Making Clots and Breaking Clots: Modelling Arterial Occlusion to Test Stroke Sonothrombolysis

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STATEMENT OF ORIGINALITY

The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University’s Digital Repository*, subject to the provisions of the Copyright Act 1968. * Unless an embargo has been approved for the determined period.

STATEMENT OF COLLABORATION

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers, and carried out in other institutions. I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices.

See Appendix A.

STATEMENT OF AUTHORSHIP

I hereby certify that the work embodied in this thesis contains published papers of which I am a joint author. I have included as part of the thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publications.

See Appendix A.

________________________   ________________________
Amelia Tomkins     Date
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CONTENTS

Declarations ................................................................................................................................. i
Acknowledgements .................................................................................................................... ii
Contents ...................................................................................................................................... iv
List of Figures and Tables ........................................................................................................ vii
Abbreviations ............................................................................................................................ ix
Clinical trials referenced in this thesis ..................................................................................... x

Abstract ...................................................................................................................................... 1

Chapter 1
Introduction ................................................................................................................................ 4
1.1. Stroke ................................................................................................................................... 5
1.2. Sonothrombolysis .................................................................................................................. 17
1.3. Animal models for preclinical stroke research ................................................................. 25
1.4. Aims and rationale ................................................................................................................ 36

Chapter 2
Microbubble-enhanced sonothrombolysis of middle cerebral artery occlusion ............... 38
2.1. Study aims, rationale and hypotheses ................................................................................... 39
Publication: Platelet rich clots are resistant to lysis by thrombolytic therapy in a rat model of embolic stroke .............................................................................................................. 41
2.2. Abstract ................................................................................................................................ 42
2.3. Background ........................................................................................................................... 43
2.4. Methods .................................................................................................................................. 43
2.5. Results .................................................................................................................................... 48
2.6. Discussion ............................................................................................................................ 53
2.7. Conclusion ............................................................................................................................ 55
2.8. Additional information ........................................................................................................ 56
LIST OF FIGURES AND TABLES

Chapter 1

Figure 1-1 Ischaemic core and penumbra progression.................................6
Figure 1-2 Platelet activation........................................................................9
Figure 1-3 Clotting cascade........................................................................10
Figure 1-4 Thrombolysis with tissue plasminogen activator.........................13
Figure 1-5 Microvascular occlusion.............................................................15
Figure 1-6 Mechanisms of sonothrombolysis..............................................18
Figure 1-7 The most common preclinical models of stroke.........................27
Figure 1-8 Clot delivery to the middle cerebral artery.................................28
Figure 1-9 Decision tree of different experimental clot preparations...........29
Figure 1-10 Histopathological composition of thrombi from stroke patients...30
Figure 1-11 Fibrin fibre thickness.................................................................31
Figure 1-12 Fibrin structure of platelet poor and platelet rich clots..............33

Chapter 2

Table 2-1 Experimental protocols.................................................................44
Figure 2-1 Visualisation of vascular filling and clot presence (Study 2).........47
Figure 2-2 Laser Doppler flowmetry and infarction following middle cerebral
artery occlusion with platelet rich clots (Study 1)....................................49
Figure 2-3 Laser Doppler flowmetry (LDF) of regional cerebral blood flow in
treatment groups (Study 2).................................................................50
Figure 2-4 Vascular filling and clot presence (Study 2)...............................50-52
Chapter 3

Table 3-1 Properties of SonoVue® and BR38 microbubbles

Figure 3-1 Experimental timeline

Figure 3-2 Micro-CT scan for vascular volume

Figure 3-3 Vascular volume of the ipsilateral hemisphere

Figure 3-4 Nano-CT scan of cortex

Figure 3-5 Volume of ischemic lesion

Chapter 4

Figure 4-1 Experimental timeline [A] and surgery schematic [B] of carotid artery occlusion with stenosis

Figure 4-2 Example flow trace of crush, occlusion and recanalization

Figure 4-3 Carotid artery recanalization in the setting of mild or severe stenosis

Chapter 5

Figure 5-1 Recanalization rates at varying doses of tPA

Figure 5-2 Time to sustained recanalization

Figure 5-3 Doppler flow of crush and occlusion with recanalization

Appendix C

Figure C-1 Systematic review of preclinical embolic stroke models: studies retrieved to date

viii
# ABBREVIATIONS

*Please note, this thesis uses British English unless directly citing a paper or clinical trial name.*

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACA</td>
<td>Anterior cerebral artery</td>
</tr>
<tr>
<td>ACEC</td>
<td>Animal Care and Ethics Committee</td>
</tr>
<tr>
<td>AIC</td>
<td>Acute ischaemic changes</td>
</tr>
<tr>
<td>BPU</td>
<td>Blood perfusion units (for laser Doppler flowmetry)</td>
</tr>
<tr>
<td>CCA</td>
<td>Common carotid artery</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>ECA</td>
<td>External carotid artery</td>
</tr>
<tr>
<td>GP IIb/IIa</td>
<td>Platelet glycoprotein IIb/IIa receptor</td>
</tr>
<tr>
<td>HU</td>
<td>Hounsfield units</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>ICA</td>
<td>Internal carotid artery</td>
</tr>
<tr>
<td>JLU</td>
<td>Justus-Liebig University, Giessen, Germany</td>
</tr>
<tr>
<td>kHz</td>
<td>Kilohertz</td>
</tr>
<tr>
<td>LDF</td>
<td>Laser doppler flowmetry</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
</tr>
<tr>
<td>mg/kg</td>
<td>Milligrams per kilogram of body weight</td>
</tr>
<tr>
<td>MHz</td>
<td>Megahertz</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRS</td>
<td>Modified Rankin Scale</td>
</tr>
<tr>
<td>mW/cm²</td>
<td>Milliwatts per square centimetre</td>
</tr>
<tr>
<td>NIHSS</td>
<td>National Institute of Health Stroke Score</td>
</tr>
<tr>
<td>o.d.</td>
<td>Outer diameter</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen inhibitor-1</td>
</tr>
<tr>
<td>PRC</td>
<td>Platelet rich clot</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>rCBF</td>
<td>Regional cerebral blood flow</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>SAH</td>
<td>Subarachnoid haemorrhage</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneously hypertensive rat</td>
</tr>
<tr>
<td>sICH</td>
<td>Symptomatic intracerebral haemorrhage</td>
</tr>
<tr>
<td>TCCD or TCCS</td>
<td>Transcranial color-coded Doppler/sonography</td>
</tr>
</tbody>
</table>
TCD Transcranial Doppler ultrasound

tPA or rt-PA (Recombinant) tissue plasminogen activator

TTC 2,3,5-triphenyl-tetrazolium chloride

U/S Ultrasound

UoN University of Newcastle, Australia

VV Vascular volume

VVF Vascular volume fraction

CLINICAL TRIALS REFERENCED IN THIS THESIS

Clinical trial names are defined in footnotes at first mention throughout the text.

CLOTBUST Combined Lysis Of Thrombus in Brain ischaemia using transcranial Ultrasound and Systemic tPA (Clinical sonothrombolysis Trial)

CLOTBUST-ER Combined Lysis of Thrombus With Ultrasound and Systemic Tissue Plasminogen Activator (tPA) for Emergent Revascularization in Acute Ischemic Stroke

ECASS III European Cooperative Acute Stroke Study III

ESCAPE Endovascular Treatment for Small Core and Anterior Circulation Proximal Occlusion with Emphasis on Minimizing CT to Recanalization Times

EXTEND-IA Extending the Time for Thrombolysis in Emergency Neurological Deficits - Intra-Arterial

MR CLEAN Multicenter Randomized CLinical trial of Endovascular treatment for Acute ischemic stroke in the Netherlands

NINDS National Institute of Neurological Disorders and Stroke rtPA Stroke study

REVASCAT RandomizEd Trial of reVascularizAtion With Solitaire FR® Device Versus Best mediCal Therapy in the Treatment of Acute Stroke Due to anTerior Circulation Large Vessel Occlusion Presenting Within 8 Hours of Symptom Onset

SWIFT-PRIME Solitaire With the Intention For Thrombectomy as PRIMary Endovascular Treatment

TRUMBI TRanscranial low-frequency Ultrasound-Mediated thrombolysis in Brain Ischemia (Clinical sonothrombolysis Trial)

TUCSON Transcranial Ultrasound in Clinical Sonothrombolysis (Clinical sonothrombolysis Trial)
Abstract

**Background:** Acute ischaemic stroke is caused by occlusion of a major cerebral artery and is a major cause of death and disability worldwide. Early reopening of the occluded artery (recanalization) to restore blood flow to the ischaemic tissue is the best known approach to improving patient outcome after stroke. However, the current approved thrombolytic drug, tissue plasminogen activator (tPA), causes recanalization in <50% of cases treated, indicating a need for better recanalization approaches. Recent studies have revealed benefit of endovascular intervention to remove the occluding clot, but with limited endovascular centres, improving tPA’s effect may be a better and more cost effective approach for most centres. One such approach to enhanced tPA recanalization is with the application of continuous ultrasound insonation during tPA infusion (sonothrombolysis). Small scale trials of sonothrombolysis for stroke have shown improved recanalization and patient outcome over tPA alone. Added infusion of microbubbles has also been suggested to enhance sonothrombolysis. However, much is still unknown regarding sonothrombolysis efficacy in different situations of stroke and in preclinical models of clinically relevant scenarios. Clinical trials of sonothrombolysis have thus far focused on intracranial large vessel occlusions. However, stroke is a heterogeneous condition whereby patient outcome is affected by clot compositions, the occlusion site, and vascular stenosis. Efforts to model the clinical situation have also resulted in a large variety of experimental clots used for preclinical thrombolytic testing. Preclinical studies of sonothrombolysis to-date have tended to use models with clot compositions that are more susceptible to thrombolysis than a more clinically common platelet rich clot (PRC) composition. The effect of sonothrombolysis on PRC is yet to be tested in vivo. The middle cerebral artery (MCA) is the most commonly affected vessel in stroke, yet occlusions of the internal carotid artery (ICA), often associated with vascular stenosis, are associated with poorer patient outcome and lower rates of tPA recanalization. The recanalization potential of sonothrombolysis for extracranial carotid occlusions has not previously been tested, nor the effect of varying degrees of stenosis underlying an occlusion. While recanalization of the large occluded arteries is known to be a predictor of good patient outcome, reperfusion of the ischemic tissue has recently been shown to be a better predictor of good outcome than recanalization. Perfusion deficits caused by occlusions of the microvasculature can persist even after recanalization due fibrin and platelet deposits causing a “no-reflow phenomenon”. Improving therapies to target not only large vessel recanalization, but also microvascular reperfusion should improve the overall rates of patient outcome after stroke. To date, there has only been one preclinical study of sonothrombolysis of the microvasculature that suggested a benefit of this therapy for stroke reperfusion. Sonothrombolysis ± microbubbles may therefore, be a potential therapy to target both large vessel recanalization and microvascular reperfusion. To
obtain meaningful data of the potential of sonothrombolysis for stroke, it needs to be compared against the current standard, tPA. Rodent doses of tPA are conventionally 10-fold higher than human doses, due to some evidence that the rat fibrinolytic system is less sensitive than humans. However in some models, this dose causes high rates of recanalization that are not representative of the clinical response to thrombolysis. An appropriate “human equivalent” tPA dose that models the clinical response and allows room for improvement with sonothrombolysis is not known.

**Aims:** The overall aim of this thesis was to test sonothrombolysis (± microbubbles) for recanalization in different models of stroke in rats. The individual aims of the studies were: 1) To develop a model of MCA occlusion with PRC and to test for recanalization with sonothrombolysis and microbubbles (Chapter 2), 2) To test the effect of sonothrombolysis with microbubbles on restoring microvascular patency after large vessel recanalization and to directly compare two microbubble formulations at high and low doses (Chapter 3), 3) To develop a model of extracranial carotid artery occlusion with variable stenosis and to test sonothrombolysis for recanalization in this model (Chapter 4), and 4) To compare different doses of tPA ranging from the clinical dose (0.9 mg/kg) to the conventional rat dose (10 mg/kg) in a model of carotid artery occlusion to identify a “human equivalent” tPA dose that mimics clinical recanalization rates (Chapter 5).

**Methods:** 1) Preformed PRC were injected via the extracranial carotid arteries to occlude the origin of the MCA. Treatment groups were sonothrombolysis with microbubbles (BR38), tPA alone, or control. Recanalization was monitored by laser Doppler flowmetry and macroscopic visualization of the cerebral vasculature post-mortem. 2) Microvascular occlusion was achieved in a model of thread occlusion of the MCA with recanalization. Treatment groups were sonothrombolysis with SonoVue or BR38 microbubbles at full or half doses, tPA alone, or saline control. Patency of the microvasculature was assessed by micro-computed tomography. 3) The carotid artery was crushed to injure the endothelium and stenosed with a ligature to induce local thrombosis. Following occlusion, the stenotic ligature was released for a model of mild stenosis, or left in place for a model of severe stenosis. Doppler flow was used to confirm occlusion and to monitor for recanalization. Treatment groups were sonothrombolysis or tPA-alone in both stenosis models. 4) The mild stenosis model was used for testing the recanalization rates of different tPA doses, monitored by Doppler flow. tPA doses were: clinical dose (0.9 mg/kg), 2x the clinical dose (1.8 mg/kg), 5x the clinical dose (4.5 mg/kg), or the conventionally used rat dose (10 mg/kg).

**Results:** 1) No recanalization was observed in any treatment group (sonothrombolysis with microbubbles, tPA or control) in a model of MCA occlusion with PRC. 2) Microvascular patency was restored by sonothrombolysis with microbubbles after large vessel recanalization, regardless
of microbubble formulation or dosage. tPA alone did not restore microvascular patency. 3) High rates of recanalization were observed in a model of carotid artery occlusion with a mild stenosis, regardless of treatment: sonothrombolysis or tPA alone. No recanalization was observed in the severe stenosis model, regardless of treatment. 4) The clinical tPA dose did not cause recanalization, and the conventional rat dose caused recanalization rates too high to mimic clinical recanalization in a carotid artery occlusion model. In this model, 1.8 mg/kg tPA (2x the clinical dose) more closely mimicked recanalization rates of clinical carotid artery occlusion.

