	Baseline behaviour (cylinder and grid walking);
0 d	pMCAO surgery (electrocoagulation model);
30 min	Mice were allowed to recover in an incubator for 30 minutes and returned to their home cage;
2 h	Drug treatment: intraperitoneal injection;
24 h	Drug treatment: intraperitoneal injection;
48 h	Drug treatment: intraperitoneal injection;
3 d	Behaviour (cylinder and grid walking);
7 d	Behaviour (cylinder and grid walking); Euthanasia and tissue collection.

Chapter 3

Results

This chapter presents the results of the preclinical studies performed to evaluate fingolimod to inform translation into large clinical trials. The overall chapter is divided into eight sections. The results are presented in the order in which the studies were performed.

First, we established a model of intracerebral haemorrhage and evaluated whether three administrations of fingolimod (0.5 mg/kg, at 30 min, 24, and 48 h) affected recovery 14 d after stroke. Then we proceeded to establish an ischaemic stroke model. We had planned to establish a thromboembolic model with in situ thrombin injection to block blood flow in the MCA region. However, the technical challenges discussed herein had us shift to another stroke model. We established an electrocautery stroke model, wherein the MCA is cauterised, and blood flow is permanently blocked.

The electrocautery stroke model was used to determine the best fingolimod dosage in a dose-response study. The optimal dose was evaluated in a treatment duration study to determine if an extended treatment duration of 5 or 10 d was better for stroke recovery. Lastly, the effect of an optimal fingolimod dose was evaluated in two studies on stroke models with comorbidities; one on aged mice and another on hyperlipidaemic mice.

3.1. The Effect of Fingolimod on Intracerebral Haemorrhage

The results in this section have been copied from the published manuscript, with minor adaptations, to include unpublished data collected from scoresheets.

Diaz Diaz, A. C., Shearer, J. A., Malone, K., and Waeber, C. (2021). *Acute Treatment With Fingolimod Does Not Confer Long-Term Benefit in a Mouse Model of Intracerebral Haemorrhage.* Front Pharmacol 11, 613103. DOI:10.3389/fphar.2020.613103

Survival and recovery

The intracerebral study used 91 mice distributed across four groups, male and female, with a treatment (0.5 mg/kg fingolimod) and vehicle control. Mice that were found in distress (e.g., due to weight loss >20%, significant mobility problems and poor neuro-score) were humanely euthanised (Fig. 3.1.1). Most deaths occurred within three d of surgery (Fig. 3.1.2a), with none occurring after five days. There was a significant difference in survival between all the groups ($X^2(3)=10.88$, $p=0.012$). Male mice showed no treatment-related difference ($X^2(1)=0.81$, $p=0.37$), yet there was an increase in the survival odds of fingolimod-treated compared to saline-treated female mice ($X^2(1)=4.6$, $p=0.032$). When comparing saline-treated groups, male mice had higher survival odds than females ($X^2(1)=7.23$, $p=0.007$). Fingolimod-treated mice showed no difference in survival ($X^2(1)=0.001$, $p= 0.97$).

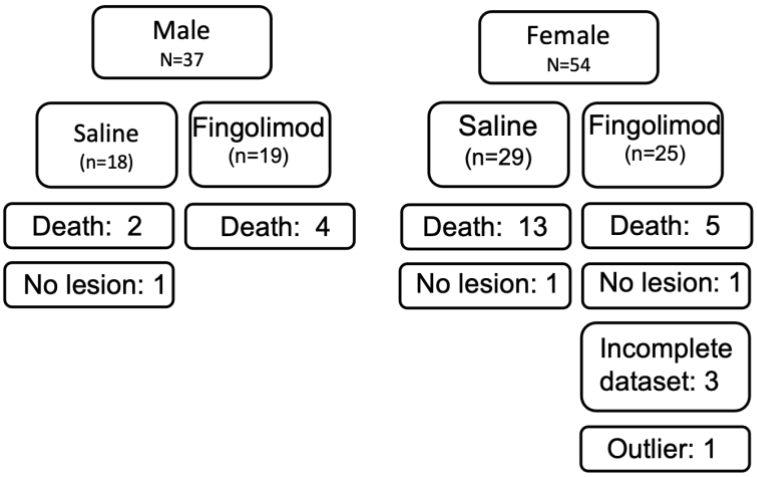

Figure 3.1.1: Mouse allocation in the intracerebral haemorrhage study: Ninety-one mice were used in the ICH study; 24 mice died before the 14-day experimental endpoint, and three were excluded from survival analysis because they did not have a lesion. Four additional mice were excluded from histology and behavioural analysis (three brains were damaged during the processing and one lesion size was an extreme outlier).

As expected, the weight of the mice varied over time (Fig. 3.1.2b), but there was no treatment effect and no interaction between time and treatment variables. Male and female mice from both treatment groups experienced a significant weight loss one day after surgery (Male Saline mean-diff=2.96g, 1.51 to 4.33, p=0.0002; M-Fingolimod mean-diff= 2.66, 1.19 to 4.12, p=0.0003; Female S mean-diff=2.48g, 1.68 to 3.47, p<0.0001; FF mean-diff= 2.21, 1.56 to 3.08, p<0.0001). Both groups of male mice regained the weight lost by day 3 (MS mean-diff=1.64g, -1.12 to 4.42, p>0.99; MF mean-diff=1.68, -0.67 to 3.96, p=0.36), but female mice took a day longer to regain the weight lost after stroke (FS mean diff=1.48, -0.03 to 3.01, p=0.055; FF mean diff=1.39, -0.63 to 3.16, p=0.25).

Fingolimod did not affect lesion volume.

Stained brain sections were scanned and quantified; mice that did not have a quantifiable lesion were excluded from the analysis (Fig. 3.1.1). Some lesion size datasets were not normally distributed; hence all lesion sizes were evaluated with a Mann-Whitney test (Fig. 3.1.3). Lesion size comparisons were carried out independently between treatment groups of the same sex because the study was not powered to detect sex-related differences. Fingolimod treatment had no significant effect on lesion sizes measured either with H&E (male diff= 0.14, -0.58 to 0.81, p=0.56, female diff=0.09, -0.31 to 0.63, p=0.88) or with NeuN staining (male diff=0.74, -0.53 to 1.57, p=0.41, female diff= 0.97, -0.79 to 1.42, p>0.99) (Fig. 3.1.2c-f). Additionally, we measured the size of the ipsilateral

to contralateral ventricle and calculated the ratio to estimate tissue loss (Barratt, Lanman and Carmichael, 2014). This ratio (measured on H&E stained sections) did not show any differences between treatment groups (male diff=-0.12, -0.42 to 0.87, p=0.51, female diff=0.14, -0.67 to 0.33, p=0.64) (Fig. 3.1.3b).

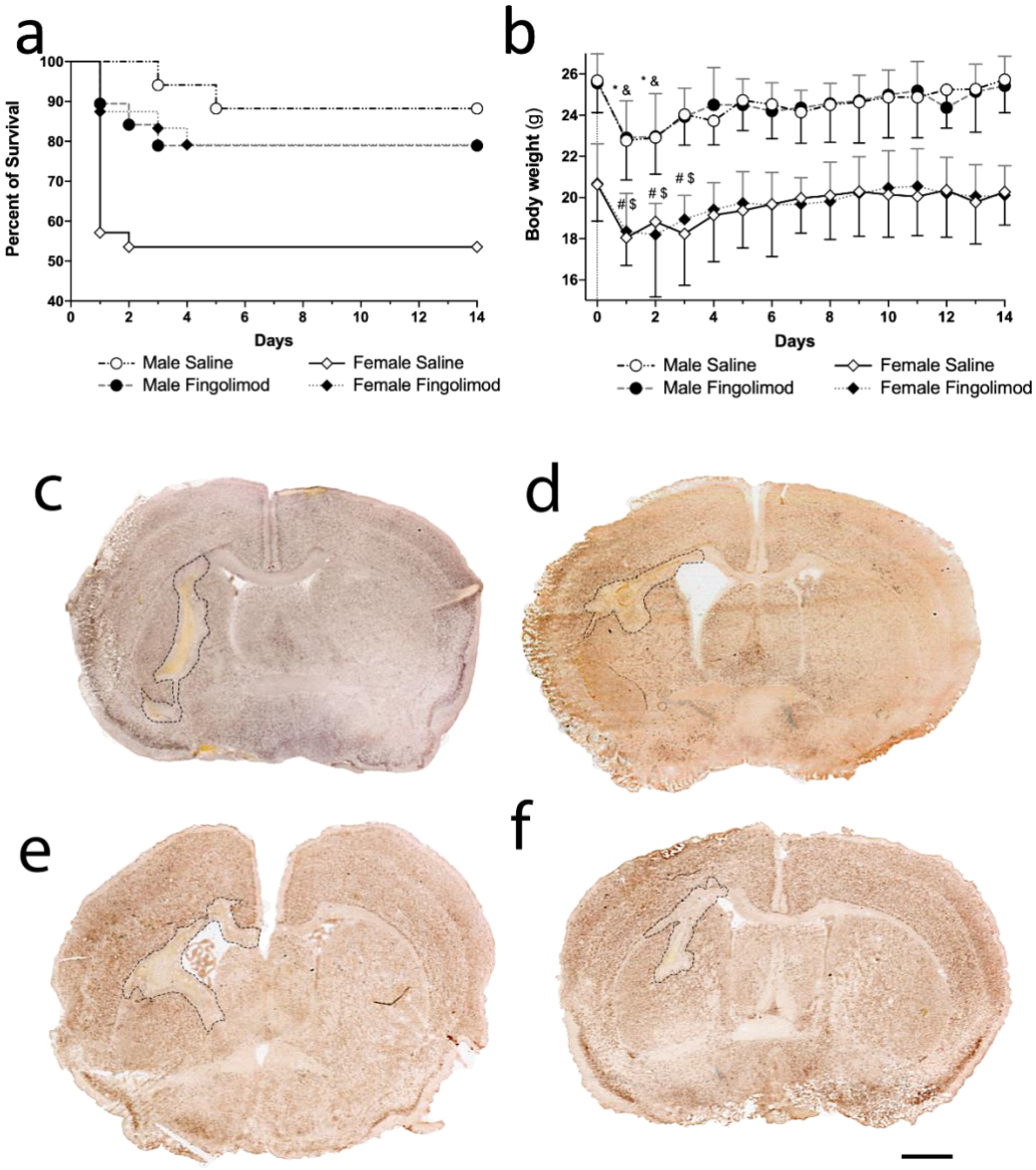

Figure 3.1.2: (a): Kaplan-Meir survival graph; female mice treated with fingolimod had a higher survival proportion than vehicle-treated mice, and vehicle-treated male mice had a higher chance of survival than their female counterparts. (b): Weights over time are shown as mean ± SD (for clarity, the fingolimod error bars are drawn above in grey, and vehicle error bars are drawn below in black). Significant differences: * male saline compared to baseline; & male fingolimod compared to baseline; # female saline compared to baseline; $ female fingolimod compared to baseline. Photomicrographs of NeuN stained section representative ICH-induced damage at 14 d in saline-(c) and fingolimod-treated male mice (d), in saline-(e) and fingolimod-treated female mice (f). All images were taken at the level of the anterior commissure. Lesions in all four tissue sections are outlined in black. Scale bar: 1 mm.

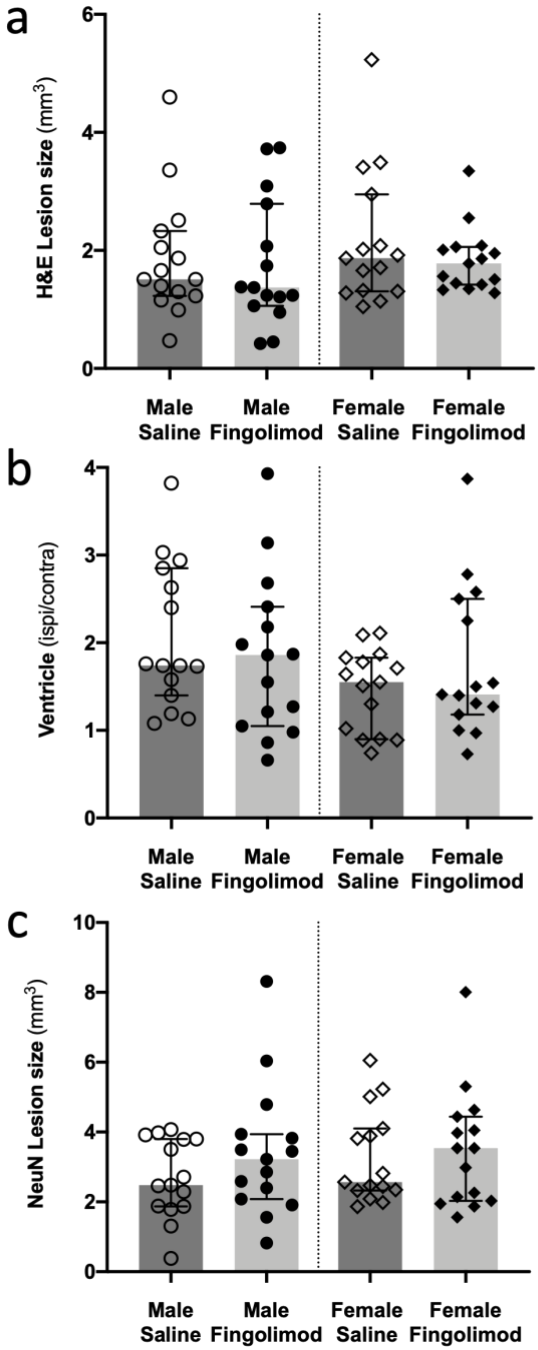

Figure 3.1.3: Histological damage quantification after intracerebral haemorrhage. Lesion size was measured on (a) NeuN-stained sections and (b) Haematoxylin/Eosin-stained sections. The ipsi- to contralateral ventricle size ratio (c) was measured on Haematoxylin/Eosin-stained sections. Values are shown as median ± 95% CI. Fingolimod treatment had no significant effect on any of these measurements.

Behavioural response to treatment

The cylinder test evaluates forelimb usage when exploring the sides of the cylinder; a score closer to 0 represents symmetrical limb usage, while a positive value suggests a predominant usage of the right limb (i.e., ipsilateral and hence not directly affected by the brain lesion). There was no effect of time ($p= 0.92$) or treatment ($p= 0.51$) on the cylinder

test scores of male mice, and there was no significant interaction between these independent variables (p= 0.08). A similar lack of effect was seen in female mice (treatment: p=0.872; time: p=0.062; interaction: p=0.073) (Fig. 3.1.4a). There was a significant improvement in the score of saline-treated females between week 1 and week 2 (mean-diff=0.13, 0.01 to 0.26, p=0.024), but not in the fingolimod group (mean-diff=0.002, -0.11 to 0.12, p>0.99).

The foot fault test score represents the agility of the mice while crossing a grid; lower ratios represent a better score (Fig. 3.1.4b). Neither time (male: p=0.31; female: p=0.57), nor treatment (male: p=0.93; female: p=0.54) affected foot fault test scores, and there was no significant interaction between these variables (male: p=0.23; female: p=0.58).

Lastly, the wire hanging test evaluates dexterity and the ability of the mice to use their forepaws to balance and walk off the wire (Fig. 3.1.4c), with higher scores indicating better recovery. There was no treatment-related difference in male mice at week one or week two (diff=-1, -2 to 1, p=0.43; diff=0, -2 to 0, p=0.63), or in female mice at either time points (diff=1, 0 to 2, p=0.22; diff=0, -1 to 0, p=0.31).

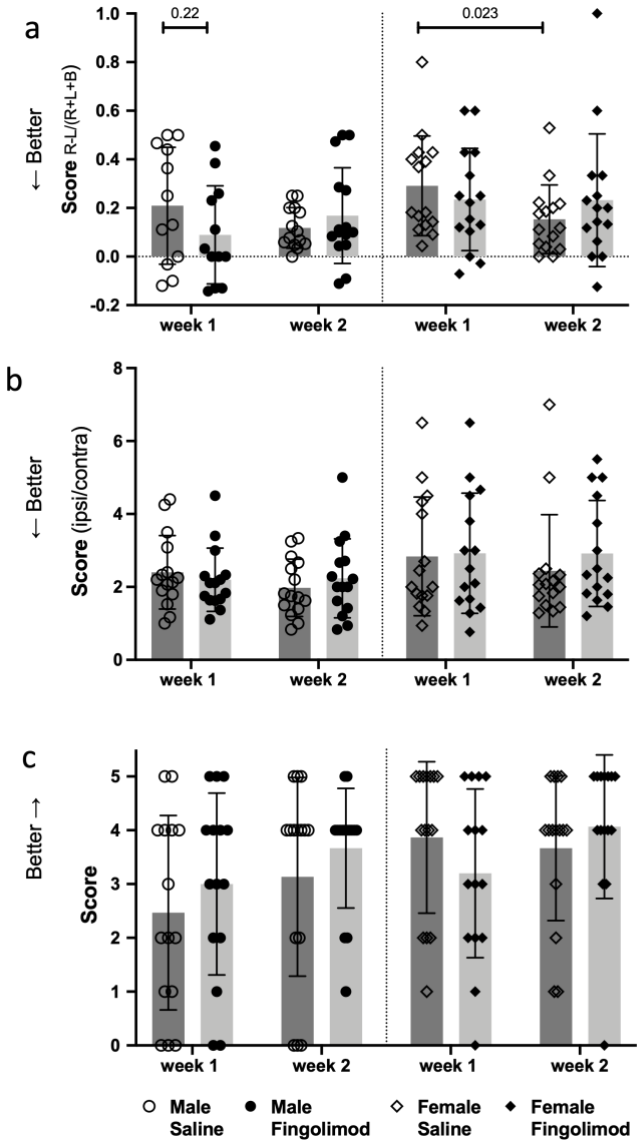

Figure 3.1.4: Behavioural test scores at two time points after intracerebral haemorrhage: (○) saline-treated males, (●) fingolimod-treated males, (◇) saline-treated females, (◆) fingolimod-treated females. Mice were assessed using the cylinder test (a): left panel males, right panel females tested 6 and 13 d after collagenase injection. They were assessed using the foot fault test (b) left panel males, right panel females tested 7 and 14 d after collagenase injection. Finally, their performance in the wire hanging test (c) left panel males, right panel females assessed on the same d as the foot fault test. Scores in (a) and (b) are shown as means ± SD, while wire hanging scores (c) are shown as medians ± 95% confidence intervals. There was no difference between the scores of saline- and fingolimod-treated mice.

Lymphocyte counts

To confirm drug delivery and the expected effect of fingolimod on circulating lymphocytes, we measured CD3+, CD4+ and CD8+ cells in blood samples. An unpaired t-test was performed to evaluate the mean difference in the lymphocyte populations between fingolimod and saline-treated mice (Fig. 3.1.5). There was a significant difference in CD3+ cell counts between fingolimod and saline-treated animals (mean-diff=321,601 cells, CI= 25,499 to 617,703, p=0.039). The CD4+ and CD8+

subpopulations were also significantly different between fingolimod and saline-treated mice with a 76% and 94% reduction respectively (CD4+ mean-diff=73,252 cells, 15,843 to 330,661, p=0.038; CD8+ mean-diff= 116,886 cells, 390.6 to 233,381, p=0.049).

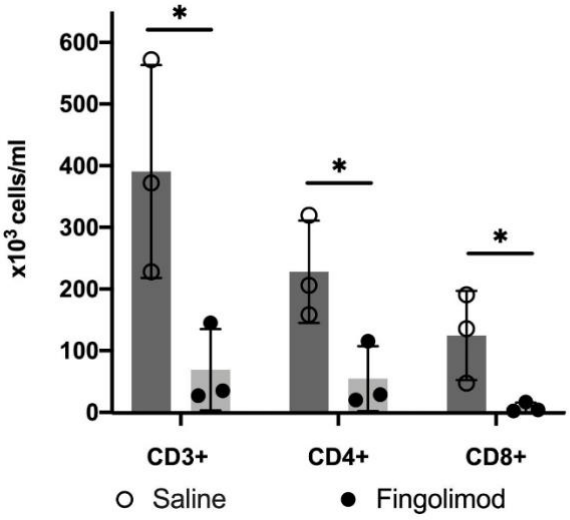

Figure 3.1.5: Fingolimod caused a significant drop in blood lymphocyte counts. (○) male saline-treated, (●) male fingolimod-treated mice (mean ± SD). Mice (n=3 per group) treated for three days with 0.5 mg/kg of fingolimod had a significantly lower CD3-, CD4- and CD8-positive lymphocyte count than mice treated with saline; p values for each comparison are indicated above the respective bars. ns p> 0.05; *p≤ 0.05; **p≤ 0.01; ***p≤ 0.001; ****p≤ 0.0001.