**Conclusions:** My results raise doubts regarding the overall efficacy of sonothrombolysis (± microbubbles) as a recanalization therapy for stroke. In different models of stroke, I determined that characteristics of clot composition and vessel stenosis will affect the success of thrombolysis (and sonothrombolysis). PRC were found to be completely resistant to microbubble-enhanced sonothrombolysis. My results suggest that sonothrombolysis will not be beneficial for this subpopulation of stroke patients. The degree of carotid artery stenosis associated with an occlusion was found to be predictive of thrombolytic success. My study was inconclusive regarding sonothrombolysis for recanalization in a model of mild stenosis and occlusion due to high recanalization rates with tPA alone. Sustained recanalization with any thrombolytic or thrombolytic-enhancer is unlikely to occur in the presence of a severe stenosis. Sonothrombolysis with microbubbles restored microvascular perfusion after mechanical recanalization of the large vessel occlusion. This effect was regardless of microbubble formulation or dose, suggesting a class effect of microbubbles to enhance sonothrombolysis, rather than individual microbubble properties or concentration. With recent evidence that reperfusion is a better predictor of good outcome than recanalization, microbubble-enhanced sonothrombolysis of microvascular occlusion is a potential approach to enhance the reperfusion clinically. Finally, the conventional tPA doses (human and rat) used preclinically, were found to be poor mimics of clinical recanalization rates in a model of carotid artery occlusion. For any thrombolytic-enhancer therapies, recanalization is an important outcome measure as it is the main mechanism by which tPA causes good patient outcome (by clot lysis and flow restoration). Using doses that mimic clinical recanalization rates may allow better translation of therapies. This finding should be considered for any future testing of thrombolytic-enhancers. Overall, this thesis presents data to indicate that sonothrombolysis (± microbubbles) is unlikely to be a suitable enhancer of tPA for large artery recanalization in stroke patients. However, there could be a potential for this strategy as an enhancer of reperfusion to improve patient outcome.
Chapter 1

1 INTRODUCTION

1.1 Stroke

1.1.1 Pathophysiology of stroke

1.1.1.1 Ischaemic core and penumbra

1.1.1.2 Arterial occlusion and thrombosis

1.1.2 Current treatment options

1.1.2.1 Thrombolysis and tissue plasminogen activator

1.1.2.2 Recanalization and reperfusion

1.1.2.3 The need for new therapies for acute stroke

1.2 Sonothrombolysis

1.2.1 Mechanisms of action of sonothrombolysis

1.2.2 Sonothrombolysis for stroke

1.2.3 Sonothrombolysis of the microvasculature

1.2.4 Sonothrombolysis of carotid artery occlusion

1.3 Animal models for preclinical stroke research

1.3.1 Thromboembolic stroke models

1.3.2 Modelling microvascular occlusion

1.3.3 Modelling carotid artery occlusion

1.3.4 Tissue plasminogen activator for preclinical research

1.4 Aims and rationale

1.4.1 Overall aim

1.4.2 Rationale for the overall aim
The primary focus of this project was to test the potential of sonothrombolysis for acute ischaemic stroke therapy. Acute ischaemic stroke occurs as the result of an occlusion of any artery supplying the brain. Hence, this project has tested sonothrombolysis in different models of arterial occlusion that result in cerebral ischaemia. This introduction will focus on the background information regarding these arterial occlusions along with what is currently known about thrombolysis and sonothrombolysis for stroke, and the pre-clinical models used to test these therapies.

1.1. Stroke

Stroke is a leading cause of death and disability worldwide. It is the second most common cause of cardiovascular death after coronary heart disease in Australia and developed countries. Stroke is a cerebrovascular event that causes damage to the brain and can lead to long-term functional and sensory impairments. There is a general rule of thirds for stroke patients: approximately a third will recover with mild or no disability, a third will be severely disabled requiring care, and a third will die. Recent stroke audits have revealed fatality rates of 12% in hospital, while over a third of patients had a disability attributed to their stroke [1, 2]. The primary aim of acute stroke therapy is to reduce patient disability and prevent death. A long term study of the Hunter region in Australia demonstrated that despite a gradual decline in stroke attack rates over a 12 year period (0.85% decline per year), case fatality rates remained the same [3]. Reasons for decline in attack rates are likely due to improvements in primary and secondary stroke prevention. This highlights the continuing burden of stroke and the need to improve on current therapies.

1.1.1. Pathophysiology of stroke

Stroke is caused by the interruption of blood flow to the brain and results in cell death and neurological deficits. There are two main types of stroke: ischaemic and haemorrhagic. Ischaemic stroke is caused by the occlusion of major arteries supplying the brain and occurs in approximately 80% of all stroke cases [4]. Haemorrhagic stroke is caused by the rupture and subsequent bleeding of cerebral vessels into the parenchyma. Due to its high incidence, ischaemic stroke is the focus of this project.

1.1.1.1. Ischaemic core and penumbra

Following occlusion of cerebral and/or carotid arteries, ischaemia and infarction of cerebral tissue occurs. Ischaemia is the reduction of blood flow sufficient enough to alter normal cellular function, while infarction describes the tissue that has died as a result of ischaemia. Brain tissue is highly sensitive to ischaemia and even brief ischaemic periods can initiate a sequence of events ultimately ending in neuronal death. This process is a complex combination of excitotoxicity, oxidative stress, inflammation and apoptosis, resulting in two characteristic regions of cellular injury: the ischaemic core, and the ischaemic penumbra [5-7]. The core is the region of greatest reduction of blood flow and within this region cell death occurs rapidly. It is predominantly
necrotic and considered out of therapeutic reach [8]. In the region beyond the core, tissue is less severely affected due to only moderate reductions in blood flow. Tissue within the penumbra may be transiently sustained due to residual flow from collateral vessels and although neurons are functionally silent, they remain metabolically active. However, this tissue does not remain viable indefinitely. Without rapid restoration of blood flow, cells within the penumbra will die causing expansion of the stroke lesion (Figure 1-1). To date, the most promising therapeutic strategy for acute stroke is the early restoration of blood flow to the ischaemic tissue to limit neuronal cell death. Early recanalization (vessel reopening) is strongly correlated with good outcome after stroke [9]. Hence, the primary goal of acute stroke therapies is to rapidly restore blood flow to the brain, thus targeting the penumbra to prevent further injury or increase in the size of the infarcted core.

A common saying in stroke medicine is “time is brain,” i.e. the quicker to treatment, the more brain to salvage (Figure 1-1). This is clearly highlighted by a systematic review estimating the quantity of brain lost per hour after stroke [10]. In patients experiencing a typical large vessel occlusion ischaemic stroke, an estimated 120 million neurons, 830 billion synapses and 714 km of myelinated fibres are lost each hour after occlusion. When compared with the normal rate of neuron loss in an aging brain, the ischaemic brain ages 3.6 years each hour recanalization is delayed. It is clear that as the time from stroke onset increases, less tissue is available to salvage.

**Figure 1-1. Ischaemic core and penumbra progression.** There are two characteristic regions of cellular injury following occlusion of a major cerebral vessel. The ischaemic core (dark red) is the area of greatest flow reductions where cell death occurs rapidly. The potentially salvageable penumbra (light red) is the area of moderate flow reductions where cell death is more gradual. Without rapid restoration of flow, the cells of the penumbra continue to die and the core region expands. Reproduced with permission from Dirnagl 1999 Trends Neurosci [5].
Early recanalization has been shown to be predictive of reduced infarct growth when salvageable penumbral tissue is present [11, 12]. Therefore, the main goal of acute stroke therapy has been to rapidly reopen the occluded vessels that cause cerebral ischaemia.

1.1.1.2. Arterial occlusion and thrombosis

Acute ischaemic stroke is the result of the occlusion of major blood vessels supplying the brain, of which the middle cerebral artery (MCA) is the most commonly affected [13]. These occlusions occur primarily due to blood clots, formed as a result of thrombosis at the site of occlusion (thrombi) or, formed distally and travelling to the cerebral vasculature (emboli). Emboli originate primarily from the heart or large arteries of the upper chest and neck.

The most common causes of stroke are cardioembolism, large-artery atherosclerosis and small vessel disease, although many strokes remain cryptogenic [14-16]. Cardioembolism commonly causes cerebral occlusions (such as occlusion of the MCA) resulting from clots forming in the heart and traveling to the brain [15, 17, 18]. The most common cause of cardioembolism is atrial fibrillation, but clots may also form as a result of cardiac arrest or abnormalities of the heart valves. Large artery atherosclerosis commonly causes carotid occlusion [19] and is a condition in which plaques build up within arteries, narrowing the vessel lumen (stenosis), thereby restricting blood flow. Stenosis of the vessel causes altered flow conditions that can lead to thrombosis. Ruptured plaques can initiate thrombosis as the blood becomes exposed to subendothelial matrix proteins and occlusion of the vessel occurs at the site of the plaque. Additionally, plaque material may break off or the local thrombus may embolise and travel upstream to the brain. Small vessel disease causes lacunar infarcts, which are small infarcts presumed to occur due to occlusion of single, small perforating arteries that supply the subcortical brain regions [20]. Lacunar infarction is associated with a higher rate of long-term survival compared with the other stroke subtypes [17]. Other events that may cause thrombosis/embolism and subsequent vessel occlusions include traumatic injury (dissection) to the blood vessels of the neck, or disorders of blood clotting.

Although the MCA is the most commonly occluded artery in stroke patients, arterial occlusion can occur in any artery. While occlusion of the MCA territory occurs in approximately 70% of cases [9, 13], it has been estimated that anywhere between 15-45% of patients with ischaemic stroke have occlusion of the internal carotid artery (ICA), either alone or in tandem with MCA occlusion [9, 13, 21-23]. Patient outcome has been linked to the site of occlusion. In particular, patients presenting with acute symptomatic ICA occlusion have a poorer long-term prognosis compared to patients with occlusion of only the MCA [19]. ICA occlusion is an independent predictor of neurological worsening after stroke [24]. Tandem occlusion of the MCA and ICA is also an independent predictor of poor clinical outcome after stroke therapy [25]. The risk of recurrent stroke and mortality is also higher for patients with ICA occlusion than for MCA occlusion [26]. The poorer outcomes for ICA and tandem occlusions are likely due to a failure to
recanalize the occluded artery and restore perfusion to the ischaemic tissue. Indeed, recanalization of the ICA with the current stroke drug, tissue plasminogen activator (tPA), is far less effective than recanalization of the MCA (13.9% versus 50.8% recanalization, respectively) [9]. This recanalization failure can, in part, be attributed to clot size, clot composition and vessel stenosis. An occlusion of the more proximal ICA requires a larger clot to block the vessel due to increasing vessel diameters as the ICA becomes more proximal. Yet, the larger the clot, the less likely clot lysis will occur [27]. This “clot burden” is also observed in cases of tandem MCA-ICA occlusion where the clot is longer, occluding multiple vessels. Prognosis for tandem occlusions is poor because treatment with the current thrombolytic therapy is often ineffective likely due to larger clots. Additionally, clot composition is related to the source of the thrombosis. For example, clots forming at atherosclerotic lesions are often more platelet-rich, and hence do not respond to fibrinolysis with tPA. The effect of clot composition on thrombolysis will be addressed further in section 1.3.1. It is also possible that an underlying stenosis, caused by an atherosclerotic plaque, affects recanalization. If a vessel is severely stenosed, the chances of sufficient tPA reaching a clot formed distal of the stenosis, is unlikely. This potential contributor to failed recanalization has not been well explored clinically, and will be addressed in Chapter 4. Hence, occlusion location, clot composition and vessel stenosis all contribute to the variable nature of stroke and the effect on patient outcome and treatment success. To understand how treatment is affected, it is necessary to understand the process of clot formation leading to occlusion.

Pathological clots, such as those that cause stroke, form as a consequence of abnormal haemostasis – the natural response to vascular injury. When vascular injury occurs, haemostasis triggers a sequence of events to form a stable haemostatic plug to repair the vessel. These events begin with the activation and aggregation of platelets [28] (Figure 1-2), and lead to a complex series of enzymatic reactions, known as the clotting cascade, leading to stable thrombus formation (Figure 1-3). These processes are summarised in Figures 1-2 and 1-3, but have been reviewed in detail elsewhere [29, 30]. For the purposes of this thesis, it is important to highlight that the clotting cascade leads to the conversion of fibrinogen to fibrin, forming a strong fibrin mesh and a stable thrombus. The development of therapeutic strategies for stroke have focused on various aspects of the thrombotic process. Prevention of thrombosis (primary and secondary) can be achieved by inhibiting platelet interactions, or elements of the thrombotic pathway to prevent clot formation. Acute stroke therapy, designed to dissolve the occluding thrombus and restore blood flow to the brain, employs the mechanism of fibrinolysis to dissolve the fibrin strands that hold the thrombus together.
Figure 1-2. Platelet aggregation in response to vascular injury. In response to vascular injury, platelets from flowing blood are captured on the injured vessel wall. This occurs through the specific interaction of the platelet glycoprotein Ib-V-IX complex with collagen-bound von Willebrand factor that is exposed on the subendothelium. Stable platelet adhesion occurs through the binding of platelet receptors to collagen and fibronectin. Adhered platelets undergo biochemical changes that lead to the high affinity interaction with adhesion proteins (including von Willebrand factor, fibrinogen and fibronectin) that cause platelets to form stable aggregates with other activated platelets, promoting thrombus growth. Reproduced with permission from Jackson 2011 Nat Med [28].
The clotting cascade occurs by three pathways and is initiated in response to platelet activation. The intrinsic pathway utilizes factors present within the blood, while the extrinsic pathway is initiated by tissue factor from the subendothelial matrix. These pathways are essentially independent of each other and involve the activation of a cascade of coagulation factors (named by roman numerals) culminating in the activation of factor X and the start of the common pathway. The common pathway culminates in the conversion of fibrinogen to fibrin by thrombin to create a fibrin mesh thrombus. Figure created by A. Tomkins, adapted from information from Martini 2004 Fundamentals of anatomy and physiology [30]
1.1.2. **CURRENT TREATMENT OPTIONS**

There are four primary areas of stroke treatment: prevention of initial stroke (primary stroke prevention), acute stroke treatment, prevention of recurrent stroke (secondary stroke prevention), and rehabilitation. While all areas of stroke therapy are important for patient outcome, I will only briefly discuss primary and secondary stroke prevention and rehabilitation, since the major focus of this thesis is on acute stroke therapies.

Primary and secondary stroke prevention involves modifying stroke risk factors. Stroke risk factors can be divided into two categories: non-modifiable (including age, gender, race and ethnicity) or modifiable. Modifiable risk factors include hypertension, diabetes, smoking, high cholesterol, atrial fibrillation, diet, physical inactivity and obesity. These are called “modifiable” as they can be controlled and limited with the taking of medications (e.g. antihypertensives, anti-platelets, anti-coagulants) or a change in lifestyle habits and are therefore the targets of primary and secondary prevention. Hypertension is the most important risk factor for ischaemic stroke and the efficacy of antihypertensive treatment is clinically well-established. Stroke incidence is reduced by up to 40% in patients using anti-hypertensive treatment [31]. Following an ischaemic event, there is an increased risk of recurrent stroke. Secondary prevention relies on the same principles of primary stroke prevention to modify underlying stroke risk factors, with the use of blood pressure control, antiplatelet agents, lipid lowering agents, anticoagulation for atrial fibrillation, and carotid endarterectomy and/or stenting for carotid stenosis. Studies have demonstrated that these interventions can reduce the risk of recurrent stroke by up to 90% [32].

Rehabilitation strategies generally involve task-oriented training and there is some evidence to show that this assists in functional recovery after stroke [33]. Impairments following MCA and/or carotid artery occlusion can be both motor and/or sensory and may restrict function in muscle movement or mobility, or affect speech and language, vision, sensation and cognition. Rehabilitation aims to recover these functional and sensory impairments and patients work with trained therapists (e.g. physiotherapists, speech therapists, etc.) to achieve this goal.

Acute stroke therapy aims to restore blood flow to the ischaemic tissue (reperfusion) by the rapid reopening of occluded vessels (recanalization). Restoration of perfusion can potentially salvage the ischaemic tissue that has not yet infarcted, thereby preventing expansion of the lesion of neuronal cell death. There is a large amount of evidence to confirm that early restoration of flow results in smaller infarct sizes and improved clinical outcome, highlighting the importance of this approach [9, 11, 12, 34, 35]. Recanalization can be achieved in two ways: thrombolytic drugs or endovascular interventional approaches. To date, the only approved thrombolytic therapy for stroke is intravenous tPA. Thrombolysis and enhanced thrombolytic therapies are the major focus of this project, and will be addressed in more detail in section 1.1.2.1. and 1.3. Endovascular approaches to revascularisation utilize intraluminal embolus retrieval devices. Until recently, the
benefits of such approaches were controversial [36]. However, 5 recent clinical trials incorporating new generation devices and more selective patient inclusion, revealed significant benefit of intervention to standard treatment alone [37-41]. While tPA still remains the current standard therapy for recanalization, these trials reveal that alternate revascularisation approaches are achievable and beneficial.

There are several outcomes assessed with regards to effectiveness of stroke therapies, primarily patient outcome and vessel recanalization. The ultimate goal of all therapies is to improve patient outcome, and in the setting of stroke this involves reducing impairment and disability and restoring functional and cognitive ability. These parameters are measured by scoring systems including, but not limited to, the National Institute of Health Stroke Score (NIHSS) to measure neurological deficits and the modified Rankin Scale (mRS) to measure long-term levels of disability [42]. These scoring scales give indications of either neurological deficits on presentation with stroke and post-treatment (NIHSS) or overall function and disability during short- and long-term follow-up (mRS). Successful recanalization provides evidence that the benefits of therapy are related to the primary mechanism of the thrombolytic, i.e. the lysis of the occluding thrombus.

1.1.2.1. Thrombolysis and tissue plasminogen activator

The rationale for tPA thrombolysis in acute stroke is based on the primary cause of most ischaemic strokes: the occluding thrombus. The occluding thrombus is composed of a fibrin mesh and tPA initiates fibrinolysis by the activation of plasminogen, a single-chain glycoprotein, to plasmin which cleaves fibrinogen and fibrin (Figure 1-4). tPA is a serine protease released endogenously in response to thrombosis. It is primarily sourced from endothelial cells in the intravascular space. Clots are composed of fibrin monomers that are cross-linked through lysine side chains. tPA binds to the lysine side chains of the cross-linked fibrin monomers of a thrombus, activating plasminogen and catalysing the conversion of plasminogen to plasmin – the major enzyme for clot lysis. This activation only occurs in the region of the thrombus, thereby minimising activation of circulating plasminogen and non-specific fibrinolysis. Plasmin splits fibrinogen and fibrin into fibrin degradation products, resulting in clot lysis [43]. During physiologic fibrinolysis, circulating plasmin is inhibited by α2-antiplasmin (Figure 1-4). This restricts fibrinolysis by plasmin to the thrombus because the lysine binding sites of plasminogen (and therefore plasmin) bind to the same fibrin binding sites as α2-antiplasmin. Hence, fibrin-bound plasmin is relatively protected from α2-antiplasmin inhibition [44]. Thrombolytic agents such as urokinase or streptokinase activate both circulating and fibrin-bound plasminogen, leading to high circulating levels of plasmin and risks of non-specific bleeding. Alteplase, a manufactured recombinant form of endogenous tPA and the current stroke therapy, is more fibrin-selective, acquiring a high affinity for plasminogen after binding to fibrin. This leads to more
localized fibrinolysis than other thrombolytic drugs such as urokinase and streptokinase. Any further reference to tPA in this thesis refers to recombinant tPA (alteplase) unless otherwise stated.

tPA has been approved for use out to 4.5 hours after stroke onset at a dose of 0.9 mg/kg. The first trial to clearly show a benefit of therapy with tPA treatment was the NINDS\textsuperscript{1} trial in which tPA was delivered at 0.9 mg/kg within 3 hours of symptom onset \cite{45}. It is from this trial that the clinical dose of tPA was chosen and the original treatment window of 3 hours was approved. Compared to its use for thrombolysis in myocardial infarction, tPA is more than 3 times as effective in acute ischaemic stroke at reducing death and disability \cite{46}. For every 1000 patients treated with tPA, an additional 125 stroke patients can avoid death and disability, compared to 39 for myocardial infarction. The ECASS III\textsuperscript{2} trial \cite{47} went on to demonstrate that tPA was effective out to 4.5 hours after stroke, prompting a reassessment of the time to treatment window. This study showed a 7% absolute improvement in the primary outcome of disability at 3 months as well as no increase in the rates of symptomatic intracerebral haemorrhage (sICH) or mortality beyond 3 hours as compared with the NINDS trial. This trial has led to the approved time for

\textbf{Figure 1-4. Thrombolysis with tissue plasminogen activator (tPA).} When a clot forms, plasminogen is incorporated into the clot where it remains inactive. tPA catalyses the conversion of plasminogen to plasmin which cleaves fibrin molecules, dissolving the clot. PAI-1 is released by platelets to inhibit the actions of tPA. Circulating α\textsubscript{2}-antiplasmin inhibits unbound plasmin, restricting fibrinolysis to the fibrin thrombus. Figure created by A. Tomkins.

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\textsuperscript{1} National Institute of Neurological Disorders and Stroke rt-PA Stroke Study
\textsuperscript{2} European Cooperative Acute Stroke Study III
treatment to be extended to 4.5 hours after stroke onset in most countries. There is also evidence that tPA may be beneficial in some patients out to 6 hours post stroke onset [48] however the efficacy of tPA to prevent brain damage decreases with time and the earlier the intervention, the more likelihood of good clinical outcome [47, 49-51].

Functional and cognitive recovery of patients (clinical outcome) after stroke is the most important outcome of stroke therapy and has been clearly demonstrated to be improved with recanalization therapy [45, 47, 52, 53]. The original tPA trial revealed that patients treated with tPA were 30% more likely to have no or minimal disability at 3 months [45]. Patients with stable early recanalization have a 50% chance of favourable outcome at 3 months compared to 33% chance for those with early reocclusion [54]. Those patients with complete recanalization before the end of 1 hour tPA infusion are 3.5 times more likely to achieve favourable outcome at 3 months [55]. It is clear that early and complete recanalization is a predictor of good outcome and this has been confirmed with meta-analysis [9].

A major concern of tPA is intracerebral bleeding and increased mortality risks and these outcomes are therefore important safety measures and/or end points for clinical trials. Indeed thrombolytic trials have been terminated due to increased rates of sICH [56]. tPA has been shown to increase cerebral haemorrhage, oedema, cell death and blood brain barrier disruption, but treatment with intravenous tPA is 10 times more likely to help than to harm eligible patients with acute ischaemic stroke [57]. The NINDS trial revealed a 6.4% rate of sICH within 36 hours in patients treated with tPA, compared to 0.6% in the placebo control group \(p<0.001\) [45]. However, subsequent trials have reported lower rates of sICH (e.g. ECASS III reports 2.4% in the treatment group [47]). These differences are likely due to the definitions of sICH. NINDS reported any sICH as a deterioration of neurological status accompanying any blood on scans. Yet, this does not distinguish from those patients with large stroke and deterioration due to oedema and any capillary extravasation even within the infarct core. Subsequent trials and improved imaging technologies have been able to better classify sICH. Even so, NINDS determined that the absolute risk of haemorrhage was outweighed by the overall improvement in clinical outcome at 3 months. This study also revealed a non-significant reduction in mortality at 3 months from placebo control (17 versus 21%, respectively; \(p=0.3\)). A meta-analysis [53] revealed that although the number of deaths within 7 days was increased in tPA treated patients, by the final follow-up, mortality was no longer significantly different from controls. Although concerns of haemorrhage are ever present for thrombolytic therapy, the overall benefit outweighs the risk.

1.1.2.2. Recanalization and reperfusion

The primary mechanism of thrombolytic therapy is to reopen the occluded arteries, yet the terminology to describe vascular reopening has become a little confusing. “Recanalization” and “reperfusion” have been used in both clinical and preclinical studies to describe revascularisation
events and are often used interchangeably [58, 59]. Yet, there are two distinct events that occur in revascularisation: recanalization of the primary occlusive site, and reperfusion of the distal ischaemic tissue [58, 59]. Distinguishing between these events may be clinically important because, for example, recanalization of the major artery may be achieved but distal clot embolization or microvascular thrombosis may limit reperfusion of the ischaemic tissue ultimately affecting neurological recovery. Conversely, reperfusion may be achieved despite lack of recanalization due to the recruitment of collateral vessels leading to restoration of flow [11, 60].

Although the primary mechanism of stroke therapies is early recanalization, there is growing evidence that recanalization of the primary occluded artery does not always constitute tissue reperfusion [11, 59, 61]. Migration of emboli, secondary thrombosis or swelling of the intima may cause a “no reflow phenomenon”, and although the large vessels recanalize, microvessels may remain occluded (Figure 1-5) [62, 63]. This “no-reflow phenomenon” was first described for stroke by Ames et al [64] and results from fibrin deposition in areas of stagnant flow [65, 66]. Multiple closely related factors contribute to the “no reflow phenomenon”, including oedema,

Figure 1-5. Microvascular occlusion. Modified Carstair’s Stain for fibrin and platelets of rat cerebral tissue after embolic occlusion the middle cerebral artery (preliminary data, unpublished). Fibrin and platelet deposits are observed in the microvessels (red arrowheads) of the ischaemic tissue. Platelets are stained blue (blue arrow, insert), while fibrin is stained pink/red (pink arrow, insert). Ischaemic tissue is observed to the left of the image as the area of paler staining and damaged tissue, compared with the intact tissue of the non-ischaemic area in the top right of the image.
microvascular damage and microvascular obstruction [11]. Additionally, the primary clot may fragment and migrate to occlude distal cerebral arterial branches [67]. There is now growing evidence that reperfusion may be a better indicator of good outcome and reduced infarct size in patients with a salvageable penumbra rather than recanalization [11, 61, 68].

1.1.2.3. The need for new therapies for acute stroke

Despite its effectiveness, tPA has its limitations with recanalization achieved in less than 50% of patients with MCA occlusion treated with tPA [9]. For ICA occlusion, these numbers are even less, with only 10-30% achieving complete recanalization [21, 69]. The National Stroke Audit (2013) revealed that in Australia, only 7% of all patients with ischaemic stroke received thrombolytic treatment [1]. For patients arriving within 3 hours of symptom onset, 15% received tPA and of those arriving within 4.5 hours, 13% received treatment. Although these numbers have increased since the 2009 audit (3% treated overall, 10% within 3 hours of symptom onset [70]), it is still a very small proportion of all patients presenting with ischaemic stroke. However, the implementation of stroke units, ambulance triage, and public awareness, has increased the numbers treated in some centres to as much as 30%. The biggest factors affecting the total numbers of patients receiving treatment are the time window of treatment (within 4.5 hours of stroke onset) and the exclusion criteria of contraindications that may cause increased risk of bleeding. Yet there is evidence to suggest that these contraindications should not always exclude patients from therapy [71, 72]. Additionally, of those treated with tPA, less than half will successfully recanalize [9]. Since recanalization and reperfusion are very strong predictors of good outcome, there is need to address the reasons for failed recanalization.

Exact reasons for failed thrombolysis in all cases are not clearly understood, but there is evidence that the site of occlusion, clot composition, and vessel stenosis correlate with tPA efficacy. Occlusions of the MCA are more susceptible to thrombolytic lysis, while ICA occlusions have very low recanalization rates (as discussed in section 1.1.1.2). Occlusions of the MCA M2 segment, M1 segment or ICA have recanalization rates of 44%, 22% and <10-14% respectively [9, 55]. Patients with persisting occlusions of the MCA or terminal ICA (i.e. no recanalization) have only a 10% chance of complete recovery at 3 months [54]. The thrombi causing stroke are also highly variable with regards to their composition [16, 73, 74]. In particular, differing proportions of platelets, fibrin and erythrocytes affect thrombolytic efficacy. This will be discussed further in section 1.3.1. Additionally, narrowing of the vessel caused by atherosclerotic plaques leads to changes in blood flow and may limit penetration of tPA into the occluding thrombus. Again, this has been discussed in section 1.1.1.2. Individually, or in combination, these factors may affect thrombolysis and the number of patients responding to therapy.

There are two primary areas for improving on current therapies: improved therapies for those patients who are ineligible for thrombolysis with tPA, or enhancing the efficacy of current
thrombolytic therapy, to which more than half the patients treated do not respond. This project will investigate ways of enhancing tPA efficacy by application of ultrasound in a process called sonothrombolysis.

1.2. SONOTHROMBOLYSIS

Sonothrombolysis is the application of ultrasound to an area of thrombosis to enhance clot lysis and has been used as a potential enhancer of thrombolytic therapy for stroke. The concept of sonothrombolysis dates back to the 1970’s when ultrasound was applied to the thrombosed iliofemoral arteries of dogs [75, 76]. The first evidence of sonothrombolysis for stroke was in the late 1990’s when application of ultrasound through skull bone in vitro promoted clot lysis [77, 78]. Since then, a number of small scale clinical trials have demonstrated efficacy of ultrasound at enhancing tPA recanalization and clinical outcome [79-84]. It has also been demonstrated that the added infusion of microbubbles can further enhance the effects of sonothrombolysis [85-89]. In general, sonothrombolysis refers to the application of ultrasound in conjunction with thrombolytic therapy, such as tPA, while sonolysis refers to clot lysis by ultrasound application alone [90-92]. Although these terms have been used interchangeably in the literature, these will be the definitions used for this thesis.

1.2.1. MECHANISMS OF ACTION OF SONOTHROMBOLYSIS

Despite much research, the mechanisms of sonothrombolysis for stroke are still poorly understood. The studies of sonothrombolysis mechanisms of action, as outline below, have all been performed in vitro and few in the presence of human skull bone. Additionally, while these mechanisms seem the most likely to cause sonothrombolysis, it should be noted that no mechanistic studies have trialled the exact ultrasound parameters used in clinical stroke trials (i.e. 2-MHz, 720 mW/cm²). It is known that ultrasound affects biological tissue by three principle mechanisms: cavitation, radiation forces, and heat generation. It is postulated that the mechanical effects of ultrasound (cavitation and radiation forces) primarily cause accelerated thrombolysis, while heat generation is an unwanted side effect of low frequency ultrasound. There are a range of mechanisms by which ultrasound is believed to enhance tPA clot lysis, including acoustic cavitation, microstreaming, and alteration of the fibrin structure leading to increased flow through the thrombus and increased tPA uptake and binding [93-100]. These mechanisms act in combination with each other and can promote thrombolysis with and without additional thrombolytic therapy (sonothrombolysis or sonolysis).