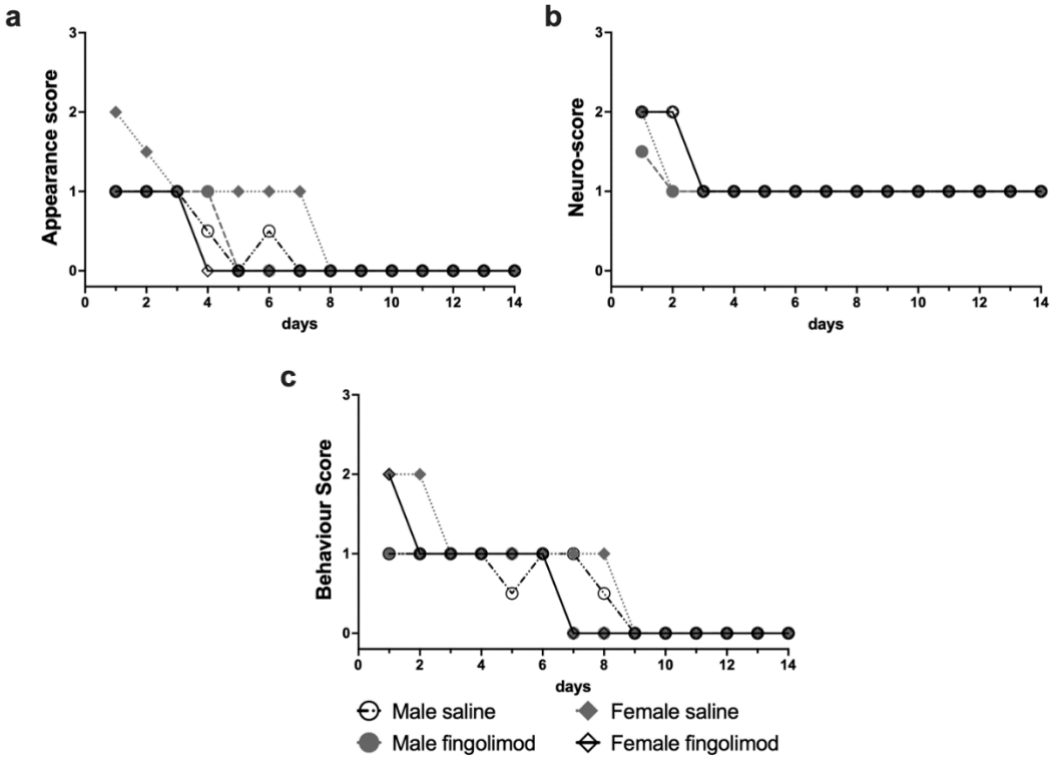

Figure 3.1.6: Scoresheet results for intracerebral haemorrhage study: (○) saline-treated males, (●) fingolimod-treated males, (◇) saline-treated females, (◆) fingolimod-treated females. (a) Appearance scores over time, female mice were slower to recover and regain the ability to groom themselves. (b) Behaviour scores: Male and female saline-treated mice were slower to recover to a normal behaviour score. (c) Neurological score: all mice show a neurological deficit 14 d after ICH. The median score is plotted without error bars for clarity.

Scoresheet data

In addition to the published data, we collected daily scores after surgery to monitor recovery. The appearance, behaviour, and neuro-scores were collected and analysed by repeated-measures ANOVA with Greenhouse-Geisser correction for non-parametric data. No effect was associated with treatment in any of the scores (Fig. 3.1.6). Bonferroni-corrected multiple comparisons revealed no effect between treatment groups. Both male and female mice recovered at a similar rate in appearance and behaviour scores, and no differences were observed in the neuro-scores.

3.2. Effects of Fingolimod on Ischaemic Stroke in a Dose-Response Study

Survival and recovery

In a permanent focal ischaemic stroke model, fifty-three mice were used to study a dose-response with fingolimod. Five mice were humanely euthanised during surgery either due to complications with the electrocautery procedure or variations in the MCA anatomy that would lead to smaller lesions. The surviving 48 mice were randomised into the three treatment groups with a final n = 16 per group. All mice recovered well, and none required euthanasia in the 1-week observation period after surgery.

Effect of fingolimod on lesion size and hemispheric volume

Brain sections were stained with Cresyl violet. The stained sections were used to measure lesion size and hemispheric volumes (Fig. 3.2.1). There was no difference in lesion size between the treatment groups (Fig. 3.2.2a). There was no difference between treatment groups when comparing hemispheric volume changes (Fig. 3.2.2b).

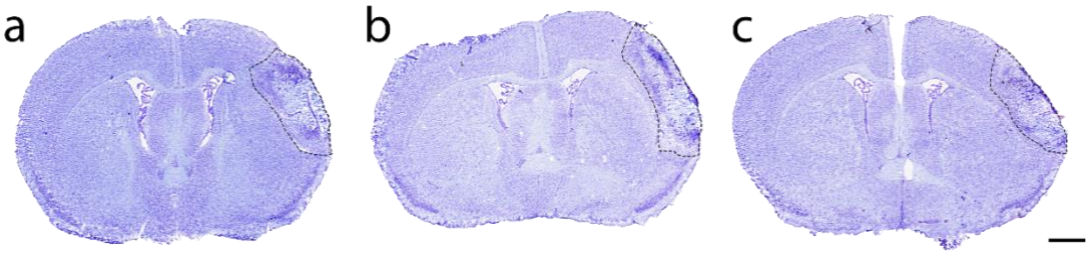

Figure 3.2.1: Representative imaged of Cresyl violet stained brain sections from (a) saline, (b) 0.5 mg/kg fingolimod, and (c) 1.0 mg/kg fingolimod treated mice. Scale bar 1 mm.

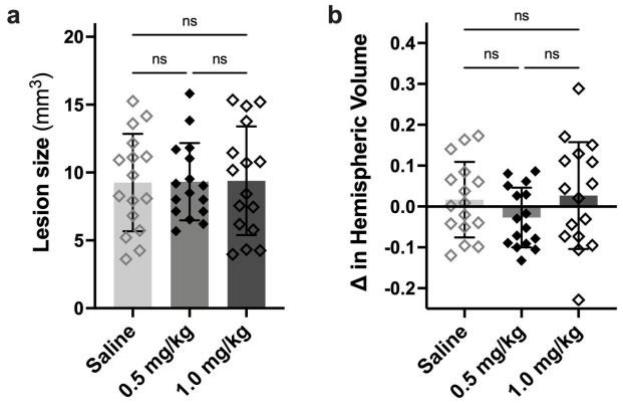

Figure 3.2.2: (a) Lesion size measured in mm^3 from Cresyl violet stained brain sections showed no difference between treatment groups. (b) Difference between the ipsi- and contralateral hemispheric volume showed neither oedema nor atrophy in any of the groups; n=16 for all groups (mean ± SD); ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Effect of fingolimod on behaviour

Cylinder scores were analysed by two-way ANOVA and showed no associated-treatment effect ($p=0.334$). Mice treated with a lower and higher dose of fingolimod both scored significantly worse than baseline on day 3 ($p=0.017$, $p<0.0001$), and they had a significant recovery by day 7 compared to day-3 scores ($p=0.011$, $p<0.0001$). There was no difference over time in the saline-treated mice, and no significant improvement was observed when comparing treatment groups (Fig. 3.2.3a).

The results for the foot fault test revealed that all mice scored significantly worse than baseline on days 3 and 7 (Fig. 3.2.3b). The wire hanging test did not show any differences between the treatment groups over time or within treatment groups (Fig. 3.2.3c) and was not used in subsequent studies.

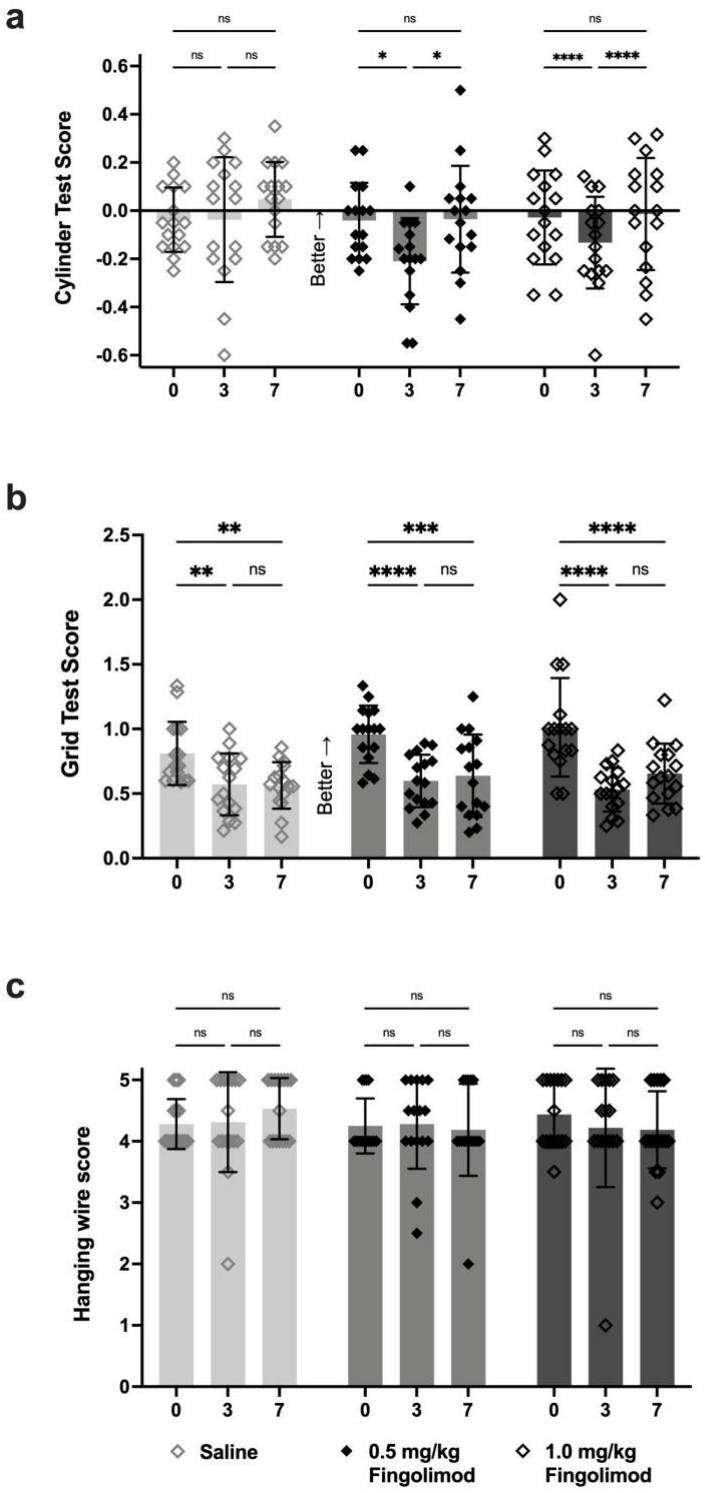

Figure 3.2.3: Dose-response study behaviour test results comparing saline, 0.5 mg/kg fingolimod and 1 mg/kg fingolimod treatment at baseline (i.e., before stroke), 3 and 5 d after stroke with arrows representing the direction for better performance (a) Cylinder test results: saline-treated mice show almost no deficit, and both fingolimod-treated groups developed a behavioural deficit after stroke that resolved by day 7. (b) Grid walking scores: mice in all groups developed a measurable behavioural deficit at three days after stroke. (c) Hanging wire test: there was no difference in the test results between groups over time. Mean ± SD, n=16 for all groups; ns $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

Supplementary data collected

Although the studies were not powered to use these measurements as indicators of the effect of fingolimod, the data collected from the post-procedure scoresheets were used to monitor recovery. Mice, as expected, had a significant drop in body weight after the stroke compared to baseline (saline p=0.003; 0.5 mg/kg p=0.0003; 1.0 mg/kg p<0.0001) (Fig. 3.2.4a). All mice recovered from their post stroke weight loss at different rates. Bonferroni multiple comparisons showed that the low dose group (0.5 mg/kg) regained significant weight by day two (p=0.040), the higher dose (1.0 mg/kg) recovered by day four (p<0.0001) and saline by day six (p<0.0001) after stroke.

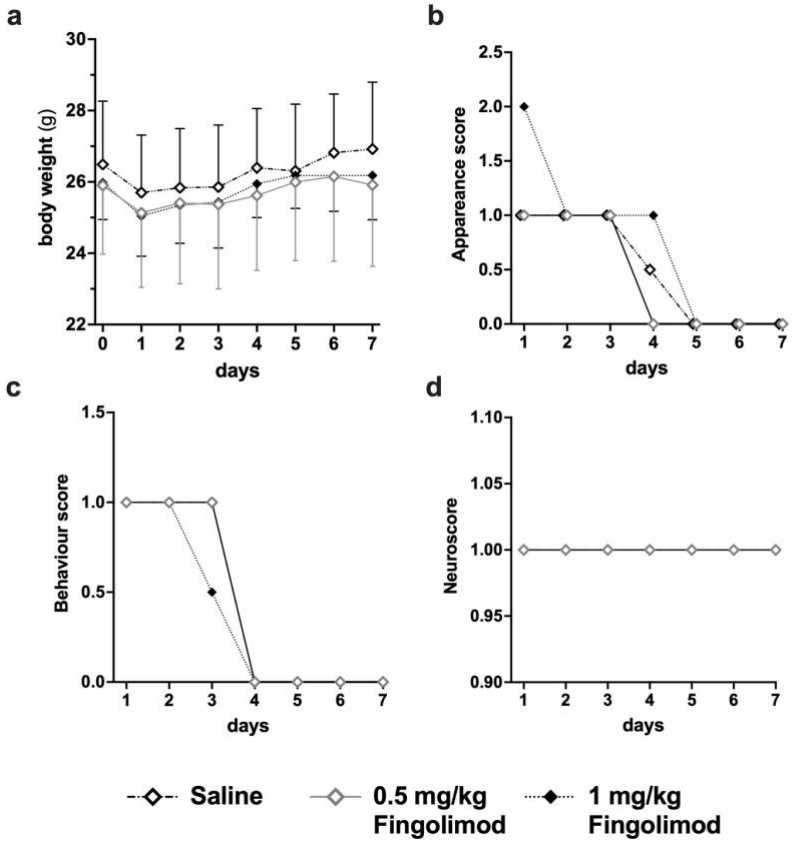

Figure 3.2.4: (a) Body weight over time, measured starting on the day of surgery. Fingolimod-treated mice regained weight faster than saline-treated mice, irrespective of concentration (mean ± SD). One directional error bar for clarity: saline upper, 0.5 mg/kg light grey lower and 1.0 mg/kg black down. (b) Appearance score indicating low dose mice recovered faster than both higher dose and saline groups; (c) Behaviour score showing mice in both fingolimod treatment groups recovered faster than control groups; (d) Neuro-score there was no difference between groups. Median without error bars for clarity in figures b, c and d.

The other secondary data collected were appearance, behaviour, and neurological scores. The data were analysed by two-way ANOVA with Greenhouse-Geisser correction for non-parametric data. Appearance scores (Fig. 3.2.4b) showed that mice treated with 0.5 mg/kg fingolimod recovered a day faster (day 1 vs day 4 p=0.004) than saline (day 1

vs day 5 p=0.0004) and 1.0mg/kg fingolimod treated mice (day 1 vs day 5 p< 0.0001). Behaviour scores revealed that both fingolimod treated groups recovered faster (day 1 vs day 3: 0.5 mg/kg p=0.0002; 1.0 mg/kg p=0.0004) than saline-treated mice which recovered by day 4 (p=0.0004) (Fig. 3.2.4c). Lastly, the neuro-score did not reveal any differences between the treatment groups or any changes in the recovery over time (Fig. 3.2.4d).

3.3. Effects of Anaesthetic Delivery Gas on Stroke Lesion Size

Sixteen C57BL/6 mice were used for this study. Four mice were euthanised before surgical recovery due to complications or unsuitable MCA anatomy, and twelve mice were operated under isoflurane anaesthesia using 100% oxygen or 70% nitrogen: 30% oxygen mixture as carrier gas (n=6 per group).

There was no difference in the lesion sizes between the two study groups (p=0.69) (Fig. 3.3.2). There was no difference in the cylinder or the grid walking test scores between groups. However, the grid test from both groups showed a functional deficit after stroke (Fig. 3.3.3).

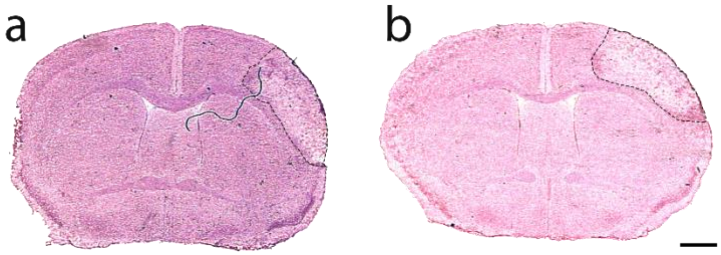

Figure 3.3.1: Representative image of H&E stained brain sections from mice operated under (a) 100% oxygen and (b) 70% nitrogen: 30% oxygen mixture. Scale bar 1 mm.

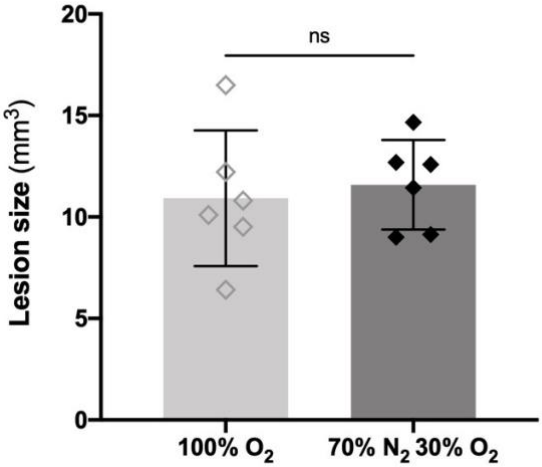

Figure 3.3.2: Lesion size measurements. There was no difference in the lesion sizes in the anaesthetic delivery gas study comparing 100% oxygen or 70% nitrogen: 30% oxygen mixture. O_2: Oxygen; N_2: Nitrogen. Mean ± SD, n=6, ns p> 0.05.

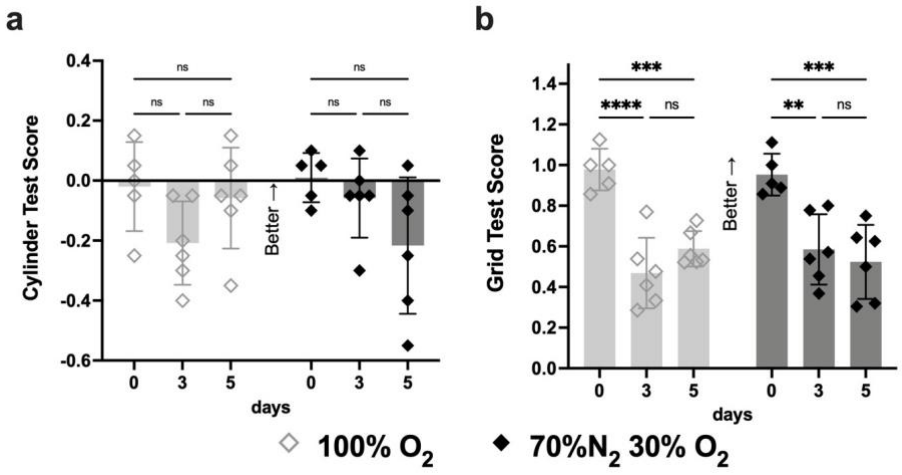

Figure 3.3.3: Behaviour results for the anaesthetic delivery gas baseline, 3 and 5 d after stroke with arrows representing the direction for better performance (a) Cylinder scores: show no significant deficit or recovery in both groups. (b) Grid walking score: mice in both groups performed significantly worse than baseline at 3 and 5 d after stroke. O_2: Oxygen; N_2: Nitrogen. Mean ± SD, n=6, ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

The scoresheet data monitoring recovery after stroke showed that mice in the 100% O_2 group did not have a significant weight drop following the surgery; however, mice in the gas mixture had a significantly lower weight than baseline on day 2 ($p=0.0315$) (Fig. 3.3.4a). The appearance of mice in the 100% O_2 group was generally better than those in the mixture group, and the repeated measures two-way ANOVA with Greenhouse-Geisser correction for non-parametric data revealed that the delivery gas ($p=0.009$) and time ($p<0.0001$) had significant effects on appearance (Fig. 3.3.4b). Similarly, behaviour scores were affected by time ($p<0.0001$), the interaction of time and delivery gas ($p=0.01$), but not delivery gas individually. Bonferroni corrected multiple comparisons revealed that the mice in the mixed gas performed significantly worse than the 100% O_2 on day two ($p=0.05$) (Fig. 3.3.4c). Lastly, the neuro-score analysis showed no difference between groups (Fig. 3.3.4d).

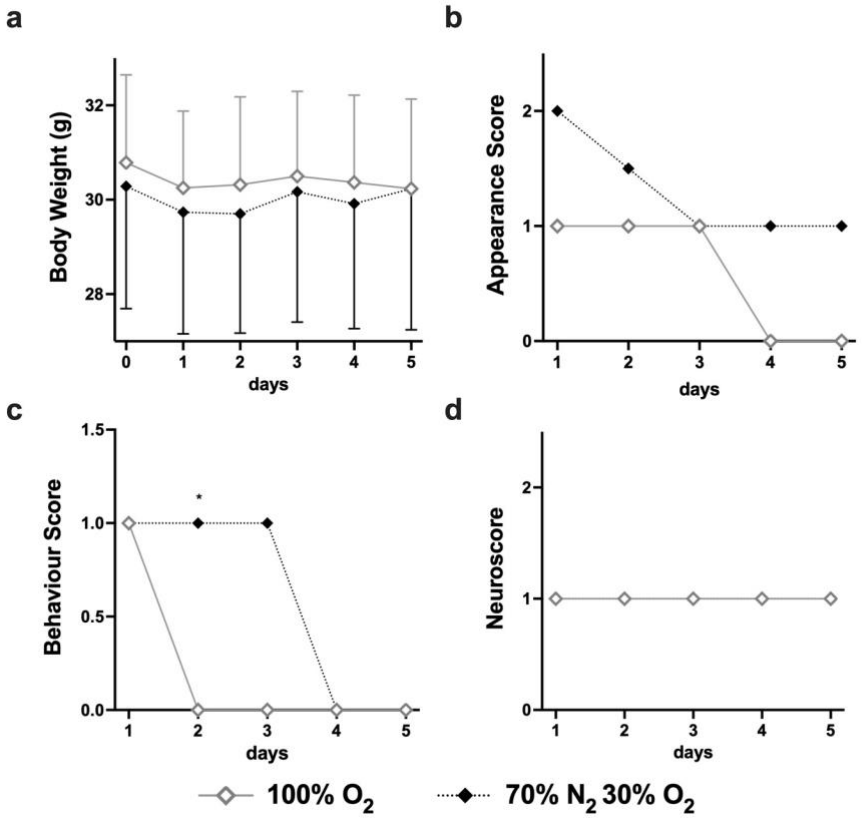

Figure 3.3.4: Scoresheet results n=6 per group (a) Mouse body weight over time (mean ± SD) error bars in one direction for clarity above 100% O_2 group and below for the mixed gas; (b) Appearance scores; (c) Behaviour scores showing mice in the mixture group performing significantly worse than 100% oxygen; (d) Neuro-score: the data for both treatment groups overlaps. O_2: Oxygen; N_2: Nitrogen. Median score without error bars for clarity in figures b, c and d; ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Effects on secondary outcomes measures showing that oxygen is protective suggest that oxygen might have interfered with the fingolimod treatment effect in the dose-response study. Thus, all the subsequent experiments were carried out using a mixture of 70% nitrogen and 30% oxygen for the anaesthetic delivery.

3.4. Effects of Fingolimod on Ischaemic Stroke in a Dose-Response Study Under 30% Oxygen

The data in this section are part of a published manuscript in Frontiers in Pharmacology.

Diaz Diaz, A.C., Malone, K., Shearer, J., Moore, A.C. and Waeber, C., 2022. Preclinical Evaluation of Fingolimod in Rodent Models of Stroke with Age or Atherosclerosis as Comorbidities. Frontiers in Pharmacology. [online] Available at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.920449/>.

The re-evaluation of the dose-response study under 70% oxygen involved the use of 49 mice. During the surgical procedure, three mice were euthanised, another two mice were excluded from analysis as they had no ischaemic lesion, and one mouse was excluded due to damaged histological samples. There were 15 mice in the saline and 14 mice in the 0.5 mg/kg and 1.0 mg/kg fingolimod groups.

The lesion size was measured using H&E and NeuN immunohistochemistry stained sections (Fig. 3.4.1). The data were analysed using one-way ANOVA with Bonferroni corrected multiple comparisons. The lesion size measurements showed that saline-treated mice had significantly smaller lesion sizes compared to 0.5 mg/kg fingolimod-treated mice (H&E $p=0.039$; NeuN $p=0.025$). There was no difference between 1.0 mg/kg fingolimod compared to saline and or 0.5 mg/kg fingolimod for both histological stains (H&E saline vs. 1.0 mg/kg $p=0.119$, 0.5 mg/kg vs. 1.0 mg/kg $p>0.9999$; NeuN saline vs. 1.0 mg/kg $p=0.079$, 0.5 mg/kg vs. 1.0 mg/kg $p>0.9999$) (Fig. 3.4.2a).

The change in hemispheric volume analysis identified that saline-treated mice had a significantly greater degree of atrophy compared to 0.5 mg/kg fingolimod-treated mice (H&E $p=0.042$; NeuN $p=0.040$) (Fig. 3.4.2b), There was no difference between 1.0 mg/kg fingolimod and the saline or 0.5 mg/kg fingolimod groups. (H&E: saline vs. 1.0 mg/kg $p>0.999$; 0.5 mg/kg vs. 1.0 mg/kg $p=0.267$; NeuN: saline vs. 1.0 mg/kg $p=0.463$; 0.5 mg/kg vs. 1.0 mg/kg $p=0.40$.)

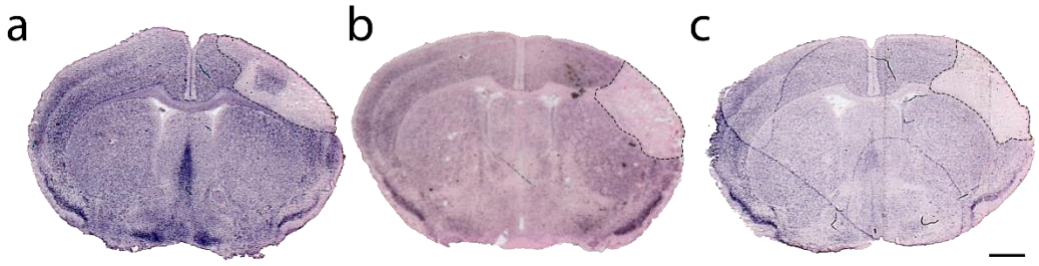

Figure 3.4.1: Representative images of brain section stained by immunohistochemistry with NeuN. (a) saline, (b) 0.5 mg/kg fingolimod (c) 1.0 mg/kg fingolimod treated mice. Scale bar 1 mm.

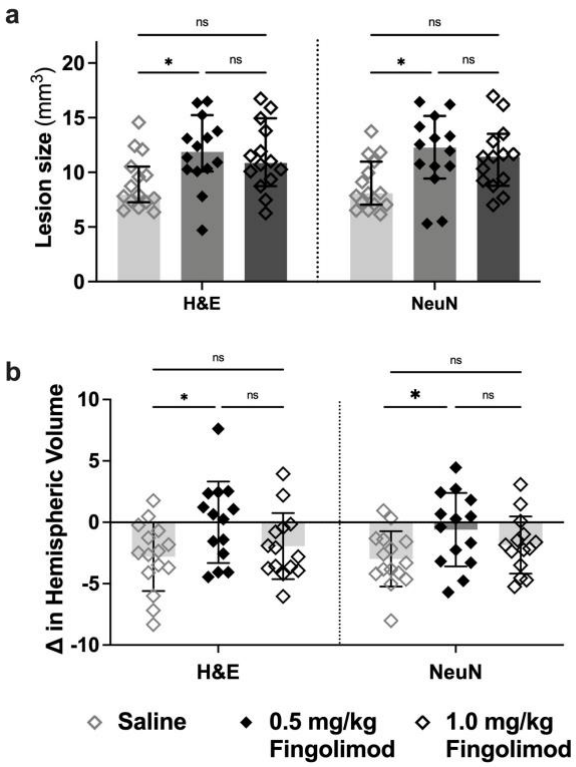

Figure 3.4.2: (a) Lesion size (mm^3): 0.5 mg/kg treated mice had a significantly larger lesion size compared to saline; the 1 mg/kg treated mice showed no difference between the saline or 0.5 mg kg treated mice. (b) Change in hemispheric volumes: saline-treated mice had significantly more atrophy than 0.5 mg/kg treated mice, which was no different than 1 mg/kg treated mice. Data is organised by staining method, H&E to the left and NeuN to the right. Mean ± SD ◇ Saline (n=15), ◆ 0.5 mg/kg fingolimod (n=14), and ◇ 1.0 mg/kg fingolimod (n=14). ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Behavioural data were analysed using two-way ANOVA with Bonferroni corrected multiple comparisons. The cylinder test multiple comparisons showed that mice treated with saline and 0.5 mg/kg fingolimod showed significantly worse performance on days 3 and 7 when compared to baseline (saline p=0.002, p=0.014; 0.5mg/kg p=0.034, p=0.005); and 1.0 mg/kg fingolimod group only scored significantly worse on day 3 (p=0.0004) when compared to baseline (day 7 p=0.16) (Fig. 3.4.3a). The grid walking test multiple comparisons showed a significantly worse performance when comparing baseline to days

3 and 7 for all treatment groups (Fig. 3.4.3b).

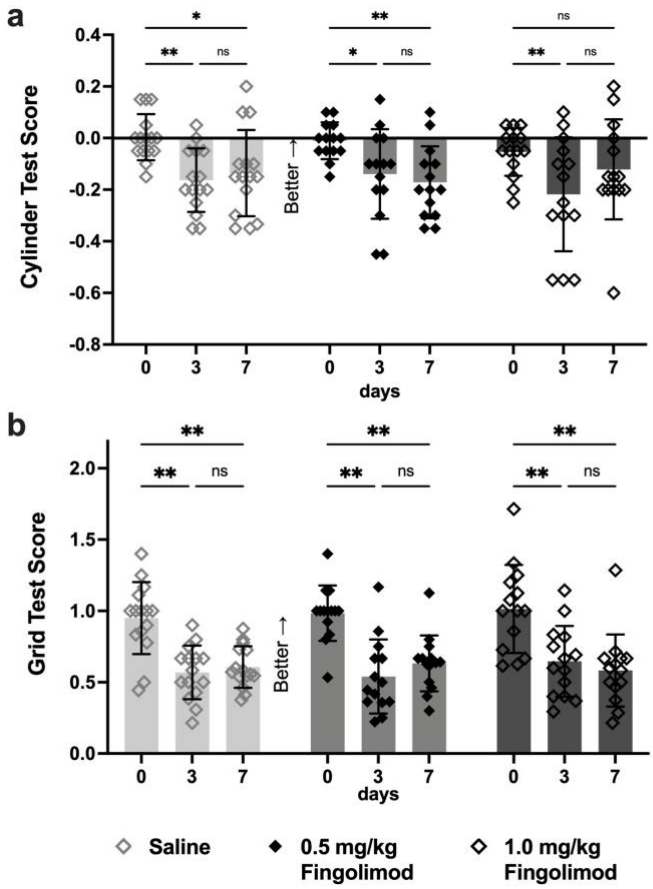

Figure 3.4.3: Behaviour score data from dose-response study under lower oxygen levels. Data shows baseline, 3 and 7 d after stroke with arrows representing the direction of improved (better) performance. (a) Cylinder test scores for saline and 0.5 mg/kg fingolimod treated mice performed worse than baseline three and seven days after stroke, and 1 mg/kg treated mice performed significantly worse three days after stroke but not seven days after stroke. (b) Grid walking scores: mice from all groups performed significantly worse at 3 and 7 days compared to baseline. Mean ± SD. (◇) Saline (n=15); (◆) 0.5 mg/kg fingolimod (n=14); (◇)1.0 mg/kg fingolimod (n=14). ns $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

Supplementary to the primary data, we collected weight, behaviour, appearance and neurological scores as part of the daily monitoring of mice after stroke. Mice from all treatment groups lost significant weight after stroke compared to baseline (Fig. 3.4.4a). There was no difference between treatment groups in the amount of weight loss or recovery. Ultimately, all mice significantly improved their weight on day 7 after stroke compared to their baseline scores (saline p=0.018, 0.5 mg/kg p=0.003, 1.0 mg/kg p=0.033). The appearance scores in figure 3.4.4b revealed no difference between treatment groups and that all mice, irrespective of treatment group, improved their grooming behaviour by day three compared to their day one scores (saline p=0.021, 0.5 mg/kg p=0.019, 1.0 mg/kg p=0.019).

The daily behaviour scores suggested different recovery rates between treatment

groups, yet there was no statistical significance (Fig. 3.4.4c). Fingolimod treated groups significantly improved by day four compared to their day one scores (0.5 mg/kg p=0.019, 1.0 mg/kg p=0.019), and saline-treated mice took longer to significantly improve (p=0.037). Lastly, the neuro-score remained unchanged irrespective of treatment (Fig. 3.4.4d).

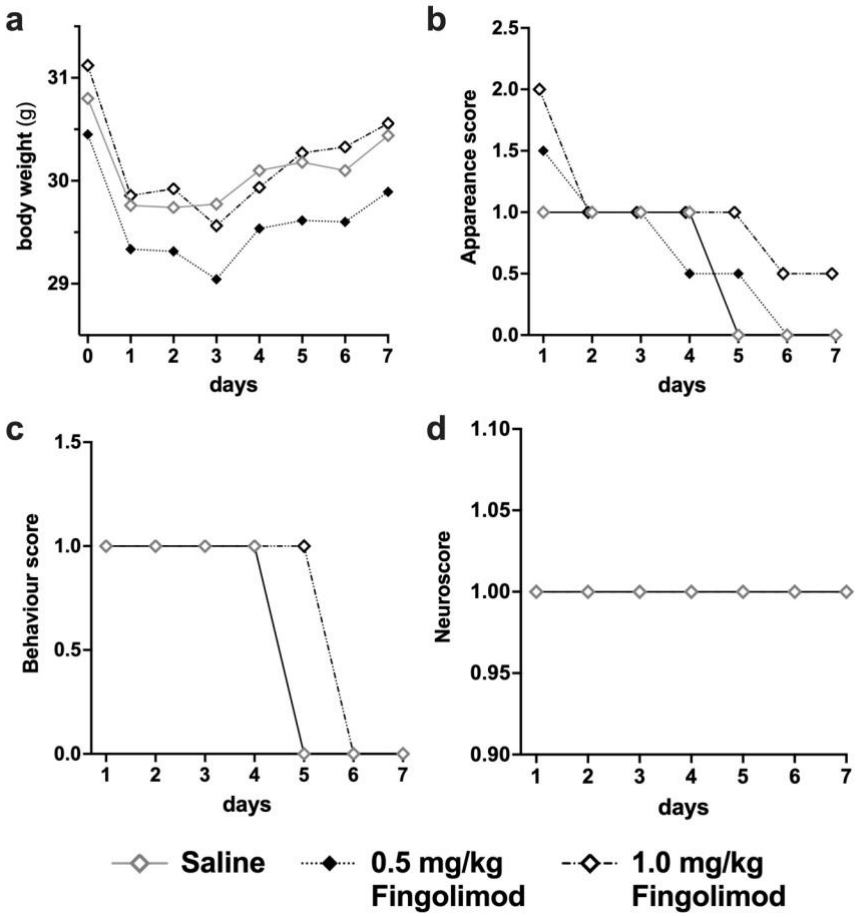

Figure 3.4.4: Scoresheet data median scores; (a) Body weight: all treatment groups experienced a significant weight loss after stroke that did not improve until day 7 (mean ± SD). (b) Appearance improved by day three without influence from treatments. (c) Behaviour scores: there was no difference in recovery between treatment groups. (d) Neuro-scores: remained unchanged from day one through day 7 for all groups. (◇) Saline (n=15), (◆) 0.5 mg/kg fingolimod (n=14), (◇) 1.0 mg/kg fingolimod (n=14). Median score without error bars for clarity. ns p> 0.05; *p≤ 0.05; **p≤ 0.01; ***p≤ 0.001; ****p≤ 0.0001.

Additionally, the number of lymphocytes were measured at the end of the study to confirm that the fingolimod treatment had been effective. Three-day treatment with fingolimod caused a significant drop in lymphocytes in both 0.5 mg/kg and 1.0 mg/kg treatment groups compared to saline-treated mice (Fig. 3.4.5). There was no difference in the lymphocyte counts between the two different fingolimod doses.

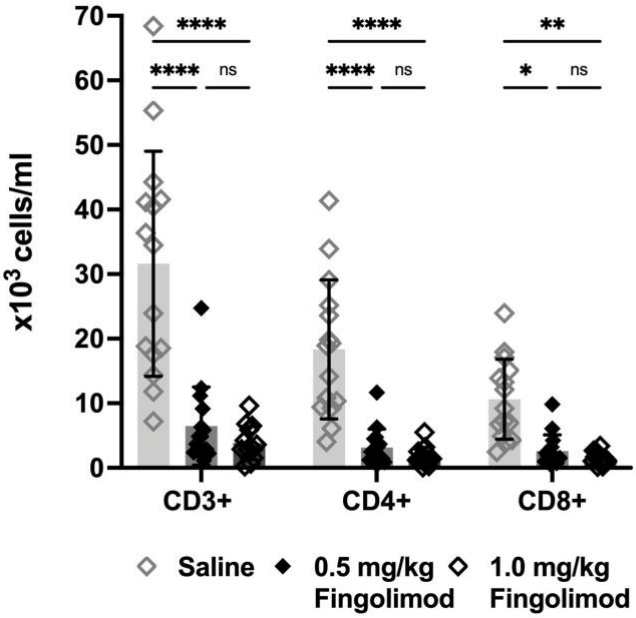

Figure 3.4.5: Fingolimod-treated mice from either dose had significantly lower CD3-, CD4-, and CD8-positive lymphocyte counts compared to saline-treated mice. (◇) Saline (n=15), (◆) 0.5 mg/kg fingolimod (n=15), (◇) 1.0 mg/kg fingolimod (n=14). Mean ± SD ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

3.5. Effect of a Longer Fingolimod Treatment Duration on Ischaemic Stroke

The data in this section are part of a published manuscript in Frontiers in Pharmacology.

Diaz Diaz, A.C., Malone, K., Shearer, J., Moore, A.C. and Waeber, C., 2022. Preclinical Evaluation of Fingolimod in Rodent Models of Stroke with Age or Atherosclerosis as Comorbidities. Frontiers in Pharmacology. [online] Available at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.920449/>.

The evaluation of the effect of different fingolimod treatment durations involved 51 mice. Three mice were euthanised during surgery, and the surviving 48 mice were randomly allocated to either saline, 5-day, or 10-day fingolimod for an n=16 per group.

Lesion size was measured from NeuN stained sections (Fig. 3.5.1). There were no differences between treatment groups in lesion sizes or atrophy (Fig. 3.5.2a & b). The grid test scores (Fig. 3.5.2c) were analysed by two-way ANOVA and showed no treatment effect. Bonferroni corrected within-group comparisons showed that saline-treated mice scored significantly worse on days 2, 5 and 10 after stroke (p=0.002, p=0.001, p=0.0006). On the other hand, 5-day fingolimod-treated mice only scored worse on day two compared to their baseline score (p=0.0004, p=0.056, p=0.439), and 10-day fingolimod-treated mice showed no difference at any time post-stroke compared to baseline (p=0.18, p=0.058, p=0.14).

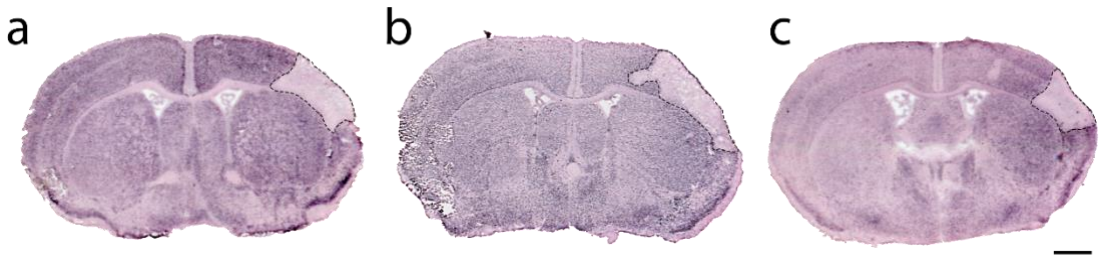

Figure 3.5.1: Representative images of brain sections stained with immunohistochemistry to detect NeuN: (a) saline; (b) 5 d fingolimod and 5 d saline; and (c) 10 d fingolimod. Scale bar 1 mm.

The supplemental scoresheet data for body weight, appearance, behaviour, and neuro-

score (Fig. 3.5.3) were analysed by two-way ANOVA. Bonferroni corrected comparison showed no differences in body weight between the treatment groups. Within-group comparisons revealed that all mice had a significantly lower weight 24 h after surgery compared to their starting weight (saline: p=0.001, 5-days fingolimod: p=0.005, 10-days fingolimod: p=0.008) (Fig. 3.5.3a). By day two, the mice in the 10-day treatment group were no longer significantly lighter, as opposed to the saline and the 5-day fingolimod groups that remained significantly lighter than baseline (saline: p=0.0004, 5-days fingolimod: p=0.011, 10-days fingolimod: p=0.080).

The appearance scores revealed no differences between groups (Fig. 3.5.3b). Within-group comparisons showed that mice in the 5-day fingolimod-treated group had a significant improvement by day five compared to day one scores (p=0.001), the 10-day fingolimod-treated group improved by day six (p=0.012) and saline by day seven (p=0.001). The behaviour scores showed that all but the 10-day fingolimod-treated group scored better than day one at the end of the study without differences between the groups (saline: p=0.024, 5 days fingolimod: p=0.004, 10 days fingolimod: p=0.256). Lastly, the neuro-scores did not change over time within treatment groups.