Acoustic cavitation is a broad term that describes the action of bubbles in a sonic field [101]. When ultrasound of sufficient amplitude is applied to fluid, such as blood, it causes the partly dissolved gases to form small bubbles. These microbubbles vibrate in response to the absorption of the ultrasonic energy. Stable cavitation refers to the controlled oscillation of microbubbles...
within the ultrasonic field, and can cause disruption of the fibrin mesh [94]. At higher ultrasound energies, microbubbles may undergo inertial cavitation, whereby the bubbles rapidly and transiently expand followed by a violent collapse. Inertial cavitation can result in mechanical disruption of the clot as a result of this violent collapse [96]. The higher energies needed for inertial cavitation are not always achieved in stroke sonothrombolysis due to the large attenuation of energy when ultrasound is passed through the human skull bone [102]. It is therefore believed that stable cavitation plays the major role in clot dissolution for stroke sonothrombolysis, promoting additional mechanisms such as microstreaming [94] (Figure 1-6).

Microstreaming is the promotion of fluid movement around a thrombus [97]. Following thrombotic occlusion of a vessel, blood flow around the thrombus becomes stagnant consequently slowing or preventing tPA from reaching the target thrombus. Ultrasound induction of fluid motion around the thrombus aids in the mixing of tPA into areas of otherwise stagnant flow. This increases the concentration of tPA that is in contact with the thrombus and increases the likelihood of thrombolysis (Figure 1-6). Cavitation-induced microstreaming can cause mechanical
disruption to the outer surface of the thrombus due to the cavitation-induced growth and collapse of the microbubbles [98]. In this way, thrombolysis due to cavitation-induced microstreaming does not require the activation of fibrinolytic pathways, but is enhanced by the presence of tPA.

Ultrasound reversibly alters the fibrin structure of the clot, affecting flow and tPA binding [93]. Reversible disaggregation of fibrin fibres alters flow resistance through the clot. Under constant pressure, flow is accelerated in an ultrasonic field and it has been suggested that the fibrin structure is altered to reduce flow resistance [99]. Increases in flow through the clot are observed with increasing ultrasound power intensities (1-2.3 W/cm²) [99], yet such an effect is likely to be limited in stroke sonothrombolysis due to the limits of FDA-approval of transcranial ultrasound power output (<750 mW/cm²) and the power attenuation of up to 90% through the skull bone [102-105]. As flow through the clot increases, so too does transport of tPA. Additionally, changes in clot structure cause the creation of more tPA binding sites as fibrin fibres become more accessible [99, 100]. In vitro studies have indicated increased rates of tPA uptake and binding in ultrasonic fields as well as deeper penetration of tPA into the thrombus [95, 100]. Ultrasound does not affect the functional activity or the structural integrity of tPA [106], and so the effects of ultrasound on tPA uptake and binding within the thrombus are related to the structural changes of the thrombus and the mechanical effects of the ultrasound (Figure 1-6). Although direct investigation of endogenous plasminogen binding in the presence of ultrasound has not been published, it is conceivable to expect that the structural changes of the fibrin clot that promotes tPA uptake and the generation of more tPA binding sites, is also true for circulating plasminogen.

Plasminogen and tPA bind to fibrin via their lysine-binding sites and the complex formed between plasminogen, tPA and fibrin generates plasmin that degrades fibrin. As fibrin is degraded, new C-terminal lysines are created, providing more binding sites for circulating plasminogen and tPA and creating a positive feedback mechanism for fibrinolysis. Additionally, the interaction between α2-antiplasmin (the major plasmin inhibitor (Figure 1-4)) and plasmin relies partly on the lysine-binding sites of plasmin. Hence, fibrin-bound plasmin is protected from inhibition [107]. Therefore, the known effect of ultrasound on the clot structure and tPA binding, likely extends to the increased binding of plasminogen that, in conjunction with increased tPA binding, enhances fibrin-bound plasmin generation that is protected from α2-antiplasmin. These effects could contribute to the enhanced fibrinolysis observed in the presence of ultrasound.

Systemically infused artificial microbubbles are used as contrast agents for diagnostic purposes, and there is some evidence that they can enhance the effects of sonothrombolysis for stroke treatment [85, 88, 89, 108]. When exposed to ultrasound, these micrometre-sized gaseous bubbles expand and produce stable cavitation with stronger reflected echoes and are therefore commonly used as contrast agents to enhance ultrasound signals for diagnostics. Enhanced sonothrombolysis with microbubbles is believed to be caused by the cavitation effects of the microbubbles [109].
It is hypothesised that administration of microbubbles dramatically lowers the threshold for ultrasound-induced cavitation due to the presence of the pre-existing microbubbles. The required energies for bubble production in situ, cannot always be achieved for stroke sonothrombolysis due to attenuation of energy through the skull. Yet, when microbubbles are infused systemically, the energy requirements for producing stable cavitation are lowered and the enhancement of thrombolysis by ultrasound is increased [98].

1.2.2. SONOTHROMBOLYSIS FOR STROKE

Initial clinical trials of sonothrombolysis for stroke have generated promising results of significantly increased recanalization rates and clinical recovery compared to tPA treatment alone. The first clinical report of sonothrombolysis for stroke was a serendipitous observation of increased recanalization rates and dramatic clinical recovery in patients treated with tPA and continuously monitored with diagnostic (2-MHz) transcranial Doppler (TCD) [79]. Recanalization rates were higher than those expected from studies of tPA alone, leading to the first randomised clinical trial of sonothrombolysis for stroke [80]. The CLOTBUST\textsuperscript{1} trial was the first multicentre randomised clinical trial of sonothrombolysis [80]. It was a phase 2 trial with a predetermined sample size (n=63/group) and a primary endpoint of complete recanalization as assessed by TCD, or dramatic clinical recovery (improvement by $\geq$10 NIHSS points) within 2 hours of tPA bolus. Secondary endpoints were recovery at 24 hours, and favourable outcome or death at 3 months. All patients had acute ischaemic stroke of the MCA and were treated with intravenous tPA within 3 hours of symptom onset with either continuous 2-MHz TCD ultrasound or placebo monitoring. The primary endpoints were achieved by 49% of the target group compared with 30% of control ($p=0.03$). Although this study was not designed to investigate long-term outcome, there was a trend towards better favourable outcomes in the target group (42% target, 29% control; $p=0.20$). The initial “serendipitous” study and the Phase 2 clinical trial (CLOTBUST) indicate the positive effects of sonothrombolysis for acute stroke treatment.

There have now been a variety of small clinical trials of sonothrombolysis for stroke. These trials have investigated sonothrombolysis with TCD [80] and transcranial color-coded duplex (TCCD) ultrasound systems [81, 82] as well as microbubble-enhanced sonothrombolysis [87-89]. Meta-analyses have suggested that sonothrombolysis with TCD or TCCD, with or without microbubbles is safe and effective for acute ischaemic stroke [83, 84]. The analyses revealed an almost 3-fold increase in the likelihood of complete recanalization at 2 hours post-tPA onset with sonothrombolysis compared with tPA therapy alone [83, 84]. Complete recanalization rates for tPA+TCD were 37.2%, tPA+TCCD 26.9%, and tPA alone 17.2% [84]. Administration of microbubbles is also associated with a higher likelihood of complete recanalization compared to

\textsuperscript{1} Combined Lysis Of Thrombus in Brain ischaemia using transcranial Ultrasound and Systemic tPA
tPA alone (53.6% versus 36.6%) [84]. At 24 hours, sonothrombolysis was still associated with a >3-fold increased likelihood of complete recanalization at 24 hours, however only 4 of 10 studies reported this outcome [83]. The likelihood of functional independence at 3 months was 2-fold higher with sonothrombolysis (mRS 0-1 [84] or 0-2 [83]), with rates of functional independence at 40.7% for tPA+TCD studies compared to 20.6% for tPA alone [84]. TCCD, when analysed alone, did not reach significantly different increases of complete recanalization rates (26.9%) or functional independence (22.2%) compared to tPA alone (17.2% recanalization and 20.6% functional independence). This was most likely due to the small group sizes in these studies (n=7-11 patients in treatment groups in studies insonating with TCCD versus n=12-63 patients in treatment groups in studies insonating with TCD). While meta-analyses suggest patient improvement with sonothrombolysis over tPA alone, the conclusions drawn are limited to the number and quality of the studies included. These studies were small, many non-randomised and some using historical tPA control data. Larger scale, multicentre and randomised clinical trials are required to confirm sonothrombolytic efficacy as a treatment for acute ischaemic stroke. One such trial, CLOTBUST-ER⁴, a multi-centre, randomised, Phase III trial with a predetermined sample size of 824 patients, has been recruiting during the course of this thesis and will be discussed in relation to my studies in Chapter 6.

Despite some successful studies with regards to improving recanalization and functional outcome, there are also still safety concerns of sonothrombolysis therapy with regards to increased rates of haemorrhage and mortality. These are particularly highlighted by the TRUMBI³ trial of low frequency ultrasound [110], which was terminated early due to high rates of sICH. Initially, TRUMBI found that very low kHz (<40-kHz) produced intolerable tinnitus and therefore this frequency was withdrawn from clinical testing [103]. The subsequent mid-kHz frequency study used pulsed-ultrasound at a frequency of 300-kHz continued for 30 minutes post-tPA infusion (total insonation time = 90 minutes). However, these parameters also produced deleterious effects and TRUMBI was terminated after enrolment of only 26 patients. There was no evidence of efficacy of early recanalization, no effect on clinical outcomes at 3 months, and a 36% rate of sICH in the target group was observed. High rates of atypical haemorrhages were found in ventricular or subarachnoid spaces, in remote parenchymal locations distant from the infarct core, and in areas that were unaffected by ischaemia. Mechanisms of this high rate of haemorrhage are unclear. It is possible that the production of standing waves due to reflection within the skull, as well as the mechanical action of ultrasound on small vessels and endothelial disruption contributed to these deleterious outcomes [104]. Abnormal permeability of the blood-brain barrier

⁴ Combined Lysis of Thrombus With Ultrasound and Systemic Tissue Plasminogen Activator (tPA) for Emergent Revascularization in Acute Ischemic Stroke
³ TRanscranial low-frequency Ultrasound-Mediated thrombolysis in Brain Ischemia
is another potential cause of haemorrhagic transformation [111, 112]. TRUMBI clearly highlights the safety concerns of sonothrombolysis, but also the necessity to better understand the mechanisms of sonothrombolysis as an acute stroke therapy.

Despite the concerns of TRUMBI, clinical trials of sonothrombolysis at diagnostic ultrasound frequencies (~2-MHz) generally report sICH rates similar to tPA alone. This indicates that ultrasound at higher frequencies does not increase haemorrhage risk and that the likely cause of increased haemorrhage in TRUMBI was the low frequency insonation. CLOTBUST reported sICH rates of 4.8% for both tPA and ultrasound+tPA groups [80]. The meta-analysis by Tsivgoulis et al reported overall sICH rates of 2.9% for tPA treated patients and 3.8% for tPA+TCD treated [84]. Higher rates of sICH were reported with TCCD insonation (11.1%), but group sizes were small (n=7-11) and no significant difference was determined from tPA alone. Rates of complete recanalization and functional independence, while improved over tPA therapy alone, are still <50% of patients [84]. As mentioned, these clinical trials have been small and hence provide essentially “pilot” data regarding this potential therapy. Choice of the optimal parameters for maximum efficacy of sonothrombolysis for stroke therapy while limiting the safety concerns, are still a long way from being completely understood.

The importance of preclinical testing of any new stroke therapy is clearly highlighted by the results of TRUMBI. The design of the TRUMBI study originated from *in vitro* evidence that low frequencies are up to 50% more effective at enhancing thrombolysis than diagnostic frequencies [77, 78, 113, 114]. Additionally, ultrasound applied through temporal bone at diagnostic frequencies (2 to 4-MHz) in an *in vitro* model results in lost energy and subsequent failure of clot lysis [105]. The only animal model of low-frequency ultrasound cited by TRUMBI demonstrated reduced infarct volumes in a rat embolic model when treated with sonothrombolysis compared to no therapy or tPA alone [56], and similar rates of haemorrhage in the ultrasound group compared to the tPA group. Yet, there were a number of limitations to this study. The data analysed was pooled from two experiments at two centres in two different thromboembolic stroke models using different clot types (this variable of embolic models is discussed further in section 1.3.1.). The ultrasound frequency was also substantially lower than that used for TRUMBI (25.57 Hz versus 300-kHz used for TRUMBI). Additionally, it is difficult to interpret the safety outcomes of a rat model when correlated to humans due to differences of skull sizes. This is particularly important given a likely cause of haemorrhage in TRUMBI was from standing waves. Standing waves are generated when ultrasound waves are reflected internally within the skull. These reflections cause interactions of one ultrasound wave with another altering the wave patterns. This interaction is directly related to the physical dimensions of the skull and the frequency of the ultrasound waves. Given the vast difference in skull size between rat and human skull, it is unlikely that such a mechanism of haemorrhage could be predicted in a rat model when using clinical diagnostic
frequencies. Although the in vivo study by Daffertshofer et al [56] suggested a potential for low-frequency ultrasound treatment, subsequent preclinical studies have further highlighted the detrimental effects of low frequency ultrasound for stroke therapy [112, 115]. The failed translation of low frequency sonothrombolysis to the clinic indicates a need for clinical trials to be based on more rigorous preclinical testing with investigation of more outcomes, such as long-term outcome, blood brain barrier integrity and haemorrhage rates. Additional consideration should also be given to the use of different animal species and strains to include larger mammals that may better represent human skull size.