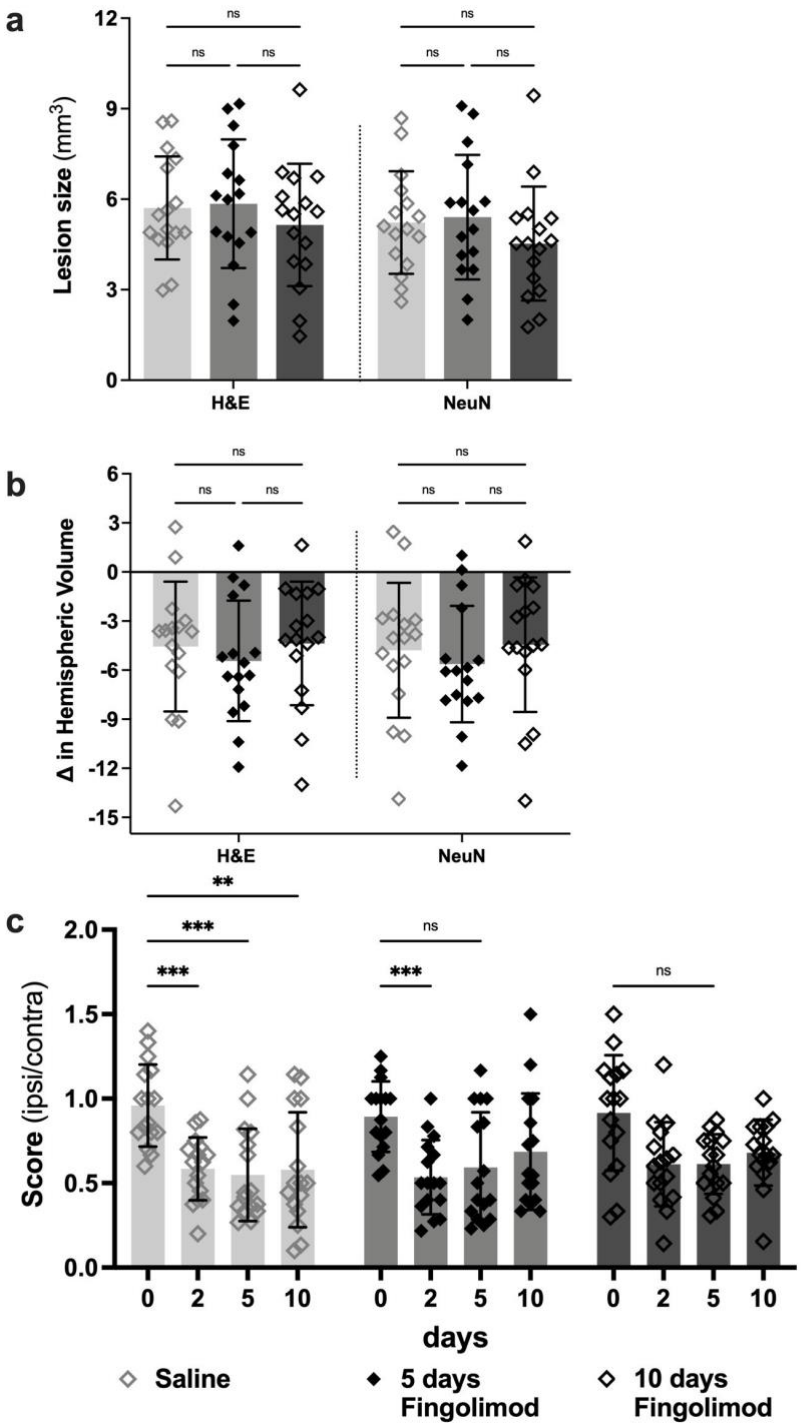

Figure 3.5.2: (a) Lesion size: there was no difference in the lesion size between the treatment groups. (b) Change in hemispheric volumes: all mice had significant atrophy without differences between groups. (c) Grid scores: saline-treated mice performed worse than baseline on days 3, 5 and 10; mice treated with fingolimod for five days perfumed worse than baseline two days after strokes, and mice treated with fingolimod for 10 days did not perform worse than baseline after stroke. (◇)Saline (n=16); (◆)5-days fingolimod (n=16); (◇)10-days fingolimod (n=16). Mean ± SD, ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

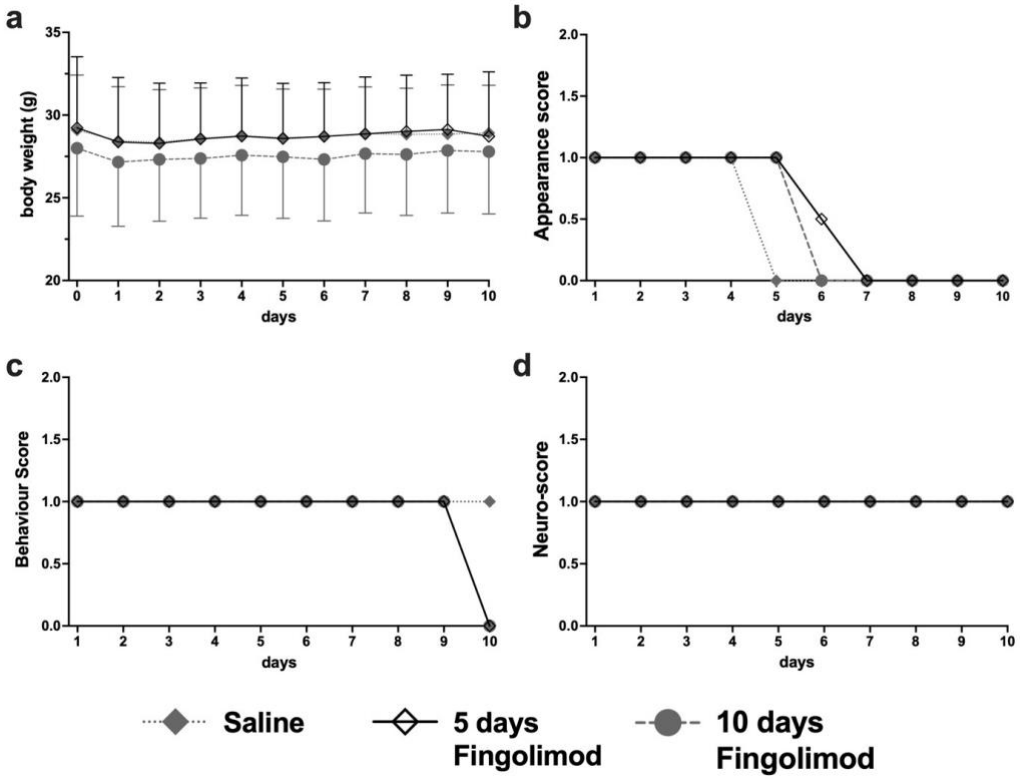

Figure 3.5.3: Scoresheet graphical report. (a) Body weight comparison between treatment groups revealed no difference. Mean with SD draw in one direction for clarity, above: saline grey, and 5-day black; below 10-day grey. (b) Appearance: there was a significant improvement in the score of the 5-day fingolimod group by day five, by day six for the 10-day treatment and by day 7 for the saline group. (c) Behaviour: all mice in the 10-day fingolimod treatment group improved their behaviour scores by day ten after stroke. (d) Neuro-score: there was no difference between treatment groups. (◇)Saline (n=16); (◆) 5-days fingolimod (n=16); (●)10-days fingolimod (n=16). Median scores without error bars for clarity in figures b, c and d.

Additionally, the number of circulating CD3+, CD4+ and CD8+ lymphocyte counts was measured at the end of the study. Fingolimod treated mice from both treatment durations had significantly lower CD3+, CD4+ and CD8+ cells in blood compared to the saline-treated mice (Fig. 3.5.4). Mice receiving 10 days of fingolimod had significantly lower CD3+ cells compared to mice treated for 5 days, and there was no difference in the CD4+ and CD8+ cell counts.

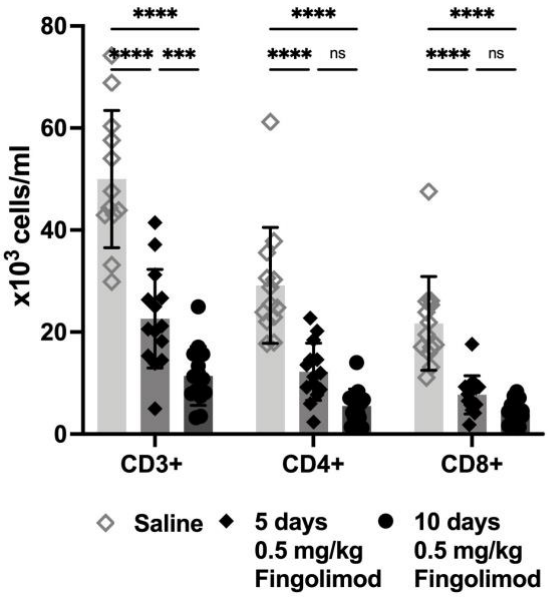

Figure 3.5.4: Fingolimod-treated mice that received 5 days or 10 days of daily doses with 0.5 mg/kg fingolimod had significantly lower CD3+, CD4+ and CD8+ lymphocyte counts compared to saline-treated mice. The 10-day treated mice had a significantly lower count of CD3+ cells compared to the 5-day treated mice, yet there was no difference in the CD4+ and CD8+ cell counts. (◇) Saline (n=13); (◆) 5-days fingolimod (n=14); (●) 10-days fingolimod (n=14). Mean ± SD ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

3.6. Effect of Stroke Comorbidities

The data in this section are part of a published manuscript in Frontiers in Pharmacology.

Diaz Diaz, A.C., Malone, K., Shearer, J., Moore, A.C. and Waeber, C., 2022. Preclinical Evaluation of Fingolimod in Rodent Models of Stroke with Age or Atherosclerosis as Comorbidities. Frontiers in Pharmacology. [online] Available at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.920449/>.

3.6.1. Effect of Fingolimod Treatment on Ischaemia in Aged Mice

Survival and recovery

The evaluation of fingolimod for the treatment of stroke in aged mice involved 33 male C57BL/6NCrl mice at 59 weeks of age. Mice were allowed to acclimatise prior to any procedure, and during the first week in the new facility, one mouse was euthanised. The remaining 32 mice were 72-73 weeks (18 months) of age at the time of the experimental procedures. During surgery, two mice were euthanised due to surgical complications. The recovering mice were randomised into saline or 0.5 mg/kg treatment groups.

Three mice were euthanised at 2, 4 and 5 days during the 7-day observation period because they reached the cut-off score for a humane endpoint; they all belonged to the saline group. A Log-rank test of the survival curve revealed a trend for improved survival odds for mice treated with fingolimod ($p=0.072$) (Fig. 3.6.1.1). Two other mice were excluded during the data analysis from each treatment group due to evidence of excessive bleeding in the brain sections (M=22/03 #07 and #33), leaving 11 mice in the saline group and 14 in the fingolimod group.

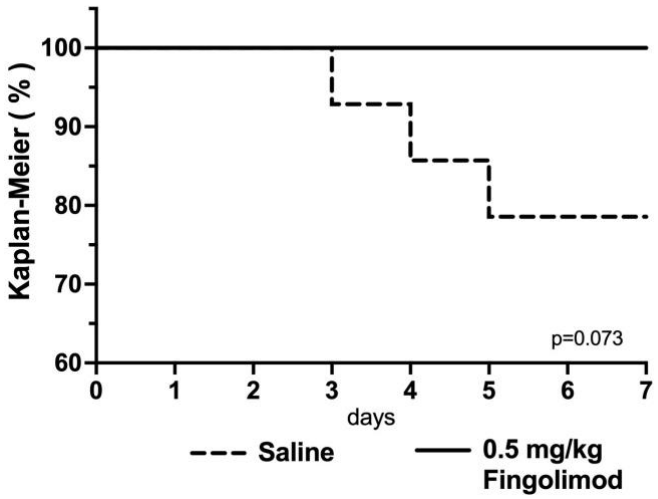

Figure 3.6.1.1: Kaplan Meier survival curve of aged mice undergoing unilateral permanent distal MCA occlusion. Mice were treated for three days with saline or 0.5 mg/kg fingolimod. p=0.0730.
◇ Saline (n=11), ◆ fingolimod (n=14)

Effects on lesion size

Brains collected from saline and fingolimod treated mice sectioned and stained with H&E or NeuN (Fig. 3.6.1.3) showed no difference in the lesion size between treatment groups (Fig. 3.6.1.2a). The hemispheric volume change showed a significant difference between treatment groups, with fingolimod-treated mice having a larger reduction of the ipsilateral hemisphere volume, suggesting increased hemispheric atrophy in this group (Fig. 3.6.1.2b).

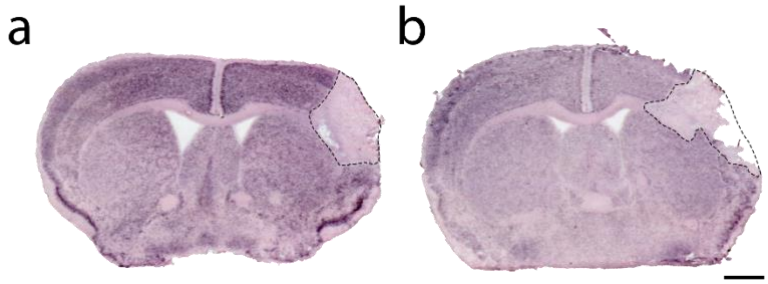

Figure 3.6.1.3: Representative images of aged mice treated with (a) saline and (b) fingolimod stained by immunohistochemistry for NeuN. Scale bar 1 mm.

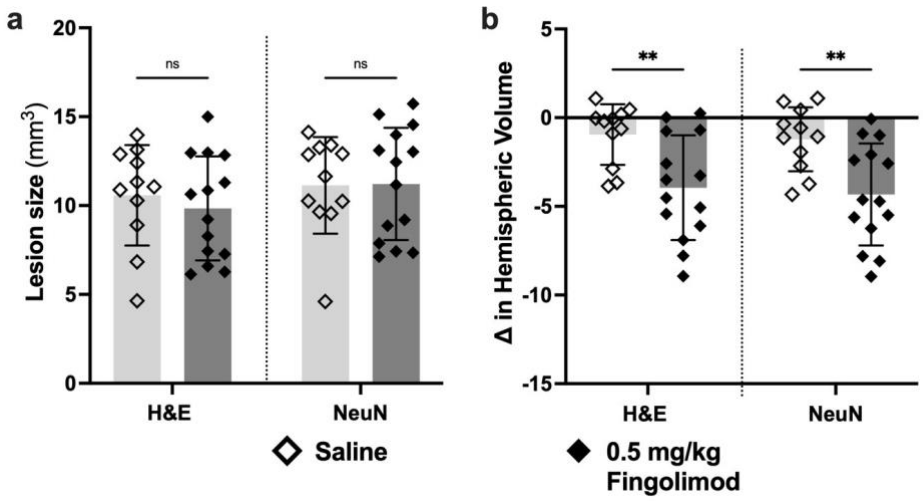

Figure 3.6.1.2: (a) Lesion size showed no difference between aged mice treated with saline or fingolimod. (b) Change in hemisphere volumes: fingolimod treated aged mice experienced significant atrophy compared to saline-treated aged mice (H&E p=0.004, NeuN p=0.003). (◇)Saline (n=11), (◆)fingolimod (n=14). Mean ± SD, ns p> 0.05; *p≤ 0.05; **p≤ 0.01; ***p≤ 0.001; ****p≤ 0.0001.

Behaviour effects

The cylinder test did not expose any differences in the recovery between treatment groups (Fig. 3.6.1.4a). The two-way ANOVA post-hoc multiple comparisons showed no difference in performance between baseline and day 3; saline and fingolimod treatment groups performed significantly worse than their baseline measurement on day 7.

The foot fault analysis with two-way ANOVA with post-hoc multiple comparisons showed that saline-treated mice performed significantly worse on day 3 (p=0.013) and day 7 when compared to baseline (p<0.0001). Fingolimod treated mice also performed significantly worse on days 3 and 7 when compared to baseline (p<0.0001; p=0.003); however, mice in the saline group performed significantly worse than fingolimod treated mice on day 7 (p=0.016) (Fig. 3.6.1.4b).

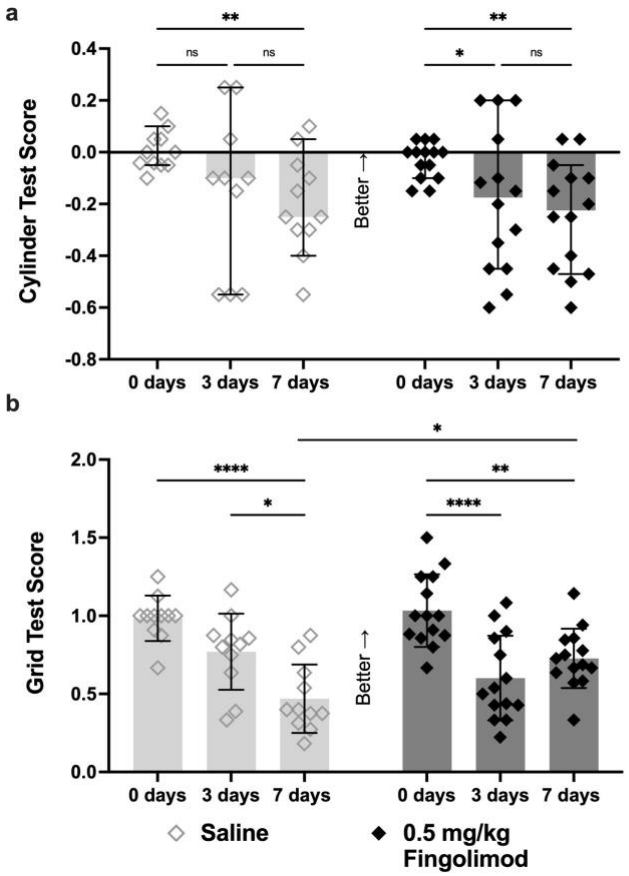

Figure 3.6.1.4: Aged behaviour tests: (a) Cylinder test showed no difference between the treatment groups. Saline-treated aged mice performed worse than baseline on day seven, and fingolimod-treated aged mice performed worse than baseline on days three and seven. (b) Grid test: fingolimod treated aged mice performed better than saline-treated mice on day seven. Saline-treated aged mice performed worse than baseline on day three and significantly worse than day seven. Fingolimod treated aged mice performed worse than baseline on days three and seven. (◇)Saline (n=11), (◆)fingolimod (n=14). Mean ± SD, ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Supplementary results

The supplementary data collected from the scoresheets involved the weight, appearance, behaviour, and neuro-scores of all mice, including mice that did not survive the 7-day observation period (Fig. 3.6.1.5). The weights of the mice were analysed by mixed-effect analysis due to missing values of the mice that were euthanised, with Bonferroni corrected multiple comparisons. Comparison between groups revealed no differences in any of the parameters.

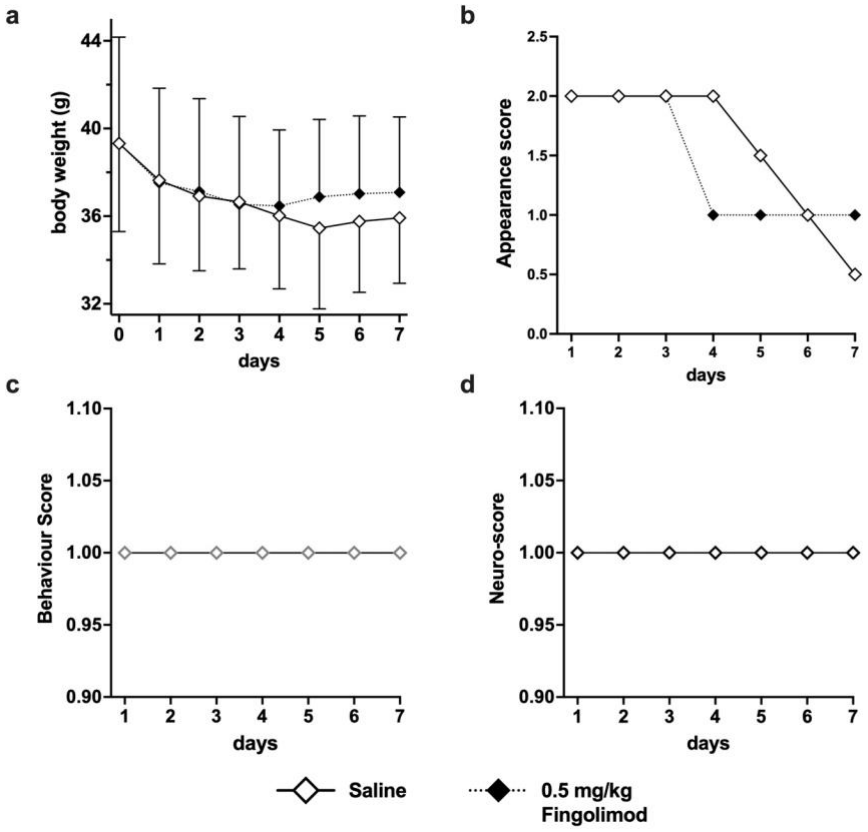

Figure 3.6.1.5: Scoresheet data (a) Body weight over time: there was no difference in weight loss or recovery between treatment groups. Mean ± SD with fingolimod error bar above and saline error bar below for clarity. There was no difference in scores between groups in the (b) Appearance, (c) Behaviour or (d) Neuro-scores. Median without error bars for clarity. (◇) Saline (n=11) and (◆) fingolimod (n=14).

Within-group comparisons revealed that saline-treated mice were significantly lighter than baseline throughout the seven days. The mice showed a continuous decline in their weight following surgery, which is reflected in the significant differences between day one and day six (p=0.037). Fingolimod treated mice had a significant weight loss between baseline and day seven without a protracted weight loss (day one vs day two p=>0.9999) (Fig. 3.6.1.5a). Appearance scores showed a difference in the recovery over time wherein mice in the fingolimod group significantly improved by day 5 (p=0.021), and mice treated with saline had a significant improvement by day six (p=0.013) compared to their day one scores (Fig. 3.6.1.5b). Behaviour and neuro-score analysis revealed no difference in the recovery rates between treatment groups (Fig. 3.6.1.5c & d).