The added infusion of microbubbles during sonothrombolysis therapy has been suggested to further improve recanalization and clinical outcomes in pilot clinical trials. In a study by Molina et al [88], complete recanalization rates at 2 hours post-sonothrombolysis were significantly increased when galactose-based microbubbles (Levovist) were infused (54.5% complete recanalization after sonothrombolysis with microbubbles versus 40.8% and 23.9% for sonothrombolysis without microbubbles and tPA alone, respectively; \(p=0.038\)). When compared to historical controls, Alexandrov et al [85] reported a similar improvement of recanalization with infusion of perflutren-lipid microbubbles, and Perren et al [89] reported recanalization improvement with infusion of sulphur hexafluoride microbubbles (SonoVue). Yet, despite improved recanalization rates, improvements of clinical outcome over standard tPA treatment remain to be confirmed. While most studies report trends of improved clinical outcome, all remain non-significant [85, 87-89]. This is largely because clinical scores were secondary outcomes of the studies and because group sizes were small. The largest study had 36-38 patients per treatment group [88], while all other trials had <15 patients per group [85, 87, 89]. Molina et al [88] report the strongest trend towards improved clinical outcome in sonothrombolysis with microbubble groups compared to sonothrombolysis without microbubbles and tPA alone. A >4 point NIHSS improvement at 24 hours was observed in 55% of the sonothrombolysis with microbubbles group versus 41% and 31% in the sonothrombolysis without microbubbles and tPA alone groups, respectively (\(p=0.065\)). Three month mRS≤2 was observed in 56% of the sonothrombolysis with microbubbles group versus 47% and 32% in the sonothrombolysis without microbubbles and tPA alone groups (\(p=0.073\)). Limiting the degree of impairment and disability after stroke is the ultimate goal of any improved therapy. Hence, a confirmed benefit over tPA alone is still needed to justify sonothrombolysis with microbubbles for clinical use. The trends reported are suggestive of a potential benefit to this approach, but much is still unknown regarding microbubble-enhanced sonothrombolysis, including optimal microbubble formulation and dose for maximum efficacy and safety. It is not known if recanalization rates are related to the microbubble formulation as no direct comparisons of microbubble formulations for stroke sonothrombolysis have yet been performed. Sonothrombolysis with galactose-based, perflutren lipid, and sulphur hexafluoride
Microbubbles have all been reported to improve recanalization rates in clinical stroke. However, Larrue et al [86] reported no improvement over tPA alone for galactose-based microbubbles and this trial was terminated early. Additionally, doses of microbubbles have only been directly compared in one study. The TUCSON\textsuperscript{6} trial involved testing of two dose tiers of a perflutren-lipid microbubble formulation (MRX-801). Interestingly, the lower dose resulted in higher sustained recanalization and clinical recovery rates than both high dose MRX-801+ultrasound+tPA and tPA-only [87]. TUCSON was also terminated early due to high rates of sICH in the high dose tier (3/11 sICH, compared to 0/24 for all remaining patients). Whether the higher microbubble dose was directly related to the increased rates of sICH is not completely clear, but this study highlighted both the need for microbubble dose responses and caution for potential increases to sICH rates. Meta-analyses have concluded that microbubble-enhanced sonothrombolysis is both safe and effective for acute stroke therapy [83, 84]. Yet, despite positive data regarding recanalization efficacy of sonothrombolysis with or without microbubbles for stroke, there is still much to be understood regarding optimal parameters for safe and effective microbubble sonothrombolysis for stroke.

1.2.3. SONOTHROMBOLYSIS OF THE MICROVASCULATURE

Following recanalization of major cerebral vessels in stroke, the microvasculature may remain occluded resulting in areas of hypoperfusion. This phenomenon, known as the “no-reflow phenomenon”, results from fibrin and platelet deposition in areas of stagnant flow and is currently not directly treated in stroke patients (this has been discussed in section 1.1.2.2). Recent evidence has revealed that reperfusion is a better predictor of good patient outcome over large vessel recanalization alone [11, 61, 68], highlighting the need to consider this outcome when treating stroke. Preclinical studies demonstrating the possible efficacy of microbubble-enhanced sonothrombolysis to improve microvascular patency are limited and this idea is yet to be tested clinically. Fatar et al investigated 2-MHz ultrasound and SonoVue microbubbles in a rat model of permanent MCAo to evaluate possible adverse bio-effects of microbubble-enhanced sonothrombolysis [116]. They observed no deleterious effects but found that the infarct sizes were unexpectedly smaller in treated animals despite no recanalization of the MCA. This study suggested the potential beneficial effects of ultrasound on the microcirculation and correlates with clinical reports of reduced infarct when reperfusion is achieved [11]. Nedelmann et al investigated sonothrombolysis of the microvasculature in a rat model of cerebral hypoperfusion following large vessel recanalization [117]. Using micro- and nano-CT technology, it was demonstrated that sonothrombolysis with SonoVue microbubbles completely reversed the microcirculatory impairment caused by large vessel occlusion and the resultant stagnant flow. These preclinical studies of this phenomenon suggest the potential for microbubble-enhanced sonothrombolysis to

\textsuperscript{6} Transcranial Ultrasound in Clinical SONothrombolysis
improve microvascular circulation after ischaemic stroke. However, these were small studies (n=3 per group for Nedelmann et al) and confirmation of this effect is required both in larger preclinical studies and clinically. With further testing of the cerebral reperfusion efficacy of microbubble-enhanced sonothrombolysis, and the need for enhancing clinical reperfusion in combination with large vessel recanalization, sonothrombolysis of the microvasculature could become a potential combinatorial treatment to recanalize both large and small vessels in patients with stroke.

1.2.4. SONOTHROMBOLYSIS OF CAROTID ARTERY OCCLUSION

To date, direct investigation of sonothrombolysis for carotid artery occlusions has not been studied clinically. Some clinical sonothrombolysis trials have reported intracranial ICA occlusion within cohorts, but no study of extracranial carotid sonothrombolysis has been made. In clinical trials of stroke sonothrombolysis, occlusion of the ICA are either not reported [81, 85, 110], or were allocated equally between treatment groups [82, 88] and referred to intracranial, distal ICA occlusion. Equal distribution between groups is because occlusion of the carotid artery is a known variable to negatively affect recanalization rates. The effect of sonothrombolysis of the intracranial carotid occlusions versus the MCA occlusions was not considered in these studies. Two sonothrombolysis trials have made mention of the response of carotid occlusion to sonothrombolysis. In the CLOTBUST trial, tandem MCA and distal ICA obstruction was observed in 27% of the control group and 38% of the ultrasound group. Complete recanalization of the MCA was reported as less likely among patients who had ipsilateral obstruction of the ICA [80]. Perren et al reported 3 patients (of 26) with concomitant distal ICA and proximal MCA occlusion treated with either sonothrombolysis or sonothrombolysis with microbubbles [89]. Of these patients, 2 had no recanalization at 60 minutes, and 1 had only partial recanalization. Yet for intracranial occlusions, limitations of sonothrombolysis apply relating to the presence of the skull. Ultrasound power is substantially attenuated through the skull bone [104, 105], but increasing ultrasound power is associated with greater clot lysis [118]. Additionally, low frequency ultrasound can enhance clot lysis [77, 118], but increases the rates of sICH due to the propagation of standing waves in the skull [104, 110]. Finally, a proportion of patients do not have an appropriate “window” of the temporal bone – where the bone is too thick – to even allow sonothrombolysis [119]. While these factors that can limit intracranial sonothrombolysis efficacy and safety, they would be negated in an extracranial occlusion. The potential to optimise sonothrombolysis parameters without having to account for the skull, could lead to an effective therapy for extracranial carotid occlusion.
1.3. ANIMAL MODELS FOR PRECLINICAL STROKE RESEARCH

Sonothrombolysis seems a promising approach to improving stroke therapy, but optimal parameters for efficacy and safety still require research. In vitro studies can provide mechanistic insights into the therapy, while in vivo studies utilizing animal models provide combined efficacy and safety data in a whole animal system. Rigorous preclinical testing can ultimately minimise patient risk by determining optimal parameters for efficacy while identifying any potential safety concerns prior to clinical implementation of a treatment. The need for preclinical testing for sonothrombolysis is particularly highlighted by the TRUMBI trial, in which significantly increased rates of sICH were observed and the trial terminated [110]. This trial cited limited preclinical work that would justify their chosen ultrasound parameters. However, it should also be noted that the causes of sICH identified in the TRUMBI trial were likely related to the physical geometry of the skull and wave frequency (i.e. the propagation of standing waves). Such effects would not have been identified in rodent models given the skull size. So, while efficacy of sonothrombolysis may be determined in the commonly used rodent models, true safety investigation likely requires larger animals. For this thesis, I tested sonothrombolysis efficacy, and hence chose to use rodent models. Any future study of safety should also include larger animal models.

There are a large number of animal models of stroke used (Figure 1-7) and the choice of an appropriate model is dependent on the intended outcome of the study [120]. Due to the heterogeneity of human stroke, there is no single model that is able to encompass and mimic all the aspects and variables of human ischaemic stroke. Different occlusive sites will cause differing degrees of ischaemia and infarction, as well as differing effects on functional outcome related to the cerebral territory affected. Thrombolytic efficacy is also affected by the site of occlusion related to the occlusive clot size and/or vessel stenosis, as well as the composition of the occluding clot. In order to benefit the maximal number of patients, all of these variables need to be considered when developing or investigating potential stroke therapies. For studies testing thrombolytic agents and thrombolytic enhancement therapies in which the primary outcome is clot lysis, the model needs to mimic the human condition of occlusion with the presence of life-like clots. For this thesis, I will be testing the thrombolytic potential of sonothrombolysis. I will also be testing sonothrombolysis at different occlusive sites (MCA, microvasculature, carotid occlusion). The vast number of different stroke models have been reviewed elsewhere [120, 121], hence I will review only the models used for this thesis.
Figure 1-7. The most common preclinical models of stroke. A large variety of models are used for preclinical stroke research. The outcomes of the study dictate the chosen model. For permanent occlusions, ligation or clipping of the artery, thread occlusion, or embolism with artificial emboli (e.g. silicone macrospheres) can be used. For testing ischaemia/reperfusion, the thread occlusion model is most commonly used. Testing of thrombolytics requires the presence of an occlusive clot which can be achieved by injecting a preformed embolus into the cerebral vasculature, or by injecting coagulants at the site of the MCA origin, or directly into the distal branches. Other thrombotic models include photothrombosis, electrocoagulation, FeCl₃ or endothelin-1 application to the vascular endothelium to induce thrombosis. Figure created by A. Tomkins
1.3.1. THROMBOEMBOLIC STROKE MODELS
Models of thromboembolic stroke contain a clot positioned within the cerebral vasculature of experimental animals. The most common target of preclinical models is the MCA due to the fact that the majority of ischaemic strokes occur by occlusion of the MCA [13]. Thromboembolic stroke models are performed by either clot formation \textit{ex vivo}, or \textit{in situ}. Clots prepared \textit{ex vivo} are injected by intraluminal catheter via the carotid arteries to the MCA [122, 123] (Figure 1-8). Injection of clots at the carotid bifurcation is commonly performed, but can result in variable occlusions as the clots can travel into the anterior or posterior cerebral artery or other branching arteries of the circle of Willis. Site specific delivery of a thrombus to the origin of the MCA can be achieved with the aid of laser Doppler flowmetry (LDF) and results in more consistent infarction [124]. This technique is described in more detail in section 2.4.2. Clot formation \textit{in situ} can be performed by the direct injection of thrombin in to the distal MCA [125], or by a thrombin-filled catheter inserted in the same way as for \textit{ex vivo} clot delivery [126, 127].

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1-8.png}
\caption{Clot delivery to the middle cerebral artery (MCA). Schematic of a method of clot delivery for embolic MCA occlusion in rats. A catheter is inserted to the origin of the MCA via the extracranial carotid arteries. For embolic stroke, \textit{ex vivo} prepared clots can be injected via this catheter, or the catheter filled with thrombin and blood withdrawn \textit{in situ}, allowing clotting in the catheter before injection back in to the cerebral vasculature.}
\end{figure}
Thromboembolic stroke models have both advantages and disadvantages. The specific advantage of the design of these models is their clinical relevance in mimicking of the cause of stroke seen in the largest proportion of patients. Consequently, these models permit the study of thrombolytic processes and efficacy. Disadvantages of this model include frequent brain haemorrhages and generally higher mortality rates, although the biggest disadvantage of this model is its large variability [128]. Variability occurs both within and between studies and is primarily related to the chosen clot and its subsequent composition that ultimately affects its susceptibility to thrombolysis. Additionally, infarct sizes and locations can be variable and often multifocal depending on size and numbers of clots introduced, where they are introduced, and their final lodgement location.

Figure 1-9. Decision tree of different experimental clot preparations. Major clot preparations used for experimental embolic stroke in rats. Data generated from an ongoing systematic review of embolic stroke models (unpublished, Appendix C).
By far the biggest factor affecting embolic model variability is the choice of clot for occlusion. A review of the literature has revealed at least 8 different “clot recipes” in rat models alone (Figure 1-9), with multiple others used in rabbits and other species (unpublished, Appendix C). Differing recipes and the addition of different clotting factors, such as thrombin, results in variable clot compositions relating to the fibrin mesh and the incorporation of erythrocytes and platelet aggregates. These differences in composition affect the mechanical, physical, and chemical properties of the clot, ultimately affecting its susceptibility to tPA lysis. Considering that differing experimental protocols result in different clot compositions, it is also important to understand the nature of clot composition in clinical stroke. Clot choice for preclinical stroke research will influence the outcomes of recanalization and clot lysis. Appropriate experimental clots should be chosen to mimic the clinical condition and therapeutic strategies being tested.

Human stroke clots are highly variable compositionally. Stroke clots are primarily made up of erythrocytes, platelets, fibrin and white blood cells. While these components are found in most clots, the proportions of each vary greatly between patients (Figure 1-10). Recent histological studies of clots surgically retrieved from stroke patients have shown that no two stroke clots are compositionally the same [16, 73, 74]. These studies indicate that clots are generally high in fibrin and platelet content with low amounts of erythrocytes. The presence of fibrin indicates the suitability of fibrinolytic agents (i.e. tPA) for clot lysis, but structural differences in fibrin clots can cause differing susceptibility to lysis. These differences relate to the incorporation of platelets into the structure, as well as clot retraction and tightening of the fibrin mesh with age that prevents tPA penetrating into the clot. Surgically retrieved clots rich in erythrocytes were

![Figure 1-10. Histopathological composition of thrombi from stroke patients.](image)

Graphical representation of the proportion of red blood cells (RBCs), fibrin and platelets in blood clots surgically retrieved from patients with acute ischaemic stroke of different causes (LAA = large artery atherosclerosis, CE = cardioembolism, DIS = dissection, UNK = unknown). Reproduced with permission from Niesten 2014 Plos One [16].
uncommon [73, 74]. The clots retrieved from these studies were often from patients treated with tPA prior to endovascular clot retrieval. Erythrocyte-rich clots may be uncommon in these histological studies because they are more susceptible to earlier lysis. Additionally, the source of the occlusive clot was previously believed to affect clot composition (e.g. large artery atherosclerosis or cardioembolism), yet these histological studies found no correlation between clot composition and stroke aetiology (Figure 1-10) [16, 73, 74, 129]. Given the heterogeneous nature of clinical stroke and resultant clot composition, the efficacy of any new therapeutic regime should always be tested and confirmed in multiple models of stroke and stroke clot composition.