Lastly, we evaluated the number of CD3+, CD4+ and CD8+ lymphocyte counts in the blood of the aged mice at the end of the study (Fig. 3.6.1.4). Mice in the fingolimod-treated groups had significantly lower lymphocyte counts compared to saline-treated mice seven days after stroke.

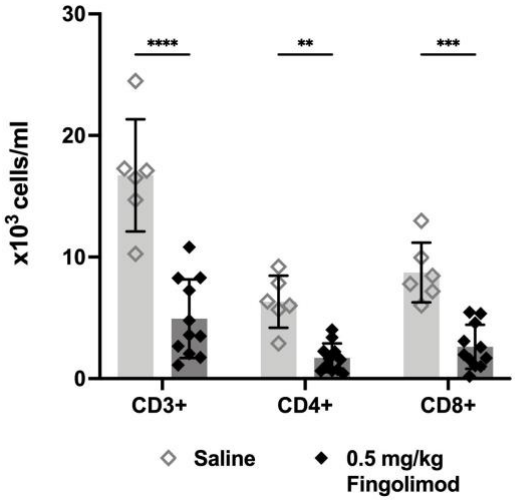

Figure 3.6.1.4: Fingolimod-treated aged mice had significantly lower CD3+, CD4+ and CD8+ lymphocyte counts compared to saline-treated aged mice. Mean ± SD ns p> 0.05; *p≤ 0.05; **p≤ 0.01; ***p≤ 0.001; ****p≤ 0.0001. (◇) Saline (n=6); (◆) 0.5 mg/kg fingolimod (n=11).

3.6.2. Effect of Fingolimod Treatment on Ischaemia in Hyperlipidaemic Mice

Survival and Recovery

The study evaluating the effect of fingolimod on stroke in mice with hyperlipidaemia included forty ApoE$^{-/-}$ mice. Mice were started on the 12-week high-fat diet at eight weeks, and stroke was induced at 20 weeks. Two mice were euthanised due to malocclusion and fighting wounds throughout the high-fat diet feeding period. Mice had to be separated for fighting, causing some to be singly housed, and some mice developed dermatitis that cleared after topical Calamine solution. Lastly, seven mice were euthanised at the time of surgery due to damage to the middle cerebral artery and uncontrollable bleeding. The remaining 31 mice were randomised into treatment groups, returned to their home cage and switched to standard feed. All mice recovered well during the 7-day observation period, and none required euthanasia (Saline n=16, 0.5 mg/kg fingolimod n=15).

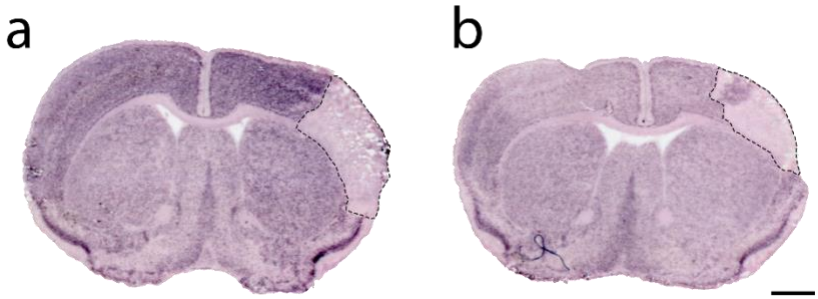

Figure 3.6.2.1: Representative images of ApoE mice after 12 weeks of high-fat diet and seven days after stroke treated with (a) saline or (b) fingolimod. Scale bar 1 mm.

Lesion size effects

The lesion size measurements revealed that fingolimod-treated mice had a significantly smaller lesion size than the saline mice (H&E p=0.019, NeuN p=0.004) (Fig. 3.6.2.2a). There was no difference in the hemispheric volume ratios between treatment groups (Fig. 3.6.2.2b).

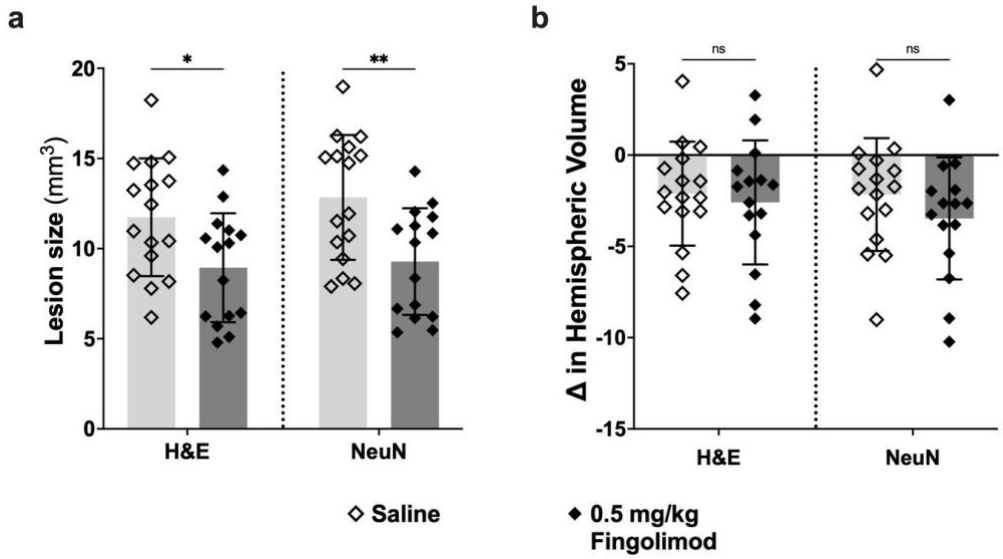

Figure 3.6.2.2: (a) Lesion size: fingolimod-treated mice had a significantly smaller lesion size. (b) Change in hemispheric volumes: there was no difference between groups. Mean ± SD (◇) Saline (n=11); (◆) fingolimod (n=14); ns p> 0.05; *p≤ 0.05; **p≤ 0.01; ***p≤ 0.001; ****p≤ 0.0001.

Behaviour effects

The cylinder test scores in the hyperlipidaemic mice revealed no difference over time between treatment groups or time-points. (Fig. 3.6.2.3a.) The foot fault test was analysed by two-way ANOVA Bonferroni-corrected multiple comparisons. The results showed no differences between the treatment groups; within-group comparisons showed a

significant difference in saline-treated mice at baseline compared to 3 and 7 days, but not between either 3 days or 7 days ($p=0.0004$, $p=0.0004$, $p>0.999$). There was a significant difference in the fingolimod-treated mice between baseline and day 7 ($p=0.048$) without differences in any other comparisons ($p=0.151$, $p>0.999$) (Fig. 3.6.2.3b).

Supplementary data

Mice were weighed once a week over the 12 weeks of high-fat diet feeding period, and all gained weight at a similar rate. The weight gained over the 12 weeks was compared between groups after the animals were randomised, and there were no differences in the weight gains (Fig. 3.6.2.4a). Following the stroke induction mice in both treatment groups lost significantly more weight than their baseline measurement (Fig. 3.6.2.4b). However, there was no difference in weight loss or rate of weight recovery between treatment groups over the 7-day observation period.

Mice treated with fingolimod improved their daily appearance and grooming scores (Fig. 3.6.2.4c) by day 3 when compared to day 1 ($p<0.0001$); saline-treated mice scored significantly better by day 4 ($p=0.0006$). The median daily behavioural and neurological scores did not change over time, and there was no difference between the treatment groups (Fig. 3.6.2.4 d & e).

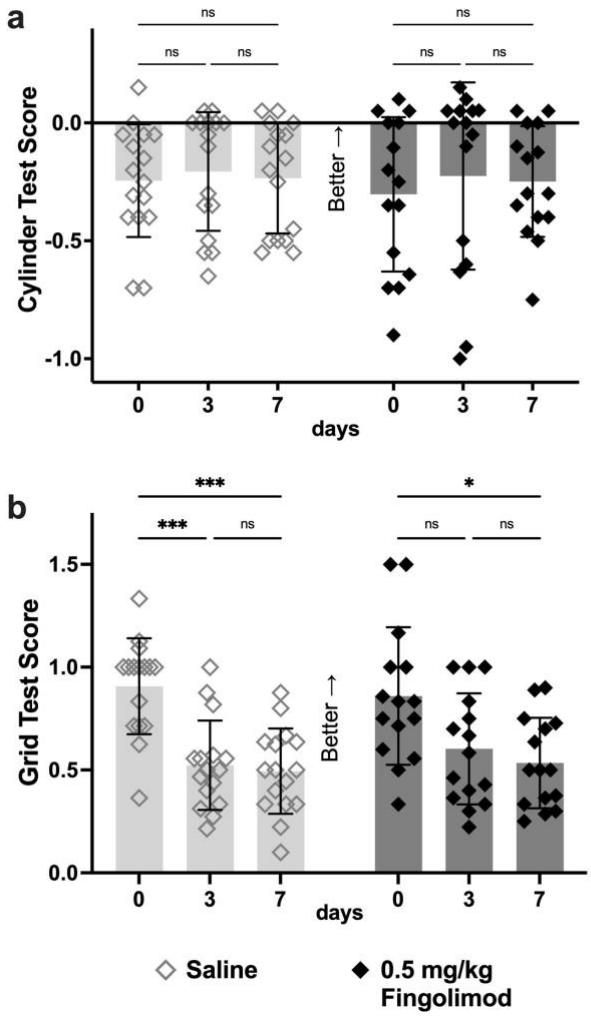

Figure 3.6.2.3: Behaviour for hyperlipidaemic study; (a) Cylinder scores showed no difference between treatment groups. (b) Grid test scores: there was no difference between treatment groups. Saline-treated mice performed significantly worse than their baseline on days three and seven, and fingolimod-treated mice performed worse than their baseline on day seven. (◇)Saline (n=11); (◆)fingolimod (n=14). Mean ± SD, ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

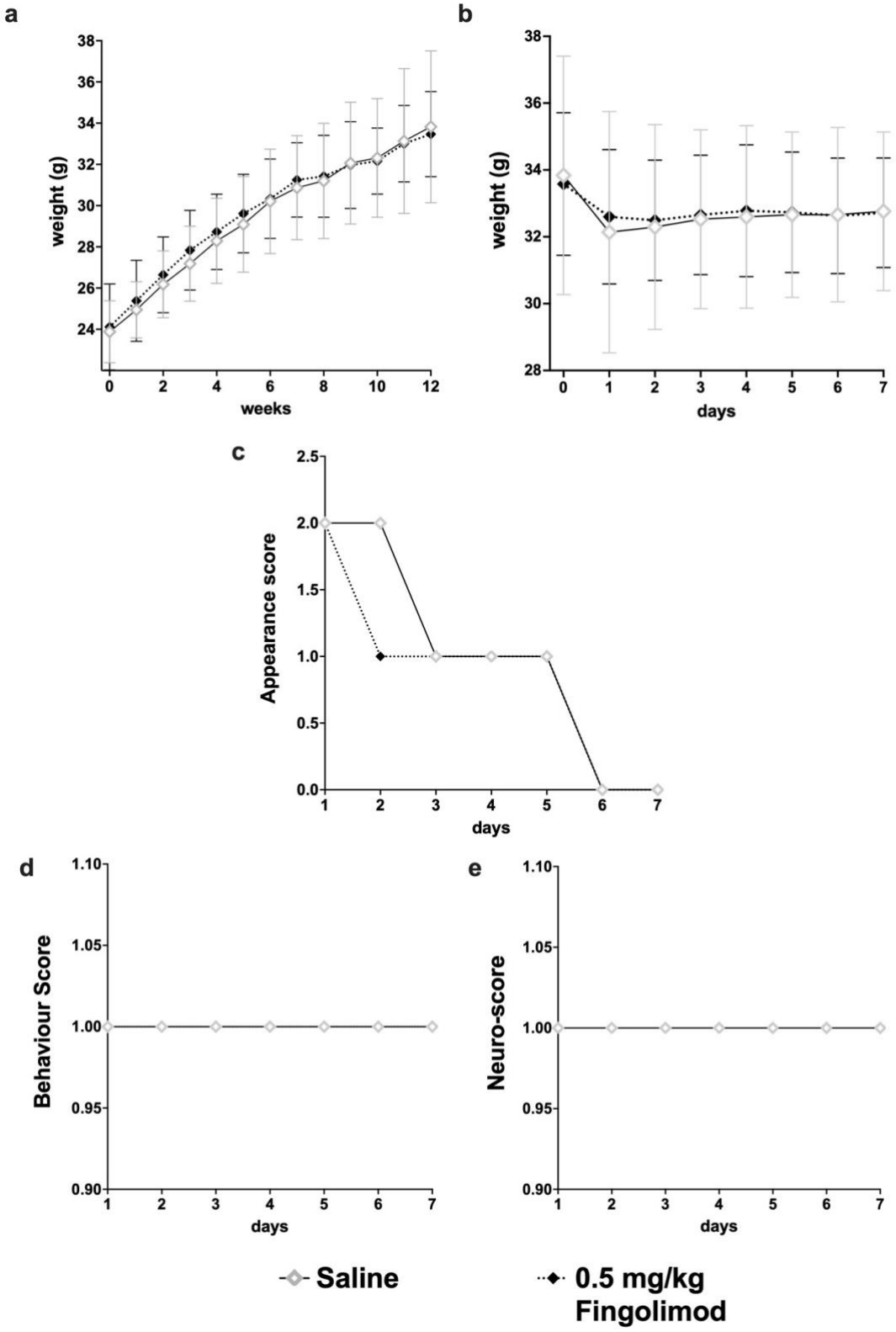

Figure 3.6.2.4: (a) 12-week weight gain graph of mice separated by treatment group during the high-fat diet feeding period; there was no difference between groups. (b) Weight measured during the post-surgery observation period showed that all mice lost significant weight after stroke without differences between treatment groups. There were no differences between groups in the (c) Appearance Scores, (d) Behaviour Scores and (e) Neuro-Score. ◇ Saline (n=11) ◆ fingolimod (n=14)

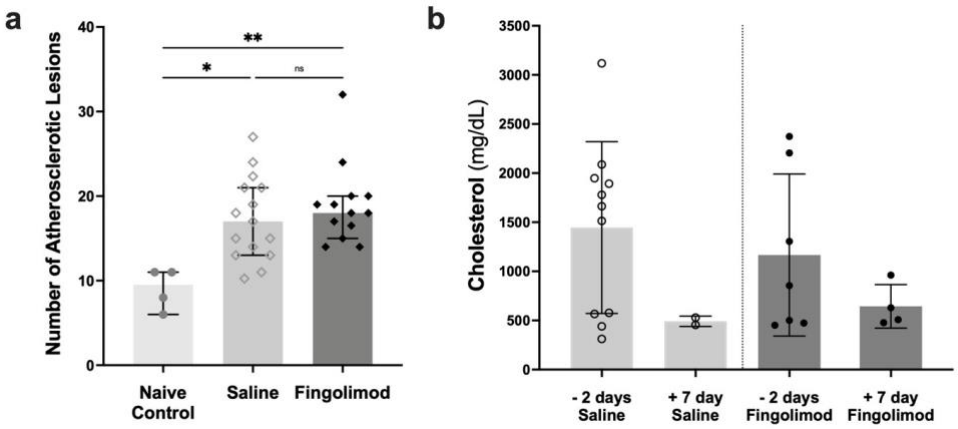

Figure 3.6.2.5: (a) Aortic lesion as expected ApoE mice fed a high-fat diet had significantly more atherosclerotic lesion than C57Bl/6 control mice (mean ± SD) and (b) Cholesterol levels: mice had elevated cholesterol levels that remained up to seven days after being switched from the high-fat diet to a normal diet (median ± 95% CI) (◇) Saline (n=11); (◆)fingolimod (n=14), ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

The atherosclerotic lesions were quantified for all mice that completed the study, plus four age-matched naive controls (C57Bl/6 mice fed a regular diet). Both treatment groups had a significantly higher number of plaques than the naive controls, and there was no difference between fingolimod and saline-treated mice (Fig. 3.6.2.5a).

The cholesterol measurements showed no differences between treatment groups or pre-surgical levels (Fig. 3.6.2.5b). Though not significant, the trend to lower cholesterols at seven days level could be associated with mice being switched back to a standard diet following surgery.

The lymphocyte counts were evaluated seven days after stroke in the fingolimod and saline-treated hyperlipidaemic mice. Fingolimod-treated mice had significantly lower counts of CD3+ cells, and there was no difference between the groups in the CD4+ and CD8+ counts.

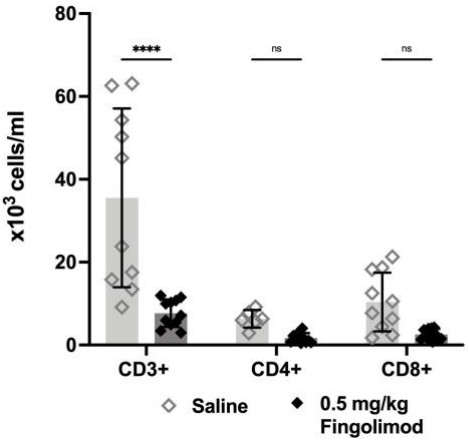

Figure 3.6.2.6: Fingolimod-treated hyperlipidaemic mice had significantly lower CD3+; there was no difference in the CD4+ and CD8+ lymphocyte counts compared to saline-treated aged mice. Mean ± SD ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$ (◇) Saline (n=10); (◆) fingolimod (n=11).

3.7. Pooled Data Analysis of Ischaemic Studies

The data in this chapter are part of a published manuscript in Frontiers in Pharmacology.

Diaz Diaz, A.C., Malone, K., Shearer, J., Moore, A.C. and Waeber, C., 2022. Preclinical Evaluation of Fingolimod in Rodent Models of Stroke with Age or Atherosclerosis as Comorbidities. Frontiers in Pharmacology. [online] Available at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.920449/>.

Focusing on the primary outcomes, we decided to pool the data from all studies and evaluate whether fingolimod would be effective in a heterogenous population more representative of human stroke patients. These pooled data encompass all the studies evaluating fingolimod in an ischaemic stroke model that used 70% N_2 with 30% O_2 as the anaesthetic delivery gas: a dose-response study; the extended treatment duration study, and studies involving aged and hyperlipidaemic mice.

There was no significant difference between lesion size and the change in hemispheric volume in the pooled data (p=0.43, p=88, respectively) (Fig. 3.8.1a). The cylinder data showed no difference between saline and fingolimod at either 3 or 7 days after stroke (p=0.97, p=0.58). However, the grid test revealed no effect on day three and a significant improvement in fingolimod-treated mice on day seven after stroke (p=0.46, p=0.029) (Fig. 3.8.1c).

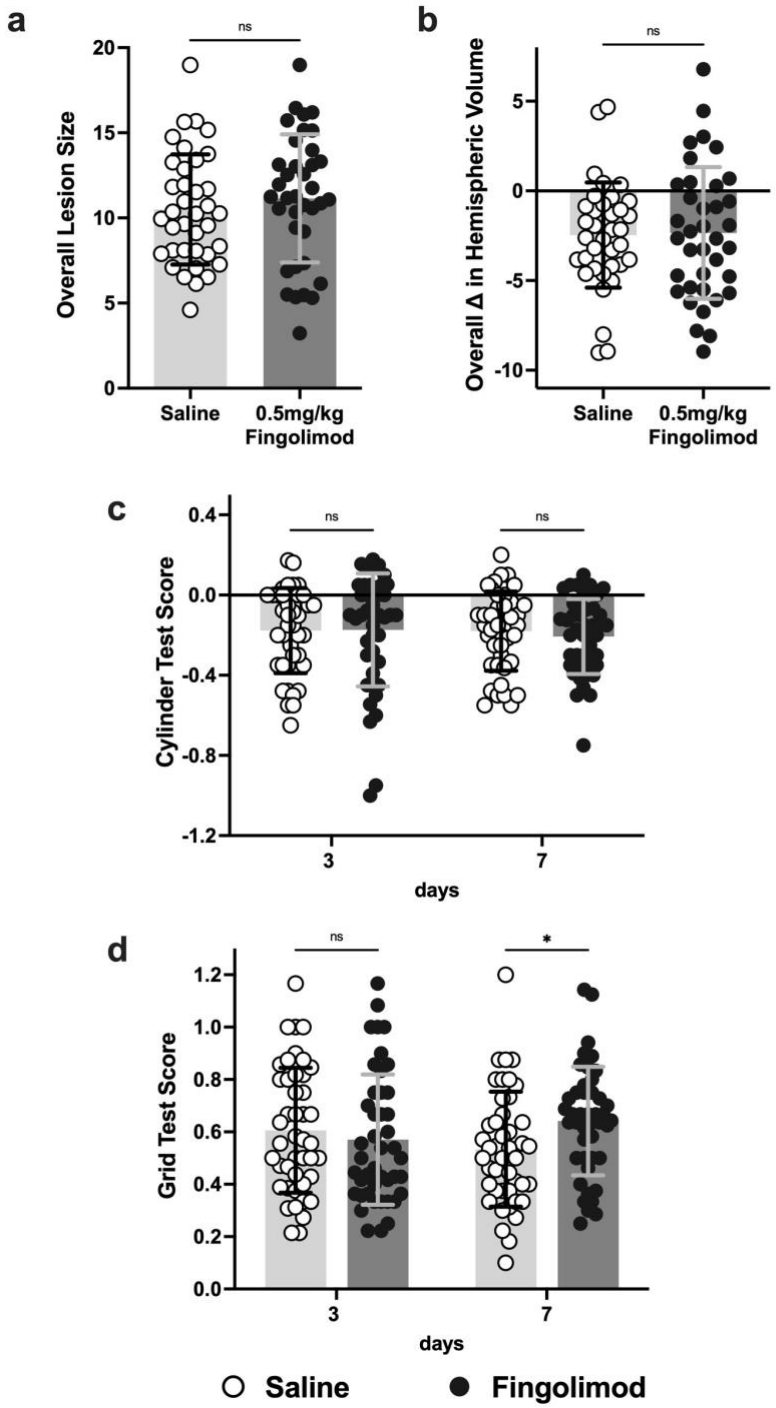

Figure 3.8.1: Summary of experimental outcomes of all young, aged, and ApoE-/- mice grouped by saline-treated and fingolimod-treated mice (a) Lesion size and (b) hemispheric volume changes were not significantly different in the summary data (○) Saline; (●) fingolimod n=37. (c) Cylinder scores on days three and seven did not differ between treatment groups; (d) Grid scores were significantly different seven days but not three days after stroke; (○) saline, (●) fingolimod n=46. ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Chapter 4

Discussion

Fingolimod is an immunomodulating drug that causes internalisation of S1P1 receptors, leading to the sequestration and degradation of immune cells. The reduction in circulating lymphocytes is hypothesised to affect the number of immune cells infiltrating into the stroke lesion, causing a reduction in the infarct size and limiting the behavioural deficits (Liesz et al., 2011; Shichita et al., 2009). The expected reduction in lymphocyte infiltration was confirmed by a reduction in the number of infiltrating CD3+ T cells into the ischaemic core (Malone et al., 2021)

Based on the hypothesis that fingolimod can reduce lesion size and improve behavioural outcomes, we aimed to evaluate the effects of fingolimod in a large preclinical studies involving an intracerebral haemorrhage study and ischaemic stroke. The ischaemic model was used for the dose-response study, treatment duration study, and the evaluation of fingolimod in two comorbid disease models. The studies performed in this large preclinical study discussed herein produced mostly neutral results.