Thrombin is often added to experimental clot preparations to initiate coagulation and to generate a more rigid fibrin mesh. It has been demonstrated that the mechanical and fibrinolytic properties of thrombin-induced clots differ greatly from spontaneously formed clots [130]. Thrombin-induced clots were described as “elastic,” returning to their original shape after bending, while spontaneously formed clots were described as “plastic,” and didn’t return to their original shape [130]. These properties of a thrombus will determine whether blood flow will cause the thrombus to deform reversibly or irreversibly, and whether it will rupture or embolise [131]. The addition of thrombin to freshly drawn blood causes the formation of compact clots with fibrin-rich cores of higher densities than erythrocyte-rich, spontaneously formed clots. Clots formed in low concentrations of thrombin form thick fibrin fibres, while high concentrations will generate thin fibres and a dense network [131, 132] (Figure 1-11). Thrombin-induced clots are also more resistant to tPA lysis than spontaneously formed clots [130]. Plasma-fibrin clots with a dense fibre arrangement made of thin fibrin fibres generated with high thrombin concentrations dissolve at a slower rate than those with a coarse fibrin structure made of thicker fibres [131]. Although thrombin plays an important role in blood coagulation, it also has profound effects on almost every aspect of vascular wall biology. Compared to erythrocyte rich clots, thrombin-induced clots

Figure 1-11. Fibrin fibre thicknesses. Scanning electron microscopy of clots formed from recalcified plasma with low thrombin concentration (A) and high thrombin concentration (B). Fibre thickness increases at lower thrombin concentrations. Magnification bar = 5 µm. Reproduced with permission from Weisel 2004 Biophys Chem [129].
result in greater degrees of blood brain barrier permeability [130]. Hence, the use of thrombin in clot preparation may be an important variable to be aware of with regards to deleterious outcomes of stroke, including intracerebral haemorrhage.

A large determinant of the final fibrin mesh structure is the incorporation of erythrocytes. Spontaneously formed clots are generally described as “erythrocyte-rich,” containing high proportions of erythrocytes within and around the fibrin mesh. These clot types are less stable and more susceptible to tPA lysis compared with other clot types [130, 133-136]. The incorporation of erythrocytes changes the fibrin structure and properties of the clot [137-139]. With increasing erythrocyte content, the average diameter of the fibrin fibres markedly increases [137] and larger fibrin fibres are associated with greater lysis [131]. Additionally, erythrocytes form large “pockets” within the mesh creating a loose fibrin structure. Hence, the efficacy of tPA lysis increases with increasing proportions of erythrocytes [136-138].

Just as erythrocytes will affect fibrin mesh structure, so too will platelets. Platelets are a key component in the initial stages of thrombosis, and therefore, should be key components when attempting to mimic physiological clots. Experimental PRC are generally formed by isolating platelet-rich plasma from citrated whole blood and recalcifying with CaCl₂ (± additional thrombin) [140-142]. PRC are heterogeneous in structure with platelet-rich and platelet poor areas and varying fibrin fibre thickness, while fibrin-rich clots are more homogeneous in structure and fibrin fibre thickness. PRC have regions of dense platelet aggregates and fibrin fibres adjacent to the aggregates are both thinner and denser than the fibres platelet-poor regions (Figure 1-12) [132, 143]. PRC are more resistant to spontaneous and thrombolytic lysis compared to other clot compositions [133-135, 143]. Resistance to fibrinolysis is related to clot structure and decreased tPA binding due to restricted access of tPA to binding sites within the dense platelet-fibrin aggregate [133]. In vitro studies demonstrate that the fibrin structure is altered in the presence of antiplatelet agents, removing fibrin from the platelet aggregate, increasing tPA binding sites and subsequent lysis rates [133]. An additional factor affecting PRC resistance to lysis is the presence of plasminogen inhibitor-1 (PAI-1) [144]. The majority of endogenous PAI-1 is contained within platelets and is released to inhibit fibrinolysis by preventing the conversion of plasminogen to plasmin by tPA (Figure 1-4). Consequently, with increasing platelet content of a thrombus, the potential amount of PAI-1 available for release increases and thrombolytic resistance increases. Histological examination of clots surgically retrieved from stroke patients has revealed platelet accumulations in the majority of clots [74]. Comparisons of experimental PRC with human stroke clots revealed that similarity to human clots increased with increasing platelet content [141]. Thrombolytic enhancers, such as sonothrombolysis, need to be tested on clots that would otherwise not lyse in the presence of tPA alone. Given the histological data of clinical stroke
clots [16, 73, 74], preclinical thrombolytic-enhancer testing, should investigate multiple models of clot compositions particularly incorporating increased proportions of platelets.

1.3.2. MODELLING MICROVASCULAR OCCLUSION

Large vessel recanalization is the major aim of current acute stroke therapies, however there is growing evidence that reperfusion of the ischaemic territory may be a better predictor of good outcome after stroke [11, 61]. Large vessel recanalization does not always restore flow to the microvasculature due to fibrin/platelet deposits during periods of stagnant flow. There is currently no direct treatment for this phenomenon and testing any thrombolytic therapies to target this effect requires a model that generates thrombosis and occlusion of the microvessels.

Microvascular occlusion has been observed after large vessel recanalization in the most common model used for stroke research, the intraluminal filament model of MCA occlusion in rodents [63, 117, 145, 146]. This model allows controlled recanalization by the insertion of a filament via the extracranial carotids to the origin of the MCA, blocking blood flow [147, 148]. Withdrawal of the filament restores flow to the MCA territory and allows control over the variable of recanalization that cannot be achieved in an embolic model. By using this method, a number of studies have demonstrated impaired microcirculatory patency after controlled occlusion/recanalization [63, 117, 145, 146]. It has been shown that fibrin and platelets deposit within the microvasculature [145], and erythrocytes become trapped within the capillaries of the ipsilateral core and penumbra.
out to at least 2 hours post-thread withdrawal [63]. Significant leukocyte accumulation also occurs within the microcirculation [146]. As the intraluminal filament model in rodents is commonly used, and results in a “no reflow” effect of the microvasculature, it is an ideal model for preclinical testing of thrombolitics and thrombolytic enhancers to treat this condition.

### 1.3.3. **Modelling Carotid Artery Occlusion**

Acute carotid occlusion is an important negative prognostic indicator for stroke, as discussed above, and improved therapies are greatly needed. For the testing of therapies, a model of carotid occlusion that mimics the clinical setting is required. Hence, as with any model for testing thrombolytic therapies and enhancers, a preclinical model of carotid artery occlusion requires a clot within the carotid artery. Additionally, stenosis of the vessel due to atherosclerosis is a common cause of carotid occlusion. Yet, the vast majority of models of carotid occlusion have been used for pathophysiological studies of the effects of the condition and have modelled occlusion by ligation or clipping of the artery [149]. Some models have created occlusions by injection of thrombin or topical application of ferric chloride to initiate thrombosis. However, these thrombi do not form in a naturally physiological manner. One advantage of using models of carotid occlusion over those of MCA occlusion, is that the carotid arteries provide access to the vessel with minimal surgical trauma, unlike the intracranial vessels that require craniectomy for direct access. This allows the use of models of natural thrombosis, such as the Folts model [150].

The Folts model was originally performed in the coronary arteries of dogs [150], but has since been used in various arteries of different species, and has recently been adapted for use in the rat carotid artery [151]. This model involves crushing the artery to produce endothelial injury and exposure of pro-thrombotic subendothelial matrix proteins to the blood. A suture or balloon cuff is used to create a controlled stenosis around the vessel at the site of injury, reducing blood flow, thereby aiding in thrombosis. As carotid artery occlusion in humans is generally caused at the site of an atherosclerotic stenosis by plaque rupture and subsequent thrombosis, the Folts model is perhaps the best mimic of a naturally occurring, physiological carotid occlusion, incorporating the variable of stenosis, for the testing of thrombolitics.

### 1.3.4. **Tissue Plasminogen Activator for Preclinical Research**

The efficacy of tPA has long been established and it has been the thrombolytic used clinically for stroke for almost two decades. However, as previously discussed, its limitations mean there is a growing field of research into thrombolytic enhancer therapies, such as sonothrombolysis. The conventional dose of tPA used for rodent stroke models is 10-fold higher than the clinical dose. This was determined from in vitro work in the early 1980’s that indicated that the rat fibrinolytic system was 10-fold less sensitive than the human system [152]. Since then, most preclinical
studies have used a dose of 10 mg/kg (compared to the 0.9 mg/kg human dose) [153]. However this dose achieves high recanalization in a number of different stroke models [154-157] and may not be indicative of the clinical recanalization response. Although two studies have shown that the clinical dose is perhaps more appropriate in different models [155, 158], a “human equivalent” dose of tPA for preclinical research, that mimics clinical recanalization rates and allows for testing of thrombolytic enhancers, is not known. This will be addressed further in Chapter 5.
1.4. AIMS AND RATIONALE

1.1 OVERALL AIM

The overall aim of this thesis is to test sonothrombolysis (± microbubbles) for recanalization and reperfusion in different models of stroke. Individual study aims and rationale are outlined in the corresponding chapter introductions.

1.2 RATIONALE FOR THE OVERALL AIM

Why recanalization and reperfusion?

It has long been understood that recanalization of an occluded artery leads to better patient outcome after stroke and current therapy and interventions aim to achieve this goal. Recent evidence has also suggested that reperfusion is a better predictor of good patient outcome than recanalization alone. Hence, the best chance of good patient outcome is following the rapid recanalization of the occluded large vessels while also restoring microvascular perfusion.

Why sonothrombolysis?

The only approved thrombolytic therapy for acute ischaemic stroke is tPA, yet a significant proportion of patients treated do not recanalize. Failed recanalization correlates with poor clinical outcome and hence, there is a need to improve tPA’s efficacy. Sonothrombolysis ± microbubbles has been shown to improve recanalization rates and clinical outcome in small scale clinical trials over tPA alone. Yet, much is still unknown regarding the potential of sonothrombolysis in different situations of stroke and in preclinical models of clinically relevant scenarios.

Why different models of stroke?

Stroke is a heterogeneous condition and a single model of stroke cannot encompass all the variables observed clinically. These variables affect the efficacy of thrombolysis and ultimately patient outcome. The composition of an occluding clot will affect thrombolytic success as will the location of the occlusion: be it intracranial or extracranial, in large vessels or the microvasculature. Additionally, extracranial occlusions often occur in regions of vascular stenosis. Using multiple models of stroke for the testing of sonothrombolysis will lead to a more encompassing understanding of the potential efficacy of this intervention and the patients that may benefit from it.
Hence, for this thesis, I developed models to test sonothrombolysis (± microbubbles) for recanalization of:

- Middle cerebral artery occlusion with platelet rich clots (Chapter 2),
- Microvascular occlusion after large vessel recanalization (Chapter 3), and
- Carotid artery occlusion with variable degrees of stenosis (Chapter 4).

Finally, I tested different doses of tPA for recanalization in a model of carotid artery occlusion to determine a “clinical equivalent” tPA dose for recanalization studies (Chapter 5). Obtaining meaningful preclinical data on sonothrombolysis is highly dependent on being able to model clinical responses to thrombolytics while allowing room for improvement with sonothrombolysis intervention. The conventionally used “rat dose” may not be the best clinical mimic for recanalization.
Chapter 2

2 MICROBUBBLE-ENHANCED SONOTHROMBOLYSIS OF MIDDLE CEREBRAL ARTERY OCCLUSION WITH PLATELET RICH CLOTS

2.1. Study aims, rationale and hypotheses

Publication: Platelet rich clots are resistant to lysis by thrombolytic therapy in a rat model of embolic stroke

2.2. Abstract

2.3. Background

2.4. Methods

2.4.1. Animals

2.4.2. Experimental design

2.4.3. Confirmation of occlusion and recanalization rates

2.4.4. Treatment groups

2.4.5. Additional outcomes

2.4.6. Inclusion/Exclusion criteria

2.4.7. Statistical analysis

2.5. Results

2.5.1. Study 1 – Investigation of the model

2.5.2. Study 2 – Thrombolytic treatment effect

2.6. Discussion

2.7. Conclusion

2.8. Additional Information

2.8.1. Funding sources

2.8.2. Author contributions

2.8.3. Competing interests
2.1 STUDY AIMS, RATIONALE AND HYPOTHESES

In this chapter, I describe a model of thromboembolic stroke with PRC. This model was developed and used to test the efficacy of microbubble-enhanced sonothrombolysis for recanalization of the MCA. The specific aims of these studies were:

**Study 1:** To investigate rates of spontaneous recanalization and infarct volumes using our new PRC model.

**Study 2:** To investigate the effect on recanalization rates of tPA therapy alone or in conjunction with ultrasound and a new microbubble formulation (BR38) in this model.

**Rationale and hypotheses: Study 1**

There are a large variety of models of embolic MCA occlusion leading to variability of recanalization and other outcomes. Variability is, in part, related to clot preparation and the final composition that affects thrombolytic efficacy. PRC are more stable and better mimics of clinical stroke clots compared to other experimental clots [141]. For study 1, I hypothesised that the PRC preparation would produce a more stable occlusion and more consistent infarction in a rat MCA occlusion model.

**Rationale and hypotheses: Study 2**

Microbubble-enhanced sonothrombolysis has been indicated as a potential stroke therapy to improve recanalization and clinical outcomes [83, 84]. Yet, much remains unknown regarding appropriate microbubble formulations for maximum efficacy of recanalization. BR38 is a new microbubble formulation that has been shown to enhance lysis of spontaneously formed clots *in vitro* [159]. Spontaneously formed clots are more susceptible to lysis and have been histologically shown to be poor mimics of clinical clots. Hence, BR38 microbubbles remain to be tested in a more clinically relevant, *in vivo* model of stroke. Given positive results of study 1, I hypothesised that BR38-enhanced sonothrombolysis could improve recanalization in this MCA occlusion model with PRC over tPA alone.
The work in this chapter has been published in Experimental and Translational Stroke Medicine.


The following statement has been accepted by all co-authors to this study. Signed statements are presented in Appendix A.

*We, the co-authors, attest that Research Higher Degree candidate, Amelia Tomkins, contributed to the paper/publication entitled *Platelet rich clots are resistant to lysis by thrombolytic therapy in a rat model of embolic stroke*, as outlined below:

- 60% Conception and design of the experiments
- 90% Surgery and experimental work
- 80% Analysis and interpretation of findings
- 80% Writing the paper and critical appraisal of the content
PLATELET RICH CLOTS ARE RESISTANT TO LYSIS BY THROMBOLYTIC THERAPY IN A RAT MODEL OF EMBOLIC STROKE

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2.2 ABSTRACT

**Background:** Early recanalization of occluded vessels in stroke is closely associated with improved clinical outcome. Microbubble-enhanced sonothrombolysis is a promising therapy to improve recanalization rates and reduce the time to recanalization. Testing any thrombolytic therapy requires a model of thromboembolic stroke, but to date these models have been highly variable with regards to clot stability. Here, we developed a model of thromboembolic stroke in rats with site-specific delivery of platelet-rich clots (PRC) to the main stem of the middle cerebral artery (MCA). This model was used in a subsequent study to test microbubble-enhanced sonothrombolysis.