This chapter will discuss the results of the studies performed, followed by an overall discussion of the findings, limitations and future directions.

4.1. Evaluation of Fingolimod in an Intracerebral Haemorrhage Model

The discussion in this section has been copied with minor adaptations from the published manuscript.

Diaz Diaz, A. C., Shearer, J. A., Malone, K., and Waeber, C. (2021). *Acute Treatment With Fingolimod Does Not Confer Long-Term Benefit in a Mouse Model of Intracerebral Haemorrhage.* Front Pharmacol 11, 613103. DOI:10.3389/fphar.2020.613103.

The main finding of these studies is the lack of effect of fingolimod treatment on either histological or behavioural outcome measures after ICH. This contrasts with the significant effect of the same fingolimod administration regimen on circulating lymphocyte counts. To the best of our knowledge, three experimental ICH studies have reported a histological and/or functional improvement after treatment with fingolimod (Rolland et al., 2013, 2011; Lu et al., 2014), with an additional study showing improvement following treatment with the more selective S1P receptor modulator siponimod (Bobinger et al., 2019) (Table 1.7). As mentioned in the introduction, there is a much stronger evidence supporting the beneficial effect of fingolimod in experimental ischaemic stroke, with 17 published articles (including 32 independent experiments) by a dozen different groups (Dang et al., 2020). It should however be noted that a small proportion of these published studies did not corroborate the effect of fingolimod in experimental ischaemic stroke.

The current study was performed with an ICH model (collagenase injection) that was used in previous studies (Rolland et al., 2013, 2011; Lu et al., 2014; Bobinger et al., 2019). The dose and administration regimen were similar to those previously found to be effective (Lu et al., 2014), although the other two studies used a higher dose of 1 mg/kg. It is unlikely that the use of a relatively lower dose accounted for the lack of effect in the present study, since this dose was sufficient to decrease the number of circulating T lymphocytes, but we cannot rule out that fingolimod exerts its effect via other mechanisms, unrelated to the adaptive immune system (Wang, Kawabori and Houkin, 2020).

It is important to mention that the outcome measures used to assess the effects of fingolimod in previous studies differed in part from the ones used here (Table 1.7) and that some of the behavioural measures often only showed a significant treatment effect on the first day after surgery. Previous studies with fingolimod used CD-1 mice. Since the immune response to brain injury varies between different rodent strains, it is possible that strain-related difference may account for the lack of effect of fingolimod in our study (Becker, 2016). Although siponimod was found effective in a collagenase injection model in C57BL/6 mice (Bobinger et al., 2019), while depleting circulating lymphocytes to the same extent, we cannot rule out the effect of fingolimod is more dependent on mouse strain than the effect of siponimod. Furthermore, since C57BL/6 mouse sub-strains show different vulnerability to ischaemic stroke (Zhao, Mulligan and Nowak, 2017), it remains possible that they also show different sensitivities to the effect of S1P receptor modulators in ICH.

One of the drawbacks of the collagenase model of ICH is due to the ability of this enzyme to induce a significant inflammatory reaction (Manaenko et al., 2011; Barratt, Lanman and Carmichael, 2014), which might be exacerbated by the possible presence of endotoxin or other contaminants in the collagenase preparation (Jahr et al., 1999; Nomura et al., 2017). It is possible that inhibition of endotoxin mediated activation of microglial cells may have accounted for some of the effects of fingolimod in collagenase-based ICH models (Lu et al., 2014; Wei et al., 2010), but it is worth pointing out that fingolimod was also found to be effective in a mouse ICH model based on autologous blood injection (Rolland et al., 2013). Another drawback of this study and of previous studies of S1P receptor agonists in ICH is that fingolimod or siponimod were administered for at most 3 days after surgery. It is therefore impossible to rule out the possibility that treating animals for the whole duration of the study (i.e., 14 days or more) would have shown a treatment effect.

Furthermore, it is possible that we were unsuccessful at detecting the true effect of fingolimod (Type II error), or that the conclusions drawn from previous studies were due to Type I errors. As mentioned, our a priori power calculation for this ICH study was based on the effect sizes and variability seen in the ischaemic stroke literature on the effects of fingolimod (Liu et al., 2012). In retrospect, we may have underestimated the variability of the outcome measures assessed in this study, and hence the group sizes required to detect an improved outcome after fingolimod treatment. However, considering the lack of even a trend toward improvement in our data, we considered that it would have been unethical to adjust group sizes and add more mice to our study.

Published studies have in common relatively smaller group sizes and the lack of proper power calculation (Table 1.7). This observation, taken together with the notion that low statistical power is more likely to be associated with false positives, would be compatible with the possibility that previous findings of a beneficial effect of fingolimod in experimental ICH may not have reflected a true effect (Button et al., 2013; Ioannidis, 2005).

Two of the previous studies of fingolimod in experimental ICH did not report mortality (Rolland et al., 2013, 2011), while only one mouse died, in the vehicle-treated group, in the third study (Lu et al., 2014). Female mice were not included in any of these studies, nor in the more recent study using siponimod (Bobinger et al., 2019).

Although we did not set out to study possible sex-related differences in the response to fingolimod, and therefore did not power our study to detect an effect of sex on outcome measures, it is difficult to ignore the observation that female mice, studied concurrently with male mice, had a significantly higher mortality than male mice, and regained weight more slowly than male mice. The vast majority of experimental ICH studies are performed in male mice (Kirkman, Allan and Parry-Jones, 2011; Liddle et al., 2020) and we are not aware of previous studies showing a poorer outcome in female rodents. In fact, in an autologous blood injection model, female mice showed significantly less oedema and their behavioural deficits recovered faster compared to male mice (Nakamura et al., 2004). Similar results were reported using the collagenase injection model (Lei et al., 2012; Xie et al., 2018). Another report showed similar brain injury in male and female mice following autologous blood injection, although only a small number of female mice were studied, and no direct comparison was performed (Jing et al., 2019).

Clinically, most epidemiological studies either show no sex difference in ICH incidence, or higher incidence in men (Gokhale, Caplan and James, 2015). Similarly, no significant sex differences in ICH mortality have been observed in most studies, while some studies show a higher age-adjusted mortality in men (Gokhale, Caplan and James, 2015). There is however evidence that female sex may be associated with poorer neurological outcome either early following ICH or later on (Ganti et al., 2013; Umeano et al., 2013). Interestingly, our study shows a significantly improved survival in female mice treated with fingolimod. Despite not observing a drug effect on histological and behavioural outcomes, the results still may suggest that fingolimod could have a beneficial effect after ICH, specifically when it is associated with a potentially worse outcome.

In conclusion, these studies reinforce the need to perform rigorous and well-powered

experimental ICH studies, following the STAIR and RIGOR guidelines (Liddle et al., 2020), and to report them according to the ARRIVE guidelines. Although the absence of effect of fingolimod in the present study seems to contradict the results of previous preclinical studies, it does not invalidate the hypothesis that S1P receptor modulators may be effective in ischaemic and haemorrhagic stroke. In fact, small proof of concept clinical trials point to an efficacy of these agents for both conditions (Zhu et al., 2015; Fu et al., 2014a; b; Tian et al., 2018). Further animal studies are therefore required to determine whether fingolimod and/or siponimod are effective in specific populations (males vs females, aged animals or animals with typical stroke comorbidities such as hypertension or hyperlipidaemia) and using different treatment regimens (longer durations and/or higher dose). Similarly, at the clinical levels, larger trials will be needed to confirm the effects seen in ICH patients.

4.2. Thromboembolic Stroke Model Setup Studies

To reach the aim of the studies and to evaluate fingolimod in a preclinically relevant stroke model, it was necessary to set up an ischaemic model in the lab. The selected mouse stroke model was first described by Orset et al. 2007, in which thrombin is injected into the middle cerebral artery to form a clot and cause a thromboembolic stroke. This stroke model was selected because it responds to tPA treatment and it can be used in combination with novel treatments to test the effects of various interventions in combination with tPA-induced recanalization. Thus, the aim was to establish this model for all of the experiments characterising the effect of fingolimod on stroke.

The establishment of the model involved the use of thirty-one animals that were not recovered from surgery (non-survival); these were mice used for the initial procedural set-up, and mice that had to be euthanised due to surgical complications. Another twenty-eight mice were recovered from surgery (survival) because they had a successful thrombin injection with a stable clot or a clot that spontaneously dissolved. The analysis evaluating for the presence of a lesion in the survival mice showed that 53% of mice with a clot did not have a detectable infarct 24 h after thrombin injection. Lastly, as part of the setup of the model, a small experiment was performed to evaluate whether thrombin concentrations and isoflurane levels had an influence over clot stability and stroke lesion size; thus, two thrombin and two isoflurane concentrations were compared, resulting in a trend suggesting that 1.5% thrombin along 2.5% isoflurane were better than the other combinations.

During the set-up, we observed that there was no clear indication that a stable clot prior to recovery would cause a stroke lesion, and that some mice with reperfusion and clot dissolution had an injury. Considering that we observed a success rate of 39% for establishing a clot and observing a lesion, and that this surgery was highly technical, with many variables affecting success, there was a concern about the number of animals required to achieve the target n=16 for each experimental group. Thus, we reconsidered using the tMCAO stroke model for all studies; not doing so would have required more than 20 animals to reach the intended number of animals per group.

There are several possible explanations for our inability to set up the thromboembolic procedure as the stroke model. The animal strain and the specific characteristics of the animals are some possible explanations because the model was initially described in Swiss mice and later adapted to C57Bl/6 mice (Campos et al., 2013; Ansar et al., 2014; Orset et al., 2007). The low success rate experienced herein matches the low rates reported in C57Bl/6 mice where 67% of the animals attempted were excluded due to anatomical variations, bleeding complications, or spontaneous reperfusion (Ansar et al., 2014). The study adapting the model to C57Bl/6 mice also reported having difficulties with clot formation and frequent reperfusion. Its authors observed a 37% rate of reperfusion within the first 20 min of occlusion in animals that had a clot (Ansar et al., 2014); this also reflects the lack of clear reporting by others on the number of mice used to successfully model stroke using this surgical procedure.

The different results between mouse strains, and the lack of consistency in clot formation in this model could also be related to vessel size and variability in brain vascularisation between mouse strains (Qian et al., 2018; Barone et al., 1993). The variability of brain vascularisation has been characterised in several mouse strains showing that differences in the number of branches of the MCA and the completeness of the circle of Willis, all variables that affect lesion size (Zhao, Mulligan and Nowak, 2017; Barone et al., 1993). Collateral blood flow contributes to smaller lesion sizes and the lack of a complete circle of Willis produces larger lesions. The variation in the vessel size could also affect the amount of thrombin necessary to form a stable clot that can block the MCA bifurcation. Nevertheless, this model has been used in C57Bl/6 mice background and even though the Swiss mice could lead to more reproducible lesions, changing the mouse strain from C57Bl/6 was not an option because that would affect proposed experiments that involve aged mice and mice with comorbidities that are commonly available on a C57Bl/6 background limiting, and it would also our ability to make historical comparisons.

The limitations for establishing this model were not well communicated in the original publication, and the first description of the model neglects to report failure rates, focusing only on reporting that stable clots cause a sustained blood flow reduction of 40-50% for up to 60 min (Orset et al., 2007). Furthermore, a meta-analysis that evaluated the use of the tMCAO from the nine centres focused on lesion sizes and alteplase (tPA) efficacy without considering the number of animals necessary to achieve those results (Orset et al., 2016). Conversely, a study characterising the tMCAO method in C57Bl/6 mice reported limitations and the low success rate experienced in line with what was

experienced while attempting to establish this stroke model (Ansar et al., 2014).

The low success rate for lesion development could also be related to the concentration of thrombin and isoflurane used during the surgical procedure. The isoflurane concentration and the length of the surgery have been shown to reduce lesion size (Hoffmann et al., 2016; Gaidhani et al., 2017), and isoflurane is protective in different stroke models. Refinement and optimisation of the surgical procedure have been recommended to reduce the duration of the procedure to limit anaesthetic exposure (Munting et al., 2019; Archer et al., 2017). On the other hand, thrombin concentrations have been characterised in multiple labs, with larger concentrations shown to produce larger lesion sizes (0.75 to 3.0 UI) (Campos et al., 2013; Ansar et al., 2014; Orset et al., 2016); however, in our hands, we observed the opposite of expected results with higher isoflurane combined with lower thrombin (1.5U) concentrations producing a larger lesion size with low overall success.

All of these difficulties cast doubt on the suitability of this model for the planned high-throughput studies, especially concerning the additional mice required to account for the excluded animals, which could lead to an unethical increase in animals needed to achieve the intended group sizes. There were also concerns over the limited time available to complete all the studies planned and the additional cost associated with purchasing and generating mice with comorbidities. Most importantly, the lack of consistency and lack of predictable outcomes made us decide against the thromboembolic stroke model for our studies.

This decision had its trade-offs because there is no other method of focal ischaemia that causes a reversible occlusion without keeping the animal under anaesthesia for the duration of the occlusions, as is the case for models that use a clip to occlude or a hook to lift the MCA to interrupt blood flow. Specifically to the tMCAO model, switching to an alternative procedure would no longer allow the use of tPA to recanalise, thus limiting the translatability of these studies into the clinic. The alternative model was the electrocautery occlusion that causes a similarly sized stroke in a similar location to the thromboembolic model. Some of the benefits to the change in model are that permanent occlusion is less surgically complex, increasing the success rate and limiting the number of additional animals to achieving the intended group sizes. One of the unexpected benefits, is that this model was preferred by the original STAIR guidelines because it is representative a larger proportion of the affected population; this is in contrast to the commonly used filament (suture) model that represents more >80% of preclinical studies and with a in usage to the 2.5–11.3% clinical frequency (Stroke Therapy Academic

Industry Roundtable (STAIR), 1999; Kahle and Bix, 2012; McBride and Zhang, 2017)

4.3. Fingolimod Dose-Response Study in the Distal Electrocautery Occlusion Model

A complete and proper preclinical characterisation is needed to validate fingolimod as a therapeutic for stroke in humans. As part of the STAIR guidelines, one of the recommended aspects to evaluate translational potential is determining the optimal dose with a dose-response study (Stroke Therapy Academic Industry Roundtable (STAIR), 1999). Thus, the first study performed was a dose-response study evaluating two doses of fingolimod (0.5 mg/kg, 1.0 mg/kg) and a vehicle control in ischaemic stroke. The characterisation comprised lesion size quantification, change in hemispheric volume seven days after stroke, and behaviour tests (cylinder, foot fault and hanging wire) before surgery and three and seven days after stroke. Additionally, daily monitoring scores were collected after surgery (weight, appearance, behaviour, and neuro-score), which could be used as an indirect indication of treatment effect.

The optimal dose was selected after the completion of three studies: the first dose-response showed neutral results, a follow-up study evaluating the effects of the anaesthetic delivery gas, and lastly, the re-evaluation of the dose-response. The last study also included the evaluation of lymphocyte counts and excluded the hanging wire test.

The first dose-response performed under anaesthesia delivered with 100% O_2 revealed no effect associated with treatment at day 7 post-stroke in the primary outcome measures of lesion size and behaviour. The daily scoring showed that mice had significant weight loss and behavioural deficits attributable to stroke, without a significant difference between treatment groups. These neutral results contradicted the expected benefit previously associated with fingolimod treatment, summarised in two meta-analyses (Dang et al., 2020; Liu et al., 2012).

In the first study showed that mice in all treatment groups had smaller lesions than previously described in studies that used the same electrocautery model (Liesz et al., 2011; Llovera et al., 2014). Because we did not alter the model, we were concerned that

the lack of effect on lesion size could be the product of a ceiling effect that left no salvageable tissue (penumbra) able to respond to treatment. The discrepancy between the observed and expected outcomes made us consider that using pure oxygen as the anaesthetic delivery gas might have led to the small lesion sizes. This is supported by studies evaluating the effect of oxygen on focal ischaemia where they observe marked improvements. Briefly, these studies have modelled stroke with microvascular clips (Shin et al., 2007) or by electrocoagulation (Veltkamp et al., 2006), and they have observed modest reductions in lesion size (10%). Studies evaluating the effect of oxygen on stroke in rats modelled by filament occlusion observe lesion size reductions of 30% or more (Flynn and Auer, 2002; Singhal et al., 2002b; a; Kim, Singhal and Lo, 2005).

As for the behaviour tests, we were concerned that the tests selected might have limited sensitivity to small lesion sizes and that the tests were operating within the lower limits of detection (Balkaya et al., 2012). We could assume that the cylinder and grid walking test had some sensitivity because mice had some changes in their scores, but the hanging wire did not detect any stroke related deficits. Thus, we decided to stop using the hanging wire test for future experiments, which was supported by the fact that previous studies have shown it has low sensitivity for detecting behavioural deficits in C57Bl/6 mice (Balkaya et al., 2012; Royl et al., 2009).

Considering the possibility that having used pure oxygen as an anaesthetic delivery gas was acting as a neuroprotectant, we decided to evaluate the differences in lesion sizes between 100% oxygen and a mixture of 70% nitrogen and 30% oxygen as the delivery gas for isoflurane. The small study involved ten mice (five per group), and it evaluated lesion size five days after stroke. The lower oxygen concentration produced a relatively larger lesion size five days after stroke induction but the difference between groups was not significant. There was a trend suggesting that pure oxygen was acting as a neuroprotective, which matches reports showing that pure oxygen reduces lesion size in stroke (Veltkamp et al., 2006; Xu et al., 2016). The results, of these admittedly underpowered studies, were enough to shift away from using 100% oxygen and to use a mixture with 70% nitrogen and 30% oxygen for all future studies.

The re-evaluation of the fingolimod dose-response study on stroke without the protective effect of oxygen was carried out under a similar conditions to the first dose-response study, including the evaluation of lesion size, hemispheric volume change, the measurement of circulating lymphocytes, cylinder and grid walking test. We found that mice treated with the lower dose of fingolimod (0.5 mg/kg) had a larger lesion size compared to saline-treated mice, and the lower dose of fingolimod was no different

compared to the higher dose. Saline-treated mice developed significant atrophy compared to mice in the 0.5 mg/kg fingolimod group; and the behaviour tests did not show any effect associated with treatment. The results of the second dose-response study do not match expected results, i.e., we observed increased lesion size, reduced atrophy and a lack of effect on behaviour outcomes, yet there was a significant drop in lymphocyte counts the only outcome measure that matched the expected results. Thus, we had to consider the different variables affecting lesion size and behavioural outcomes that could explain the neutral results experienced in the second dose-response in order to select one dose for the subsequent experiments.

Firstly, we considered that the lack of effect could be a product of a lack of sensitivity in the tests used. In the case of the lesion size, there was a concern that the measurement lacked precision because H&E staining can be nonspecific in the border of the lesion and this could be a factor in the lack of significant difference between the fingolimod treatment groups and the high variability observed (Zille et al., 2011; Popp et al., 2009). In an effort to improve the precision of lesion size measurements, we adopted neurone-specific NeuN staining as an additional staining method> The new method reduced variability, but did not affect the lack of significant difference between treatment groups.

The expected lesion size reduction was based on the meta-analyses results that have a preponderance of studies evaluating the effect of fingolimod within the first 72 h after occlusion, and only a few studies that evaluated long-term outcomes. In contrast, we assessed infarct size at seven days, and early lesion size reduction could be unrepresentative of long-term outcomes (Dang et al., 2020). As part of the natural progression of stroke, lesions expand in the first 10 to 72 h due to inflammation and oedema, and after seven days the lesions shrink as oedema and inflammation subside (Iadecola and Anrather, 2011; Popp et al., 2009; Heiss, 2012). Furthermore, one study that used MRI to evaluate lesion size at one and seven days after stroke showed a lesion size reduction in the fingolimod treated group 24 h after stroke but not at seven days (Schuhmann et al., 2016). Thus, it is difficult to know how fingolimod affected the natural progression of the lesion size without using longitudinal imaging (MRI or CT scan) to determine if at an earlier timepoints there were differences in lesion size (Ejaz et al., 2013).

In our study results, the saline-treated mice had the greatest atrophy seven days after stroke, it was significantly larger than the low dose and it was no different than the higher dose. The atrophy observed on day seven in the saline-treated mice was reminiscent of a study evaluating the progression of inflammation and atrophy in rats over multiple time-

points without any treatment; within the first 72 h, there was short-term oedema followed by measurable atrophy at 7-days post-stroke and a continued rate of atrophy until the final endpoint of 24 weeks (Rewell et al., 2017). Furthermore, atrophy reduction has been achieved with Erythropoietin (EPO), wherein rats receiving EPO showed less atrophy than controls without changes in the lesion size after stroke, similarly to the observed results in our dose-response study (Ding et al., 2010). Atrophy has also been observed in humans following stroke, and it is more pronounced within the first 90 days and continues at a lower rate (Brodtmann et al., 2020). Considering that the lower dose suppressed the development of atrophy 7 days after stroke, we selected that dose for further evaluation.

The lack of effect of fingolimod in our study could be associated with the doses selected, yet the dosage was based on previously evaluated doses. Previous studies evaluating fingolimod included doses that ranged from 0.24 to 1.0 mg/kg (Dang et al., 2020). Two studies showed a dose-dependent reduction in lesion size 24 h after stroke (Wacker, Park and Gidday, 2009; Wei, Zhiquiang and Li, 2011); and another study reported that lesion size was indistinguishable between two doses of fingolimod (0.25 mg/kg and 1.0 mg/kg), but more importantly, all of the lesion sizes reported were smaller than controls, contrary to our results wherein fingolimod treated mice had a larger lesion 7 days after stroke (Hasegawa et al., 2010). Yet, as mentioned previously, most studies have evaluated short-term effects (24-72 h endpoints), making it difficult to compare with the lesion size 7 days post-stroke in our study.

The lack of effect on lesion size could be because fingolimod does not reduce lesion size in a 7-day study (Liesz et al., 2011). There are two long-term studies evaluating fingolimod treatment 7 and 31 days after focal ischaemia (Brunkhorst et al., 2013; Liesz et al., 2011). The study using the electrocautery model showed no treatment effect on lesion size or behaviour 7 days after stroke (Liesz et al., 2011), and the study using the photo-thrombotic model showed sustained behaviour improvement, but did not report on lesion sizes 31 days after stroke (Brunkhorst et al., 2013). Both these models cause small stroke lesions that are limited to the brain cortex, thus the effect of fingolimod treatment could be undetectable in long-term studies where atrophy is likely to be a factor.

In agreement with the lack of effect on histological outcome, we did not detect a difference in the behaviour tests, even though the tests selected are commonly used (Schaar, Brenneman and Savitz, 2010). This lack of treatment effect could be associated with the restricted brain region affected and variations in sensitivity of each test depending on stroke type, stroke size, animal strains, animal age and time-points evaluated in the study (Sirén et al., 2006; Manwani et al., 2011; Rosell et al., 2013). The

deficiencies observed are potentially related to the stroke injury that was not significantly influenced by fingolimod treatment. Other studies that evaluated long-term outcomes found that fingolimod effectively reduced behavioural deficits in the cylinder and grid walking test model in thromboembolic and (Campos et al., 2013) and photo-thrombotic strokes (Brunkhorst et al., 2013); however, the study that used the electrocautery MCA occlusion did not detect any differences in the cylinder test (Liesz et al., 2011).

The argument for using a smaller dose is supported by studies that evaluated fingolimod in experimental autoimmune encephalomyelitis (EAE) and the fact that fingolimod's mechanism of action is similar in both diseases (McCann and Lawrence, 2020). Preclinical studies in EAE evaluated a range of effective treatments (0.1 to 0.9 mg/kg), and 0.4 mg/kg was identified as an optimal dose for an extended treatment in rodents (Foster et al., 2007). The optimal dose for MS patients was subsequently determined to be 0.5 mg/day. The same dose (0.5 mg/kg) has also been effective in proof of concept clinical trials for treating ischaemic and haemorrhagic stroke (Fu et al., 2014a; b). This dose has fewer adverse reactions than the other clinically evaluated doses during the MS, as opposed to choosing the higher of these two doses.

Lastly, it is important to consider that many of the previously published studies evaluating fingolimod lack randomisation, blinding and a priori power calculations, leaving open the possibility that a treatment effect is observed when there is none (Ioannidis, 2005). This study aimed to improve on those shortcomings because it was well-powered to detect a difference in lesion size greater than 30%, yet we our results did not show clear difference between the treatment groups. This could mean that our results reflect the true effects of fingolimod and that any effect observed before was the product of some bias that we controlled for with appropriate group sizes, randomisation and blinding of treatment allocation.

4.4. Evaluation of an Extended Treatment Duration of Fingolimod

Stroke has a rapid onset of symptoms and a narrow treatment window for recanalization to improve survival, and aside from preventing recurrent strokes, no specific treatments have been established for the chronic stages and the long-term recovery after ischaemia (Chamorro et al., 2016; Gomez, 2018; Liang, Yang and Jin, 2016). A long-term treatment for stroke patients with fingolimod could be beneficial if it affects later steps of the pathophysiological cascade, especially considering that the cascade has distinct stages that can be modulated effectively. The anti-inflammatory and immunomodulatory properties of fingolimod and its effects on blood-brain barrier integrity are all aspects that have been identified to be critical in the acute stages of recovery from stroke and could be positively modulated in long term treatments (Wang, Kawabori and Houkin, 2020; Li, Xu and Testai, 2016). As well as the canonical effect of reducing of circulating lymphocytes, and reducing their infiltration in to the brain (Liesz et al., 2011; Malone et al., 2021)

We aimed to determine whether an extended treatment duration was superior to a shorter treatment duration, comparing the effects of fingolimod administered for 5 days (short), 10 days (long) and a saline control group. Specially since the observed benefits in MS are due to the long-term treatment with fingolimod, and the improvements are observed after prolonged treatment durations in mice and rats (Webb et al., 2004; Foster et al., 2008). The results show that there was no significant difference in the lesion size, atrophy, and grid test between groups. However, there was an overall trend in the behaviour results suggesting that the longer treatment was better than the shorter treatment.

One of the proposed reasons so many drugs are unsuccessful in stroke clinical trials is that they were not administered long enough to sustain their neuroprotective effects, making the study of long-term treatments for stroke an area of interest (Chamorro et al., 2016). Fingolimod has been proven safe and effective for long-term administration in MS patients and using it in patients with stroke would be considered safe. The approval of this drug was product of the thorough evaluation of its effect on the EAE model in

rodents; thus, an extended treatment might improve outcomes in stroke (Brinkmann et al., 2010; Kappos et al., 2006). Some other drugs that have been evaluated for stroke in long term treatments are PNU120596 (a positive allosteric modulator of α7 nicotinic acetylcholine receptors), and 4-Phenyl-1-4-Phenyl-butyl Piperidine (PPBP; a sigma1 receptor ligand); all of them showing positive outcomes in the acute and sub-chronic stages of stroke recovery (Gaidhani and Uteshev, 2018; Harukuni et al., 1998). The expansion of the treatment duration was effective in these studies further supporting the idea that evaluating fingolimod might also produce benefits even thought that was not our case.

Out of 17 published studies recently summarised in a meta-analysis, four studies evaluated fingolimod treatment lasting longer than 3 days (Dang et al., 2020). The first study evaluated fingolimod treatment in the subacute stage of stroke by treating mice for 5 days with 1 mg/kg b.i.d. fingolimod (Brunkhorst et al., 2013). The study focused on behavioural outcomes up to 31 days after stroke, showing an improved behavioural outcomes for the duration of the study, which was associated with reduced scar formation (astrogliosis). However, the study did not report lesion sizes, perhaps because after 31 days, quantification of stroke lesion is uninformative due to atrophy (Popp et al., 2009). Similarly, Shang et al., 2019 used the photo-thrombotic stroke model and observed a fingolimod associated improvement in neurone counts and modified neurological severity scores after 7 and 14 days of fingolimod treatment (0.3 mg/kg). This study focused on the molecular aspects of stroke recovery and observed that fingolimod promoted angiogenesis and attenuated damage by its ability to polarise microglia to an M2 anti-inflammatory state and did not report lesion size or behaviour outcomes (Shang et al., 2019).

The two other studies did not last as long, they administered daily treatment for four and seven days with corresponding lesion size measurements four and seven days after stroke, and they were both part of larger studies and might not have been the primary outcomes of the overall study (Liesz et al., 2011; Shichita et al., 2009). Liesz et al., 2011 reported administering 1 mg/kg fingolimod daily, and only reported a lack of difference in lesion size while using the electrocautery model without functional outcome measures. Similarly, Shichita et al., 2009 only reported lesion sizes in a study that modelled stroke with filament occlusion and found that one and four 1 mg/kg fingolimod doses reduced lesion size 4 days after stroke compared to control animals.

The effects of fingolimod in experimental stroke may be related to various mechanisms of action; (i) functional antagonism of the S1P1 receptors on lymphocytes

that causes lymphopaenia; and (ii) other S1P receptor interactions and receptor-independent interactions directly on all the brain cells (Naseh et al., 2021). The receptor-dependent and independent effects on parenchymal cells are likely to be important in the hours to days after stroke onset; in contrast the effects on lymphocytes are effectively instantaneous because within 6 h of drug administration the lymphocyte counts have dropped significantly when compared to controls (Liesz et al., 2011). Furthermore, receptor-independent effects could happen any time.

Fingolimod leads to lymphopaenia and reduced brain inflammation by limiting the number of infiltrating cells (Rolland et al., 2013; Malone et al., 2021; Czech et al., 2009). In endothelial cells it is involved in maintaining the integrity of the blood-brain barrier, aiding in recovery by limiting brain oedema (Wang et al., 2020), in glia and neurones, fingolimod has been found to modulate microglia polarisation towards an anti-inflammatory (M2) state, and in neurones it aids survivability by preventing autophagy as observed in a model of photothrombotic stroke (Shang et al., 2019; Li et al., 2017; Hu et al., 2020).

The limitations of this study, and the dose-response studies have been the use of healthy young animals, and the limited predictive value the studies have for the translation of this drug into the clinic. Even though the updated meta-analysis validates the efficacy of fingolimod (Dang et al., 2020), there is still the need to evaluate this drug in comorbid models to improve the external validity and translatability of this drug. However, the lack of differences in our studies up to now could mean that fingolimod does not have a clinically relevant effect on lesion size. Nevertheless, whether fingolimod works on healthy young animals might be irrelevant considering that animals with comorbidities would resemble the intended treatment population, thus the need for fingolimod and other novel treatments should be focused on aged individuals that have a variety of comorbidities associated with stroke.

4.5. The Effect of Fingolimod On Stroke In Mice With Comorbidities

The disparity between the relative age of the mice used for stroke research and the fact that older individuals are affected by stroke at a higher rate than younger individuals has been identified as one of the factors affecting the translatability of the research from rodent models to patients (Dirnagl, 2006; Strazzullo et al., 2010; Haley and Lawrence, 2016). Studies performed in healthy young animals have been used to inform clinical trials that were unsuccessful at improving outcomes in humans with stroke. The age range of humans presenting with stroke commonly falls between 50-80 years of age, and aside from age as non-modifiable comorbidity (Przykaza, 2021), patients experience a variety of other comorbidities, i.e., dyslipidaemia, hyperglycaemia and diabetes, that are not frequently studied in stroke research.

The lack of characterisation of drugs in comorbid mice may contribute to the translational gap that stroke research has been experiencing. Thus, the STAIR group updated the first set of established guidelines to improve the quality of research and drug treatments for stroke by including the need to characterise drugs in comorbid models prior to advancing drugs into clinical trials (Fisher et al., 2009). Thus, we chose to focus on age (non-modifiable) and hyperlipidaemia (modifiable) to study the effects of fingolimod. The results of these studies are discussed below.

4.5.1. The Effect of Fingolimod on Aged Mice with Stroke

The study evaluating the effect of fingolimod on stroke modelled in aged mice showed that fingolimod treatment led to a behavioural improvement; however, there was unexpected the lack of effect on lesion size and atrophy because there was increased lesion and atrophy that are generally associated with poor outcomes (Rewell et al., 2017; Sirén et al., 2006). In fact, although mice treated with fingolimod had a significantly greater ipsilateral hemisphere atrophy, they showed a significant behavioural improvement in the grid test at day 7 after stroke, and a trend towards improved survival odds compared to mice treated with saline.

It is difficult to compare these results to previous literature, because no other study uses fingolimod to treat stroke in aged animals. However, there is literature evaluating the effects of systemic inflammation, immunomodulation and ageing in rodent models of stroke, which along with the known effect of fingolimod, can inform the interpretation of the observed lesion, atrophy and behavioural results.

Aged humans and mice have an underlying inflammatory state that predisposes them to worse outcomes after stroke, making inflammatory modulation by immunomodulation a compelling target for translational stroke research (Przykaza, 2021; Petcu et al., 2008; Drake et al., 2011). In young and healthy individuals, the pathophysiological cascade following stroke triggers local and systemic inflammation that stimulates reparative processes; however, excessive or prolonged inflammation can be detrimental to recovery (Kim et al., 2020). The role of a young immune system and its ability to reduce inflammation after stroke was illustrated in a study using bone marrow chimaeras to evaluate the effect of replacing the primed immune system of aged mice with the bone marrow of young mice. The replacement of the aged immune cells, which normally cause excessive inflammatory response, with the bone marrow of young mice, improved behavioural performance without affecting lesion sizes after stroke (Ritzel et al., 2018).

Manipulating the immune response in old mice has been shown to reduce stroke lesion size. These studies have produced contrasting results between young and aged animals under the same treatment conditions (Ergul et al., 2016; Ritzel et al., 2018).

Immunomodulation by splenectomy and neutrophil depletion reduced lesion size in young mice, but CD4+ T cell depletion had no effect. In contrast, aged mice had improved behavioural outcomes with splenectomy, neutrophil, and CD4+ T cell depletion but only splenectomy reduced lesion size (Chauhan et al., 2018; Roy-O'Reilly et al., 2020; Harris et al., 2020). The different effects of immune-targeted interventions in young and older mice highlight the contrasting effects of the immune system in stroke pathophysiology in young, old and comorbid mice (Candelario-Jalil and Paul, 2020).

Although our studies in young and old mice were conducted separately and were not meant to be compared directly, it is interesting to observe that vehicle-treated young mice had a smaller lesion size than vehicle-treated aged mice, while aged mice benefited behaviourally from treatment. The discrepancies in the results highlight the limitations of lesion size measurements and their correlation to behaviour improvements in stroke research, especially since lesion size measurement is an uncommon outcome in clinical trials (Saver et al., 1999), and histological lesion size measurements seem to be uninformative and insufficient to predict benefits from treatments in different ischaemic models (Balkaya and Cho, 2019).

However, atrophy might be a more informative outcome measure for preclinical stroke research since it has been used to measure long-term outcomes and recovery in humans (Brodtmann et al., 2020; Lee et al., 2010). Atrophy measures the extent of tissue loss or shrinkage of the ipsilateral hemisphere compared to the contralateral hemisphere. Furthermore, atrophy serves as a long-term study outcome that has not been used in fingolimod studies, perhaps because most of those studies evaluate short term (24 - 48 h) outcomes, and at those time-points hemispheric comparisons measure oedema. Our results show that aged mice had increased atrophy when treated with fingolimod compared to the reduced atrophy observed in young animals treated with the same dose. The reduced level of atrophy observed in the aged control mice compared to the fingolimod treated group coincides with observations made by Manwani et al. (2011), who observed that aged mice had less atrophy than young mice 30 days after stroke (Manwani et al., 2011). The observed atrophy in aged mice treated with fingolimod might be beneficial because it correlates with improved behaviour in the fingolimod treated mice compared to the saline-treated mice 7 days after stroke product of systemic immunomodulation. Similarly, there is a beneficial correlation between atrophy and recovery following stroke in humans (Lee et al., 2010).

The differences between the aged study results and those from the dose-response study in young mice could indicate that the effect of fingolimod cannot be determined by

measuring lesion size but rather by evaluating the number of surviving neurones, the scar formation, the BBB integrity and immune system (Chen, Shao and Ma, 2021; Shang et al., 2019). Fingolimod may affect oedema and atrophy, leading to improved behavioural outcomes as observed in our aged study. This is supported by a recent study that showed that aged mice treated with anti-CD4 (a subset T cells that is also reduced by fingolimod) showed behavioural improvements in male and female animals without effects on lesion size, reinforcing that behavioural improvement, as observed in our results, can be independent of lesion size (Harris et al., 2020, 2016).

One limitation of this study is that we could not perform a pilot study to determine sample size for aged mice. The sample size calculation used for this study was based on fingolimod studies that used young animals, meaning that there is the possibility of a false negative (type II error) in the lesion size measurement and that there was a treatment effect when none was statistically detected. Furthermore, a recent review found that several therapies had no significant effect in animals with comorbidities, and there is the possibility that fingolimod does not affect lesion size in this comorbid model (Schmidt-Pogoda et al., 2020).

Some of the limitations for translating and bridging preclinical results into clinical applications are based on the outcome measures and biomarkers used in preclinical studies: lesion sizes and behaviour, while the focus in the clinic is on functional improvements. This discrepancy is illustrated in a clinical trial evaluating the effect of a 3-day regimen of fingolimod, which found that the lesion size was unaffected 7-days after stroke with functional improvement in the treatment group (Fu et al., 2014b), which aligns with our results in the mice similar in age to the patients in the trial. The clinical results suggest that the treatment with fingolimod in humans leads to improved behavioural outcomes without affecting the lesion size while also observing a reduction in the lesion expansion rate by possibly modulating the inflammatory response associated with stroke (Fu et al., 2014b).

The evaluation of fingolimod in aged mice emphasises the importance of performing more studies and evaluating clinically promising treatments in aged mice, because they might perform differently than young mice, as observed in our study with fingolimod. This study also adds to the limited literature evaluating drugs in aged mice.

4.5.2. The Effect of Fingolimod on Hyperlipidaemic Mice with Stroke

Obesity and hyperlipidaemia are common comorbidities associated with stroke patients that have been identified to affect their odds of having a first stroke and also affect their recovery and survival after stroke (Virani et al., 2020). Therefore, we used a model of stroke in a comorbid model of hyperlipidaemia and atherosclerosis to evaluate fingolimod as potential treatment for stroke. Hyperlipidaemia was induced in an ApoE deficient mice fed a high-fat diet for 12 weeks. The model was validated by the measuring blood cholesterol levels and the number of atherosclerotic lesions along the aorta. Mice had elevated cholesterol and atherosclerotic plaques that matched previous reports using ApoE deficient mice fed a high-fat diet (Getz and Reardon, 2006). Furthermore, the weight gained over the 12 weeks was comparable between groups confirming that the groups were evenly distributed by randomisation.

The hyperlipidaemic study showed that fingolimod treated mice had a significantly smaller lesion size compared to saline control animals without significant differences in atrophy, or behavioural outcomes. Mice were observed and evaluated daily for weight, appearance, behaviour and neurological-score and there was no difference between groups. Even though there was a significant improvement in some outcome measures (lesion size and daily scores), these results contradict the expected all-around improvement that has been previously reported from fingolimod treatment and summarised in two meta-analyses (Dang et al., 2020; Liu et al., 2012). However, as discussed previously, the expected benefits were based on studies using healthy young animals, and introducing a comorbidity might explain the discrepancies between the expected and observed results without invalidating our study.

Inflammation is a factor that underlies both obesity and apolipoprotein genotype, causing larger strokes and worse outcomes, as observed in a mouse model of inflammation and anaphylaxis with stroke (Drake et al., 2011; Dénes, Ferenczi and Kovács, 2011). The effect of inflammation is independent of comorbidities that themselves are associated with an inflammatory state (age, hypertension, diabetes and obesity), and reducing inflammation has led to improvement in lesion sizes and overall

recovery after stroke (Dénes, Ferenczi and Kovács, 2011). Obesity is associated with a specific inflammatory profile (e.g., elevated IL-6, TNF, and other factors, known as adipokines) that is also observed in hyperlipidaemia and atherosclerosis. This inflammatory profile leads to larger lesion sizes, emphasising immunomodulation as a potential treatment modality (Maysami et al., 2015a; Aguilar-Valles et al., 2015). This means that the observed lesion size reduction is consistent with the known immunomodulatory and anti-inflammatory effects associated with fingolimod treatment (Iadecola, Buckwalter and Anrather, 2020). Nevertheless, the effects of fingolimod was not beneficial in stroke model with diabetes, in which inflammation was exacerbated by fingolimod treatment (1 mg/kg) (Li et al., 2020).

Hyperlipidaemia can be a product of a genetic mutations, diet-induced or combination of both, and all the methods lead to larger lesion sizes when compared to controls. Mice homozygous for a spontaneous null mutation of the leptin gene (ob/ob) have an increased lesion size 24 h after experimental stroke compared to heterozygous ob/- mice (Haley et al., 2017). ApoE -/- mice fed a high-fat diet — as a model of atherosclerosis and obesity — have an altered immunological system that causes larger lesion sizes than three different control animals (ApoE mice on regular diet and WT mice on regular and high-fat diet) (Herz et al., 2014). Another study has identified a delayed lesion size enlargement between 10 and 72 h after stroke in obese C57Bl6 mice (Peterson et al., 2021).