**Methods:** In Study 1 we investigated spontaneous recanalization rates of PRC *in vivo* over 4 hours and measured infarct volumes at 24 hours. In Study 2 we investigated tPA-mediated thrombolysis and microbubble-enhanced sonothrombolysis in this model.

**Results:** Study 1 demonstrated stable occlusion out to 4 hours in 5 of 7 rats. Two rats spontaneously recanalized at 40 and 70 minutes post-embolism. Infarct volumes were not significantly different in recanalized rats, 43.93 ± 15.44% of the ischaemic hemisphere, compared to 48.93 ± 3.9% in non-recanalized animals (*p*=0.7). In Study 2, recanalization was not observed in any of the groups post-treatment.

**Conclusions:** Site specific delivery of PRC to the MCA origin resulted in high rates of MCA occlusion, low rates of spontaneous clot lysis and large infarction. These PRC were highly resistant to tPA with or without microbubble-enhanced sonothrombolysis. This resistance of PRC to enhanced thrombolysis may explain recanalization failures clinically and should be an impetus to better clot-type identification and alternative recanalization methods.
2.3 Background
Currently, the only approved thrombolytic treatment for acute ischaemic stroke is recombinant tissue plasminogen activator (tPA) delivered intravenously within 4.5 hours of stroke onset [47]. However, less than 10% of all ischaemic stroke patients are eligible for therapy [160] and tPA achieves successful recanalization in less than half of those treated [9]. Achieving higher recanalization rates in a timely manner is a key goal to developing better stroke thrombolytic therapy.

Use of ultrasound to enhance recanalization with tPA is a promising approach. The first clinical report of stroke sonothrombolysis indicated increased rates of recanalization in patients receiving continuous transcranial Doppler ultrasound (TCD) monitoring during tPA therapy [79]. Several small clinical trials with perflutren-lipid and galactose-based microbubbles as enhancers of sonothrombolysis suggest that microbubbles may produce further improvements in the rates of recanalization [85, 87, 88, 161]. Despite the promise of this therapy, more than half of patients treated do not recanalize [80] and concerns have been raised regarding its safety with increased rates of haemorrhage in some studies [88, 110]. Therefore, there is still a great need for pre-clinical studies to better understand the efficacy, mechanisms and safety effects of this potential therapeutic strategy.

To test any thrombolytic therapy, a model of stroke is required that uses a life-like clot to block major cerebral arteries. Current thromboembolic models are highly variable and it is likely that this variability is related to the choice of clot and its inherent stability with regards to spontaneous and thrombolytic-induced lysis. Recent studies have demonstrated that platelet-rich clots (PRC) are more stable in vitro than other clot variations and are also more similar to clots retrieved from human stroke patients histologically [140]. These clots have yet to be tested in vivo and during sonothrombolysis.

In this article we describe a method of embolic stroke using site-specific delivery of a PRC to the origin of the middle cerebral artery (MCA). Two separate studies were performed. In study 1 our aim was to investigate rates of spontaneous recanalization and infarct volumes using our new PRC model. In study 2 we aimed to investigate the effect on recanalization rates of tPA therapy alone or in conjunction with ultrasound and a new microbubble formulation (BR38) in this model.

2.4 Methods

2.4.1 Animals
All animal experimentation was carried out in accordance with local legislation. Experiments conducted at the University of Newcastle, Australia (UoN) were in accordance with Animal Care and Ethics Committee (ACEC) guidelines (approval # A-2010-128) and in compliance with the
requirements of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Experiments conducted at Justus-Liebig University Giessen, Germany (JLU), were in accordance with the German animal protection legislation and approved by the regional ethics committee (Az. B2/257). At UoN, spontaneously hypertensive rats (SHR) aged 14-18 weeks and weighing 320-375g, were sourced from Animal Resources Centre, Perth, Australia. At JLU, 15-21 weeks old SHR weighing 290-375 g were sourced from Harlan laboratories GmbH, Netherlands. Surgeries were performed at both centres by the same investigator (AT). Anaesthesia was induced with 5% isoflurane and maintained at 1.5-3% in oxygen and nitrogen (2:1). Temperature was monitored and maintained at 37 °C throughout all experiments. SpO₂ and heart rate were also monitored at UoN (Study 1).

2.4.2 Experimental design
Study 1 was performed to determine spontaneous recanalization rates in the first 4 hours post-embolism, and 24 hour infarction volume and mortality (n=14; performed at UoN). In Study 2 we investigated the effect of tPA or tPA+ultrasound+BR38 microbubbles compared to saline control in this model (n=44; performed at JLU). The specifics of each protocol are outlined in Table 2-1.

Clot preparation was modified from the methods of Roessler et al [140]. Briefly, cardiac blood from donor animals (Study 1: n=4, Study 2: n=10) was collected with sodium citrate anticoagulant (3.2%; blood:citrate=9:1) and underwent 2-step centrifugation to obtain platelet rich plasma. Whole plasma and buffy coat were collected after an initial spin (15 minutes at 180g). The platelet-rich plasma layer and buffy coat were collected after a second fast spin (20 minutes at 1500g). PRC formation was initiated by recalcification of the platelet rich plasma + buffy coat with CaCl₂ (20 mM final concentration). The sample was drawn into a 40 cm length of PE-50 tubing (40 cm, i.d. 0.58 mm) and clamped at one end. Using an air filled syringe, pressure was forced into the unclamped end of the catheter to create clots of consistent size. This end was then also clamped to retain the pressure and incubated at 37 °C for 2 hours. The resultant clot was ejected into a dish of saline and stored at 4 °C overnight. Clot preparation was the same for both

### Table 2-1. Experimental Protocols.

<table>
<thead>
<tr>
<th>Location</th>
<th>Study 1</th>
<th>Study 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aim</td>
<td>Newcastle, Australia</td>
<td>Bad Nauheim, Germany</td>
</tr>
<tr>
<td>Secondary Outcome(s) [method]</td>
<td>Infarct Volumes (TTIC), Mortality, Neurological Deficit scores</td>
<td>Cut lysis (Inspection of the major branches of the cerebral arterial circulation)</td>
</tr>
<tr>
<td>Survival post-embolism</td>
<td>24 h</td>
<td>2 h</td>
</tr>
<tr>
<td>Treatment groups (n)</td>
<td>No treatment (n = 7)</td>
<td>Saline (n = 10), tPA (n = 10), tPA + Ultrasound + BR38 microbubbles (n = 10)</td>
</tr>
<tr>
<td>Laser Doppler Monitoring</td>
<td>Continuous</td>
<td>Discontinuous</td>
</tr>
<tr>
<td></td>
<td>To 4 h post-embolism</td>
<td>Per-embolism, Post-embolism, Pre-treatment, Post-treatment</td>
</tr>
</tbody>
</table>
studies, with the exception for Study 2 in which the clot was incubated for 5 minutes in Evans blue for visualisation within major branches of the cerebral arterial circulation at sacrifice.

The method of site-specific clot delivery was based on the method of MCA thread occlusion we use routinely [147, 162, 163] with catheter placement and clot delivery as described by DiNapoli et al [124]. Briefly, a modified catheter (PE-8, o.d. 0.35 mm, connected to silastic tubing) containing a 30 mm length of PRC was inserted into the internal carotid artery via the external carotid artery. It was advanced until a sensation of mild resistance indicated the tip was at the middle cerebral artery (MCA) origin, then retracted 1-2 mm to restore flow to the MCA allowing site-specific clot injection. The clot was injected with 30-50 µl saline and the catheter left in place for 10 minutes to allow the clot to stabilize. The catheter was then completely removed from the vessel and the surgical site sealed.

2.4.3 Confirmation of occlusion and recanalization rates
In both studies, confirmation of occlusion of the MCA after clot injection was made by laser Doppler Flowmetry (LDF). Occlusion was defined as a reduction to <50% from baseline values of regional cerebral blood flow (rCBF). The primary outcome of both studies was recanalization rates. Recanalization was defined as a return to ≥100% of baseline rCBF. Two methods of LDF monitoring were used: continuous and discontinuous.

Study 1: Continuous laser Doppler tissue perfusion monitoring was performed using a single fibre optic probe (MoorVMS-LDF with probe type VP10M200ST, Moor instruments, UK). A longitudinal incision was made along the scalp, above the midline and over bregma. The skull was thinned using a dental drill 1 mm posterior to bregma and as far lateral as possible (at the border of the temporalis muscle). The Doppler probe was affixed to the skull within a silicone probe holder. The position of the probe was inspected under the operating microscope before fixation to ensure it was not placed over large vessels that would otherwise compromise the accuracy of the readings. The recordings were taken for a minimum 20 minutes pre-embolism. Baseline rCBF was calculated as the average of a 5 minute period immediately prior to clot injection. All subsequent readings were expressed as a percentage of baseline. Continuous LDF monitoring also allowed confirmation of the initial MCA occlusion by the clot delivery catheter, followed by catheter retraction (restoring flow) and clot injection. LDF monitoring continued for the duration of observation under anaesthesia (4 hours post-embolism). Four hours of post-occlusion monitoring was based on the approved time window for clinical tPA administration to determine if these clots were stable during that period. Clinical benefit of thrombolysis beyond this time is limited [164]. Animals were then woken and returned to their cages until sacrifice at 24 hours for infarct volume analysis.
Study 2: Continuous monitoring could not be performed during ultrasound treatment for Study 2 due to the apparatus set-up, so a discontinuous approach was used [165]. Discontinuous monitoring was performed at 4 time points: pre-embolism (-30 minutes), post-embolism (10 minutes), pre-treatment (50 minutes) and post-treatment (130 minutes), using an Oxyflo2000 Microvascular Perfusion Monitor with Oxyflo XP Probe 17 mm diameter (MNP 100XP-3/15, Oxford Optronix, UK). Recordings were made as per Soehle et al [165]. Animals were sacrificed after final LDF recording for visual inspection of clot presence.

2.4.4 Treatment groups
Animals in Study 2 received intravenous injections via the tail vein of either tPA, tPA and microbubbles, or vehicle (saline) started 1 hour after embolism (n=10 per group). The tPA and microbubble group also received 60 minutes ultrasound. tPA (10 mg/kg; Actilyse™, Boehringer Ingelheim, Ingelheim, Germany) was delivered as a 10% bolus and the remainder delivered over 1 hour. Total injected volume was 2.4 ml. The dose of 10 mg/kg was used based on evidence that the rat fibrinolytic system is 10-fold less sensitive than humans [152]. A total of four 0.1 ml doses of BR38 microbubbles (Bracco Research, Switzerland) at a concentration of 4 x 10^8 bubbles/ml (10 µl BR38 diluted in 90 µl NaCl), were delivered at 15 minute intervals starting with the initial tPA bolus. BR38 are 35% perfluorobutane and 65% nitrogen in a phospholipid shell. The dose of BR38 was comparable to previously calculated doses of other microbubble formulations [117]. Transcranial colour-coded Duplex ultrasound (TCCD) was applied continuously for the duration of treatment (60 minutes). A 3-MHz diagnostic ultrasound probe was placed 40 mm above the skull (B-mode, color-Doppler functions switched on, maximum output (mechanical index of 1.7)) (Sonos 7500; Philips Ultrasound, USA). The distance between the skull and ultrasonic probe was bridged with ultrasound gel (Sonosid®; Asid Bonz, Germany) in a plastic cylinder open at both ends and the beam was aligned to expose the entire brain incorporating the circle of Willis and the occluded MCA (spectral Doppler sample volume placed in the midbrain (57 mm)).

2.4.5 Additional outcomes
For both studies, the primary outcome was LDF recanalization. Secondary outcomes were infarct volume, neurological deficit and mortality for Study 1, and visualization of PRC in the MCA post-mortem for Study 2.

For animals surviving 24 hours post-occlusion (Study 1), a series of neurological tests were performed to determine level of functional deficit following stroke. Deficit was assessed by an observer blinded to recanalization outcome with a modified Bederson scoring system [166, 167], scoring the degree of flexion of the affected limb, degree of twisting of the animal’s torso, and the ability to brace against a lateral force. Each test was given a score of 0, 1, or 2 where 0 = not affected and 2 = severely affected. These scores were totalled for a final score between 0 and 6. Mobility was also scored, 0 = mobile and 2 = immobile.
Infarction was assessed at 24 hours in Study 1. Animals were sacrificed by isoflurane followed by cardiac perfusion with cold saline (3 minutes, Peri-Star™ Pro perfusion pump, World Precision Instruments, USA). Brains were removed and placed in cold saline (4 °C), stirred gently with a magnetic stirrer. Brain slices (2 mm) were covered in 1% 2,3,5-triphenyl-tetrazolium chloride (TTC) solution and incubated at 37 °C for 10 minutes. Slices were turned and covered in fresh TTC for a further 10 minutes at 37 °C, stored overnight at room temperature in buffered formalin then photographed. Hemispheres and regions of infarct were traced using ImageJ software and infarct volumes were calculated by averaging the area of the top of the section and the area of the base of a section and multiplying by the width (volume of a trapezoid). The total infarct volume was the sum of all slices. Infarct volumes were expressed as a percentage of the ipsilateral hemisphere.

Arterial filling and clot visualisation in Study 2 was achieved by intravascular silicone infusion (Figure 2-1). Animals were sacrificed immediately following post-treatment LDF (130 minutes), and Microfil (Flow Tech, Inc., USA) was used to cast the vasculature, as previously described [117]. Briefly, the circulation was flushed with saline until the venous effluent was clear of blood. The descending aorta, subclavian arteries and both left and right external carotid arteries were ligated and Microfil, prepared to manufacturer’s instructions, was injected via the aortic arch to fill the arterial and venous cerebral circulation via internal carotid and vertebral arteries. During the injection process, excessive dilation of the aorta was avoided so as to maintain physiological pressure conditions. After 45 minutes, the Microfil formed an elastomeric gel at room temperature.

![Figure 2-1. Visualisation of vascular filling and clot presence (Study 2).](image)

Vessels were perfused post-mortem with Microfil (yellow) to visualise the vasculature and clot presence (black). All animals had clot in the major cerebral vessels after treatment. (A) Shows the vasculature from the view of the Circle of Willis, (B) shows the lateral surface of the right hemisphere. Vessels labelled are: middle cerebral artery (MCA), anterior cerebral artery (ACA) and internal carotid artery (ICA). Images of all brains can be viewed in Figure 2-4.
and the brain was removed from the skull and immersed in formalin. The Circle of Willis and the lateral surfaces of the brain were photographed for visualisation of clot presence.

2.4.6 Inclusion/Exclusion criteria
Pre-specified exclusion criteria were as follows: No appropriate LDF drop after clot injection (Study 1 & 2); spontaneous recanalization pre-treatment (Study 2); Subarachnoid haemorrhage (SAH) due incorrect catheter insertion was identified during continuous LDF monitoring (Study 1) or post-mortem (Study 1 & 2). Excluded animals in study 2 were replaced so that treatment groups contained 10 animals.