The larger lesion sizes likely the results from the underlying pathological state of obesity and associated inflammation, as well as the subsequent inflammatory response associated with stroke. The lesion size reduction that we observe in the treated ApoE-/- mice fed a high-fat diet could be a product of administering treatment during the delayed lesion expansion period that occurs between 10 and 72 h after stroke, and the immunomodulation from fingolimod by reducing circulating T cells and limiting their infiltration into the ischaemic brain (Liesz et al., 2011; Malone et al., 2021; Peterson et al., 2021). In the absence of imaging methods such as MRI, our capacity to assess the lesion size histologically at 7 days limited our ability to characterise the evolution of the ischaemic injury and how obesity affects infarct size evolution (Peterson et al., 2021).

Behavioural tests are a secondary method of evaluating recovery after stroke are that assess the impact of treatments over time. Since there was no improvement in the behavioural outcomes of the hyperlipidaemic mice over time, there is a mismatch with the observed lesion size improvement. As discussed previously in the dose-response study, one explanation for observed results could be the inherent limitations of the behaviour tests selected, including the limited ability to detect small deficits and their

change over time (Manwani and McCullough, 2011). Additionally, some animal strains, including C57Bl/6 (the genetic background of ApoE mice), are less likely to develop pronounced motor deficits that can be consistently measured; thus, limiting our ability to observe behavioural changes associated with small lesion sizes that are characteristic of the electrocautery stroke model (Manwani and McCullough, 2011; Schaar, Brenneman and Savitz, 2010). Nevertheless, C57Bl/6 mice were selected for all of the studies because of their versatility in research, and C57Bl/6 mice are readily available as aged mice and serve as a background strain for many mice with spontaneous and genetically modified genes.

It is also possible that the dose selected, based on the dose-response study, is inadequate to counteract the underlying inflammation from hyperlipidaemia and the inflammation associated with the stroke. A higher dose of fingolimod might be able to improve both lesion and behavioural outcomes. Therefore, there might be a benefit from performing dose-response studies specifically in comorbid animals to understand the interaction of the comorbidities with the drug. This applies to both age and other comorbidities, and additional research with comorbid mice could narrow the target population for clinical trials that can benefit from a treatment. For instance, excluding patients with uncontrolled diabetes could be considered based on the results of the recent preclinical study wherein fingolimod exacerbated inflammation in diabetic mice (Li et al., 2020).

Overall, these results are inconsistent with the lesion size reduction and improved behaviour after fingolimod treatment in over 17 studies performed in young mice without comorbidities. Previously published studies and meta-analyses may not predict the effect of fingolimod in an atherosclerosis model, making it possible that the expected outcomes are inaccurate and that our results reflect the true impact of fingolimod treatment.

Underlying inflammation in diabetes may lead to larger ischaemic injury (Rewell et al., 2010; Kim, Tolhurst and Cho, 2014). However, the reduction of lymphocytes with fingolimod (1.0 mg/kg) caused worse neurological outcomes after stroke in diabetic mice when compared to saline controls and led to greater inflammation (Li et al., 2020). The results observed in the aged and hyperlipidaemic models highlight the different results that can be observed under comorbid disease conditions. More research focused on comorbid animals may therefore be needed to expand the knowledge of the pathophysiology of stroke in diseased states. Furthermore, the characterisation of different treatment regimens, i.e., dosage, treatment window, and treatment duration in comorbid models, might produce robust translatable results for fingolimod and other

drugs and should be considered a necessary step prior to clinical evaluation of promising drugs.

4.6. Pooled Data

As discussed previously, two meta-analyses support the use of fingolimod for the treatment of stroke in rodent models (Dang et al., 2020; Liu et al., 2012). However, those data do not include the characterisation of fingolimod in rodents with comorbidities commonly present in humans with stroke. Thus, in an attempt to replicate the characteristics of a diverse population of stroke patients we decided to pool the data obtained from our studies and evaluate the effect of fingolimod in a heterogeneous population. Doing so we would be observing the effect of fingolimod treatment in mice that could translate to a similarly heterogeneous human population.

The lesion size, atrophy, cylinder, and grid tests data were pooled from all the studies that used 70% N_2 and 30% O_2 to generate a mixed population of individuals treated under similar conditions. The mixed population involved healthy young, young hyperlipidaemic and aged mice. Statistical comparisons between treatment groups revealed a difference in the grid test, wherein fingolimod treated mice scored better than control mice 7 days after stroke. However, there was no difference in any of the other outcome measures. The results from these pooled data increase the external validity of our results both for other preclinical studies because of the large number of animals used, and for clinical studies that involve a preponderance of individuals with comorbidities.

The reproducibility crisis has led to the proposal of many guidelines and recommendations aimed at increasing the quality of the results and their external validity (Voelkl and Würbel, 2016; Goodman, Fanelli and Ioannidis, 2016; Vollert et al., 2020). A recent meta-analysis of all the guidelines found that their focus has been on improving internal validity without addressing external validity, perhaps because factors influencing internal validity are easier to control for (Vollert et al., 2020). Internal validity covers factors that affect reproducibility, i.e., groups size calculations, blinding and randomisation, all accepted ways to improve research quality and rigour. The focus on controlling these parameters might be leading researchers to over standardise studies, thus causing poor reproducibility. The use of standardisation between labs in an effort to compare their findings has shown that the results between labs carried out under seemingly similar conditions produce different results based on different phenotypic responses to different physical environments (Kafkafi et al., 2018). Thus, it is unclear whether the efforts made by researchers to follow the proposed guidelines have produced

robustly translatable results (Vollert et al., 2020).

The pooling of the data could be considered a way of heterogenising the study and increasing the external validity of our results. The concept of heterogenised studies was proposed as a way to overcome the reproducibility crisis that preclinical research is experiencing (Richter et al., 2011; Begley and Ioannidis, 2015; Richter, 2017). It was a response to the "standardisation fallacy", a consequence of attempting to control for as many variables in one study and consequently limiting reproducibility and translatability (Voelkl and Würbel, 2016; Würbel, 2000; Voelkl et al., 2021).

Systematic heterogenisation is difficult to implement because it requires the identification of variables that can affect outcome measures and systematically vary them in the study. Time of day, feed and gut microbiota, as well as strain, sex and age of the animals have all been identified as factors affecting stroke outcomes, i.e., lesion size and behaviour (Richter et al., 2011; Richter, 2017; Bodden et al., 2019; Usui et al., 2021). For our purposes, it was not feasible to systematically vary all these variables.

Our individual "standardised" studies do not show robust results supporting the use of fingolimod in stroke yet analysing them together in a pooled heterogeneous dataset might be translatable because it can be considered batch heterogenization making the overall finding that behaviour was improved seven days after stroke more robust than each observation. However, the significant difference observed in the grid test could be a false positive, type I error. Further studies in a large heterogeneous mouse cohort could be designed and powered to determine if this is a spurious finding.

FACS and immunohistochemistry experiments were performed on samples collected from all the studies as part a study evaluating the involvement of a subset of T cells in stroke recovery and fingolimod treatment (Malone et al., 2021). The regulatory T cells (Tregs) were elevated in the ischaemic core of all mice evaluated (young, aged and hyperlipidaemic), as well as in blood and spleen. Interestingly, other studies have also observed that an increase in Tregs is associated with improvement in stroke outcomes because they are associated with an anti-inflammatory phenotype (Liesz et al., 2009), and their function is not impaired by fingolimod treatment (Haas et al., 2014; Muls et al., 2014)

Lastly, interesting as they are the observed behavioural improvement in mice treated with fingolimod supports a focus on behaviour for future studies because behaviour outcomes may be representative outcomes measure in clinical trials. Yet, these data do not include female mice, whom represent a significant proportion of the stroke patients that are also in need of effective treatment. The lack of female mice limits the external

validity of these findings as there might be unknown effect from fingolimod in ischaemic mice, as were observed in the ICH studies previously discussed.

Chapter 5

Conclusion

The goal of this thesis was to inform whether fingolimod is a good candidate for stroke treatment in large randomised clinical trials. The preclinical evidence supporting fingolimod up to now has been summarised in two meta-analyses (Dang et al., 2020; Liu et al., 2012), and the clinical data was recently summarised in a meta-analysis evaluating all the small, mostly proof of concept, clinical studies testing the effect of fingolimod on stroke patients (Bai et al., 2022). While the overall evidence is positive, preclinical research evaluating fingolimod has not met many of the STAIR guidelines. This, and the fact that drugs with incomplete characterisation have gone to fail in clinical trials makes it important to evaluate fingolimod more rigorously before any large trial is initiated.

This thesis therefore aimed to fill some of the gaps in the characterisation of fingolimod by re-evaluating fingolimod in a model of intracerebral haemorrhage using both male and female mice (Diaz Diaz et al., 2020). It also filled the gaps by evaluating the effect of fingolimod on ischaemic stroke in a series of studies: first determining an optimal dose from a dose response study, then using the optimal dose to evaluate the effect of fingolimod in aged and hyperlipidaemic mice, two common comorbidities in stroke patients, and lastly to evaluate the effect of a longer treatment duration on stroke (Diaz et al., 2022). In an effort to improve the predictive value of our results, the studies were performed with a priori sample size calculation and definition of an exclusion criteria. Randomisation and blinding were implemented, with the aim of reducing internal bias. These measures helped with the internal validity aspects, and the use of aged and hyperlipidaemic mice addressed aspects of the external validity (Ferreira et al., 2020).

As part of the goal to meet the stair guidelines we set out to use a thromboembolic model of stroke. By doing so, we could use the tissue plasminogen activator (tPa), the current approved treatment for stroke, in conjunction with fingolimod; however, our

inability to establish this model of stroke affected the translatability of the results obtained in the ischaemic studies and meeting the stair guidelines of using a clinically relevant model. Yet, the more recent STAIR guidelines shift their focus to permanent occlusion with long-term outcomes that we did achieve herein. Furthermore, there were no other methods to model stroke in mice that have consistent cortical lesion, high survivability, and highly reproducible, thus limiting our options to clips, sutures, or the method we selected, electrocautery.

Fingolimod, though observed by others to be neuroprotective, was unsuccessful in the large robust studies performed for this thesis. The intracerebral haemorrhage study involved male and female mice receiving three 0.5 mg/kg doses of fingolimod and observed for up to 14 days, and the study did not identify any lesion size or behavioural benefit associated with fingolimod treatment after 14 days (Diaz et al., 2022). The fingolimod dose-response studies in ischaemic stroke showed no clear benefit associated with treatment in the primary outcomes, other than reduced atrophy in the 7 day outcome in young mice. The treatment duration study, also in young mice, was suggestive that a longer treatment was better; however, it was not significant perhaps because young mice show a limited range of improvement. The aged mice had greater atrophy with an improved behaviour outcome 7 days after stroke, and the hyperlipidaemic mice had a smaller lesion size without a significant effect on behavioural outcomes. Lastly, the pooled data analysis of the ischaemic stroke studies showed that fingolimod improved the 7 day behavioural deficit after stroke compared to saline.

Our neutral findings are not unique, and there have been several publication showing that fingolimod is not beneficial, including one study performed under similar condition than those of this thesis (stroke in young mice modelled with electrocautery) (Liesz et al., 2011) and one in diabetic mice (Li et al., 2020). Furthermore, one recent study showed that fingolimod had no effect on lesion size irrespective of the circadian phase in which the stroke was induced (Mandeville et al., 2022). Our results also highlight the discrepancies in results between young healthy animals, and the fact that we should not extrapolate the effects of drug in young mice to comorbid mice. In our case beneficial outcomes were associated with changes in atrophy that differed from young and aged or comorbid mice and only in aged mice did the atrophy negatively correlate with improved behavioural outcomes. Lastly, our results put in perspective the difference between a well-designed and well-powered study that has a high number of included individuals, and underpowered studies as summarised by Dang et al., 2020.

Even though there were no benefits associated with fingolimod treatment, this study

provides an example of how a thorough evaluation of promising drugs can be achieved and the importance of the work. Even though our studies were successfully performed in a single laboratory, multi-centre preclinical studies are complementary approaches for evaluating promising drugs. However, a multi-centre study evaluating fingolimod for the treatment of stroke might not be warranted as the evidence against its suitability for clinical usage is growing.

This study design can be used to evaluate other drugs, and it could prevent clinical studies that are bound to fail. This study also demonstrates the importance of attempting to replicate previously published results under rigorous conditions and added complexities. This is because our studies focused on confirmatory experiments that can validate or reject potential therapeutics and are necessary to parse the false positives from the true results (Dirnagl, 2019; Tukey, 1980; Kleikers et al., 2015). The use of inclusion and exclusion criteria, sample size estimation, the use of female and comorbid mice are tools for corroborating that a drug is in fact worth investigating in large randomised clinical trials. An example of a drug that was not characterised thoroughly prior to evaluation in a large clinical trial was natalizumab (anti-CD49d) (Elkins et al., 2017; Elkind et al., 2020), and when larger confirmatory preclinical studies were performed they showed no benefits associated with natalizumab treatment, had these studies been performed earlier, and their results acknowledged the clinical trials could have been prevented (Llovera et al., 2015; Drieu et al., 2019).

This thesis had several limitations, one of them, as mentioned above, was that the original plan was to evaluate fingolimod in a thromboembolic model of stroke and evaluate the co-administration of fingolimod with tPA; however, technical difficulties led us to model stroke in a permanent model of ischaemia that could not be used in conjunction with tPA. This limitation prevented us from evaluating the effect of this combined therapy in comorbid mice thus affecting the translational value of our results. Additionally, this limited our ability to evaluate potential drug-drug interactions that could affect patients receiving tPa and fingolimod after experiencing a stroke, another aspect that would have added to the characterisation of fingolimod in order to address all of the stair guidelines (Kahle and Bix, 2012). Additionally, female mice were not included in the ischaemic studies, another aspect necessary for meeting the STAIR guidelines.

Another limitation was the high degree of variability observed in our data, which could mean that we are not observing the true effect of fingolimod, not because there is not an effect, but because the effect is hidden behind that variability (Kong et al., 2020). This, compounded by the fact that the studies lack of positive controls, further affects the

quality of the results. We did not plan for a positive control in the lesion size because we assumed that fingolimod would be effective at reducing lesion size. Recently another study from our group showed that treatment with recombinant pregnancy-specific glycoprotein-1-Fc after stroke significantly improved behavioural outcomes one and five days after stroke without any effect on lesion size in mice (Malone et al., 2022). Considering that Liesz et al., 2011 also failed to show changes in lesion size at seven days, it might not be feasible to have such a positive control for long term studies.

We were also limited to the use of histology for the evaluation of lesion size, the evaluation of the effect of fingolimod treatment with an MRI could have informed the dynamics behind the fact that some mice treated with fingolimod have no atrophy (young dose-response study), and others have greater atrophy (aged study) compared to saline control. Lastly, due to time and financial constraints, we were unable to evaluate the effect of fingolimod in diabetic mice with stroke. However, as mentioned previously, a recent study found that fingolimod was ineffective at reducing lesion size in diabetic mice, and that in fact it was detrimental (Li et al., 2020). Based in the inflammatory profile of diabetic mice we might have also been unsuccessful at observing benefits in a different diabetes model.

The progression of the inflammatory process and oedema resolution is an important aspect to research further, especially in the case where treatment of stroke is focused on modulating inflammation and immune cell infiltration into the stroke area. In the case of fingolimod, treatment could be on one hand shifting or delaying the infiltration of immune cells into the brain by the S1P1 receptor internalisation (Liesz et al., 2011; Malone et al., 2021), on the other hand fingolimod can be affecting the amount of apoptotic and necrotic tissue, hence decreasing the amount of tissue loss in the case of stroke by interfering with these pathways (Qin et al., 2017, 2019). Furthermore, S1P is involved in axonal growth, which could suggest that the effect of fingolimod in behaviour is associated with neurone sprouting (Anastasiadou and Knöll, 2016). All together fingolimod can shift lesion size measurements to secondary outcomes in preclinical studies rather than primary outcomes; and increase the focus on behaviour tests.

The evaluation of a dose-response in comorbid mice, as well as a long term study evaluating the effect of two or more fingolimod doses, and other studies focusing on the interactions of fingolimod with other drugs that patients with comorbidities are taking prior to stroke (i.e., bisoprolol, warfarin, aspirin, and atorvastatin) would be a worth evaluating. However, resources would be better spent characterising other promising drugs, as the result for fingolimod have been underwhelming in this and other well

powered studies. Furthermore, in an effort to refine clinical trials, many have proposed the use of a narrow population of participants that can benefit from a treatment (Cramer, 2010). In such a case, based on the results herein, seemingly healthy older women experiencing an intracerebral haemorrhage, and individuals without other comorbidities might be good candidates for fingolimod treatment after stroke, To widen the population, more research would be necessary in hyperlipidaemic, diabetic and hypertensive animals models, as well as female mice under these comorbid conditions. Another important factor for future preclinical studies would be the implementation of systematic heterogenisation, and multicentre studies as they could produce robust results more likely to predict clinical efficacy.

Overall, these results cast a doubt on the effectiveness of fingolimod for the treatment of stroke. However, it can still be used as a pharmacological agent as an S1P1 receptor functional antagonist to further elucidate the pathways involved in ischaemic stroke, in particular the effects of lymphocyte modulation on stroke outcome (Malone et al., 2021). It would also be important to identify whether there are factors that led to fingolimod being effective in some but not other studies, or whether study rigour was the only critical determinant.

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Appendix

A: Rodent Stroke Pain Assessment

Licensee Name:	Procedure performed:
License No:	Time:
HPRA Project Auth:	Notes:
Proyect Name	
Department:	
Contact Tel:	
Species/Strain ♀ ♂	Starting weight: _____

Nº

Date: _____

Animal ID	SC	Day 1 am	Day 1 pm	Day 2 am	Day 2 pm	Day 3 am	Day 3 pm	Day 4 am	Day 4 pm	Day 5 am	Day 5 pm	Day 6 am	Day 6 pm	Day 7 am	Day 7 pm	Day 8	Day 9	Day 10	Day 11	Day 12
Weight _____																				
5-10% weight loss	1																			
11-15% weight loss	2																			
16-20% weight loss	3																			
20% weight loss	HEP																			
Appearance	0																			
Coat slightly unkempt	1																			
Slight piloerection	2																			
Marked piloerection	3																			
Behavior	0																			
Slightly abnormal gait	1																			
Markedly abnormal gait	2																			
Significant mobility problems	3																			
Immobility >24hrs	HEP																			
Tense and nervous on handling	2																			
Markedly distressed on handling	3																			
Neuroscore (additive)	0																			
Forelimb flexion	1																			
Reduced resistance to lat push w/o circlin	2																			
Same behaviour as grade 2 w/circling	3																			
Score Adjustment : 2-5																				
If you have scored a 3 more than once, add an extra point for each 3																				
TOTAL:																				

Actions	
Score 1-3	Review frequency of monitoring
Score 4-5	Provide supplementary care, e.g. extra fluids and wet mash
Score 6-9	Review progress with veterinarian
Score 10 and above	Implement humane endpoint (**HEP**)

*The severity assessment is performed by a combination of general clinical observations (bodyweight, appearance, behavior, cage environment) together with a stroke specific neurologic evaluation (Bederson scale) which is a global neurological assessment developed to measure neurological impairments following stroke. A grading scale of 0-3 is used, with 0= normal and 3= highest level of disability. Tests include forelimb flexion, resistance to lateral push and circling behaviour.

grade 0: no observable deficit

grade 1: forelimb flexion

grade 2: decreased resistance to lateral push (and forelimb flexion) without circling

grade 3: same behaviour as grade 2, with circling

Conferences and Publications

Conferences

1. 2016 Irish Association of Pharmacologist (30-11-2016) RCSI Dublin - attendant
2. 2017 Irish Association of Pharmacologist (24-11-2017) UCD Dublin - poster presentation
3. 2018 Irish Association of Pharmacologist (30-11-2018) Belfast - poster presentation
4. 2019 Irish Association of Pharmacologist (30-11-2019) NUI - Galway - Oral presentation

Publications associated with PhD research work

Diaz Diaz, A.C., Shearer, J.A., Malone, K. and Waeber, C., 2020. Acute Treatment With Fingolimod Does Not Confer Long-Term Benefit in a Mouse Model of Intracerebral Haemorrhage. *Frontiers in pharmacology*, 11, p.613103.

Malone, K., Diaz Diaz, A.C., Shearer, J.A., Moore, A.C. and Waeber, C., 2021. The effect of fingolimod on regulatory T cells in a mouse model of brain ischaemia. *Journal of neuroinflammation*, 18(1), p.37.

Diaz Diaz, A.C., Malone, K., Shearer, J.A., Moore, A.C. and Waeber, C., 2021. Histological, behavioural and flow cytometric datasets relating to acute ischaemic stroke in young, aged and ApoE-/- mice in the presence and absence of immunomodulation with fingolimod. *Data in Brief*, 36, p.107146.

Diaz Diaz, A.C., Malone, K., Shearer, J., Moore, A.C. and Waeber, C., 2022. Preclinical Evaluation of Fingolimod in Rodent Models of Stroke with Age or Atherosclerosis as Comorbidities. *Frontiers in Pharmacology*. [online] Available at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.920449/>.