2.4.7 Statistical analysis
Data is presented as mean ± standard deviation (SD). Differences in infarct volumes between recanalized and non-recanalized animals were assessed with unpaired Student’s t-test. Statistical significance was considered to be a p-value <0.05.

2.5 RESULTS

2.5.1 Study 1 – Investigation of the model
Three animals were excluded from analysis due to either SAH (n=1) or occlusion not confirmed by LDF (n=2). SAH was identified by LDF signal and confirmed post-mortem. One animal died overnight post-embolism. Post-mortem histology revealed large infarction to be the likely cause of death. This animal was included in LDF analysis, but not infarct calculations or neuroscoring.

Five of seven animals remained occluded for the duration of LDF monitoring (4 hours post-embolism) (Figure 2-2A). Two animals spontaneously recanalized at 40 and 70 minutes post-embolism (Figure 2-2B). Infarct volumes were 48.93 ± 3.9% and 43.93 ± 15.44% of hemisphere in non-recanalized and recanalized rats, respectively. There was no statistical significance between groups (p=0.7), however the rat that recanalized earlier (40 minutes) had a smaller infarct than the one that recanalized later (70 minutes): 33.01% versus 54.85% of hemisphere, respectively. Neuroscores at 24 hours were 3 ± 2. All animals exhibited a neurological deficit.

2.5.2 Study 2 - Thrombolytic treatment effect
A total of 4 animals were excluded due to SAH (n=2, determined post-mortem), excessive bleeding pre-embolism (n=1), and catheter dislodgement during treatment (n=1). These animals were replaced so that all groups consisted of 10 animals. No animals recanalized in any treatment group in Study 2 (Figure 2-3 and 2-4). Clot was visible within the Circle of Willis of all animals except one in the ultrasound group, however clot was observed in the distal MCA of this animal (Figure 2-1 and 2-4).
Figure 2-2. Laser Doppler flowmetry and infarction following middle cerebral artery occlusion with platelet rich clots (Study 1). A total of seven rats had successful embolization of the MCA. (A) Five rats remained occluded for the duration of LDF observation (mean ± SD). One rat died overnight and was not included in TTC assessment of infarct. (B) Two rats recanalized at 40 min (black trace) and 70 min (grey trace) (raw data).
Figure 2-3. Laser Doppler flowmetry (LDF) of regional cerebral blood flow in treatment groups (Study 2). Animals underwent embolization of the middle cerebral artery (MCA) with platelet rich clot. LDF confirmed occlusion in all animals and indicated no recanalization post-treatment. Data represents the mean of n=10 per group. There were no significant differences between groups. (U/S = ultrasound; BR38 = microbubbles)

Figure 2-4. Vascular filling and clot presence (Study 2). Vessels were perfused post-mortem with Microfil (yellow) to visualise the vasculature and clot presence (black). All animals are presented. (A, page 51) shows the vasculature from the view of the circle of Willis, (B, page 52) shows the corresponding lateral surface of the right hemisphere. Vessels labelled are: middle cerebral artery (MCA), anterior cerebral artery (ACA) and internal carotid artery (ICA). Images of all brains can be viewed in the supplemental data. Treatment groups were saline, tPA or ultrasound insonation with tPA and BR38 microbubbles (U/S + tPA + BR38).
<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>tPA</th>
<th>U/S + tPA + BR38</th>
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<tbody>
<tr>
<td>MCA</td>
<td><img src="MCA.png" alt="Image" /></td>
<td><img src="tPA.png" alt="Image" /></td>
<td><img src="U/S.png" alt="Image" /></td>
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2.6 DISCUSSION
We present here an experimental model of embolic stroke using PRC. This model was developed because clots retrieved from patients often have high platelet concentrations [16, 73, 74, 129], unlike most commonly used experimental clots, which are red cell or fibrin-rich [140]. We have shown the experimental PRC to be highly resistant to spontaneous and tPA-mediated thrombolysis with or without enhancement with microbubble-enhanced sonothrombolysis. This high resistance to lysis suggests that greater levels of platelets may be an important contributing factor to failed recanalization in many stroke patients despite tPA treatment.

Our findings confirmed PRC resistance to thrombolysis with tPA, and indicate that clots enriched with platelets are highly resistant to lysis, even with the addition of ultrasound and microbubbles. Within the fibrin network of a PRC clot, large platelet aggregates are observed. These aggregates lead to increased rigidity of the clot and significantly increase time to tPA-induced lysis compared with other clots [133, 140]. Histological comparisons of experimental PRC with clots retrieved from human stroke patients have also revealed both to contain a strong cross-linked fibrin mesh that ensures mechanical stability [140]. The addition of platelets to thrombin-induced clots also increases their resistance to thrombolysis in vivo [142]. A major inhibitor of plasminogen activation is plasminogen activator inhibitor (PAI-1) released locally by platelets and vascular endothelial cells. PAI-1 has been implicated as increasing PRC resistance to tPA lysis [168, 169].

There are several putative mechanisms for the action of sonothrombolysis. These include microstreaming of blood, that enhances access of tPA into the clot, and mechanical disruption of the fibrin mesh [108]. Our results showed that microbubble-enhanced sonothrombolysis was insufficient to lyse thrombi highly enriched in platelets and this may be an important consideration when delivering reperfusion therapy to patients. While sonothrombolysis appears to enhance recanalization rates in stroke patients, there is a subset of patients (33-59%) who do not achieve complete recanalization despite the addition of sonothrombolysis with or without microbubbles [80, 82, 87, 88]. Clot composition in this subgroup is not known, however from studies of mechanically retrieved thrombi from stroke patients we do know that they are very heterogeneous and high platelet levels occur frequently in clinical stroke [16, 73, 74, 129]. Moreover it has been shown, that clot composition is correlated with outcome after 90 days [170]. Our data suggest a likely explanation for at least some of these patients with failure of recanalization is PRC resistance to thrombolysis. If this is the case, alternative approaches will be needed.

Stroke is a heterogeneous condition with no two clots compositionally the same [74] thereby resulting in differing efficacies of tPA thrombolysis. The majority of preclinical in vivo studies do not directly compare clot types. Investigating only one clot type is also a limitation of our study. However, in vitro work of PRC has shown greater resistance to thrombolysis than other clot types [133, 140], with a recent study demonstrating increased tPA resistance with increasing
platelet counts [141]. PRC are also better histological mimics of clinical thrombi retrieved from stroke patients who failed to recanalize after tPA therapy. Our study confirms PRC resistance to tPA thrombolysis in an in vivo rat model and also demonstrates resistance to enhanced sonothrombolysis. Studies of sonothrombolysis in rat models are surprisingly lacking. Most have used low frequency ultrasound or were not performed in embolic models. Studies of 10 mg/kg tPA thrombolysis in rat embolic MCA occlusion models are the closest comparisons we can achieve, and only a few report recanalization. Spontaneously formed clots with high erythrocyte content as well as thrombin induced-fibrin rich clots recanalize in >80% of cases [154, 156, 157]. These experimental clots may mimic the clots of tPA responders, but does not allow room for testing thrombolytic enhancers. Since no recanalization was observed in our study, it is reasonable to conclude that the composition of our clots has a large effect on thrombolysis. The lack of preclinical studies comparing clots, or studying sonothrombolysis at diagnostic frequencies clearly highlights a gap in our knowledge of the full impact of clot type on sonothrombolysis in vivo. It should also be noted that even with microbubble-enhanced sonothrombolysis, a large proportion of patients still do not respond to therapy [84]. Our study could provide one answer to this – that the occluding thromboemboli of these patients contain high proportions of platelets.

An important implication of these findings is that there may be a subset of patients with PRC and even the combination of ultrasound with tPA may be largely futile to cause recanalization. The clinical risk of haemorrhage with increasing tPA doses prevents testing higher doses to determine if thrombolytic recanalization can be achieved at all in this clot type. The dose of tPA we used (10 mg/kg) is already a high dose, with studies of other clot types suggesting that the clinical dose is a better mimic of the clinical response to recanalization [158]. Further increasing our tPA dose would unlikely exhibit significant increases in recanalization and a more clinically relevant interpretation of these results is that PRC do not lyse solely by the fibrinolytic mechanisms initiated by tPA and ultrasound. Early identification of patients with clots of higher platelet content might allow time to pursue alternative approaches to reperfusion, such as mechanical clot retrieval. Another consideration is the use of antiplatelet agents. Both GPIIb/IIa receptor blockers (abciximab) and cyclo-oxygenase inhibitors (aspirin) have shown promise in reducing clot lysis time and causing decreases in platelet-fibrin aggregates when applied prior to clot formation. Reduced clot lysis time has been observed when abciximab is added to pre-established clots [133], suggesting a possible therapeutic strategy. There is certainly promise in using combination antiplatelet -tPA therapy, however there is clinical concern regarding the risk of additional bleeding complications in stroke patients [171, 172]. Several imaging approaches have been used to try and identify resistance of clots to thrombolysis. MRI and CT studies of experimental and clinical stroke clots have begun to correlate signal intensities with composition of red cells and fibrin [16, 129, 173-175]. So far, studies have been able to identify red cell rich clots from other
clot types, due to the presence of iron in these cells that increases signal intensity. Clots high in red cells were shown to be more easily lysed [140]. However the current study indicates that platelet content may be a key factor affecting recanalization rates. As yet, the ability to distinguish fibrin-rich from platelet-rich clots is limited.

A limitation of this study is that we did not assess haemorrhage, a known complication of sonothrombolysis [84]. Clinical evidence suggests that TCCD may cause higher rates of haemorrhage than TCD monitoring [84]. We have previously demonstrated efficacy of microvascular recanalization using this system and parameters, and with ultrasound. Intracerebral haemorrhage was observed in only 1 of 25 animals treated with tPA+ultrasound+microbubbles, and this protocol was deemed safe for sonothrombolysis [117]. All clinical sonothrombolysis studies using TCCD have used ultrasound insonation durations of 60 minutes [81, 82, 89], which was our choice for this study. Yet the majority of TCD studies have insonated for 2 hours [80, 85, 88, 176]. Increasing insonation duration may result in greater recanalization rates, but this direct comparison has not been investigated clinically and it is unknown what the optimal duration is for maximal efficacy with minimal adverse events. Given the clinical evidence of increased haemorrhage with TCCD, any study of longer duration insonation should consider safety as an important outcome.

Our ability to determine the efficacy of BR38 microbubbles to enhance sonothrombolysis was limited in this study. BR38 microbubbles were developed to improve on the existing SonoVue™ formulation and are more stable and circulate longer in the microcirculation [177]. A previous study showed that BR38-enhanced sonothrombolysis of spontaneously formed thrombi [159], a relatively easy to lyse clot type [136, 138]. Our decision not to include a sonothrombolysis group without microbubbles was made because such experiments with diagnostic frequencies and BR38 microbubbles have not been performed in rats. There is sufficient evidence that microbubbles have greater efficacy than ultrasound+tPA alone [84]. Therefore, we chose to determine maximal effect with these bubbles, while limiting animal numbers for ethical reasons. Had an effect between groups been observed, it would have warranted further study to determine the exact effect of these microbubbles. However the PRC generated in the current study ‘overshot the mark’ and proved completely resistant to lysis, regardless of treatment.

2.7 Conclusion
Here we sought to create clots more resistant to lysis to increase the rigor of thrombolytic testing. However, this model appears to have gone too far in the other direction and has created clots that are highly resistant to even enhanced lysis with tPA, ultrasound and microbubbles. Sonothrombolysis has previously been shown to be effective in embolic models of stroke with different clot types [56, 178, 179] but our study suggests that PRC are particularly resistant to
thrombolysis even with the combination of tPA, sonothrombolysis and microbubbles. Our experimental PRC may be representative of clots seen in a subset of stroke patients who are unresponsive to conventional thrombolytic therapies. If so, imaging approaches to identify such clots may allow early pursuit of alternative methods such as mechanical clot retrieval. These clots could also provide a model for development of more rigorous therapies for these patients. Our study highlights the importance of clot structure on the efficacy of thrombolytic therapy and suggests a need for further investigation of in situ identification of clot structure to tailor treatment to the patient.

2.8 ADDITIONAL INFORMATION

2.8.1 Funding sources
Ms Amelia Tomkins was supported by a Heart Foundation Australia post-graduate scholarship co-funded by the National Stroke Foundation Australia, and by a Hunter Medical Research Institute Travel Scholarship funded by Mrs Jennie Thomas. Dr Neil Spratt was supported by a National Health and Medical Research Council (Australia) Career Development Fellowship (APP1035465).

2.8.2 Author contributions
AT, NSp and CL designed Study 1. AT, NSp and MN designed Study 2 and performed analysis. AT, NSch and LM performed animal experiments. AT and NSp drafted the manuscript. All authors read and approved the final manuscript.

2.8.3 Competing interests
None
Chapter 3

3 MICROBUBBLE-ENHANCED SONOTHROMBOLYSIS OF MICROVASCULAR OCCLUSION

3.1. Study aims, rationale and hypotheses .................................................................................. 58
Publication: *Sonothrombolysis with BR38 microbubbles improves microvascular patency in a rat model of stroke* ............................................................................................................. 60

3.2. Abstract .......................................................................................................................... 61

3.3. Introduction .................................................................................................................. 62

3.4. Methods ....................................................................................................................... 63
   3.4.1. Animal preparation .................................................................................................. 63
   3.4.2. Experimental groups ............................................................................................. 64
   3.4.3. Post-mortem preparation ....................................................................................... 65
   3.4.4. Micro- and nano-computed tomography (CT) ......................................................... 65
   3.4.5. Histology ............................................................................................................... 66
   3.4.6. Inclusion and exclusion criteria ............................................................................ 67
   3.4.7. Statistical analysis ............................................................................................... 67

3.5. Results .......................................................................................................................... 67
   3.5.1. Micro- and nano-CT ............................................................................................ 67
   3.5.2. Histology ............................................................................................................. 69

3.6. Discussion ..................................................................................................................... 69

3.7. Additional information ................................................................................................... 72
   3.7.1. Acknowledgements and funding sources .............................................................. 72
   3.7.2. Author contributions .......................................................................................... 72
   3.7.3. Competing interests .......................................................................................... 72
3.1 STUDY AIMS, RATIONALE AND HYPOTHESES

In this chapter, I describe a model of microvascular occlusion that was used to test the efficacy of microbubble-enhanced sonothrombolysis for microvascular recanalization. The specific aim of this study was:

To evaluate the treatment effects of the diagnostic agent Sonovue and a new microbubble agent, BR38, with regards to reversal of perfusion deficits after stroke caused by occlusions of the microvasculature.

Rationale and hypotheses

There is growing evidence that cerebral reperfusion may be a better indicator of good patient outcome than recanalization of the large occluded vessels [11, 61]. A “no reflow phenomenon” has been observed, whereby the microvessels of the ischaemic tissue become occluded due to fibrin and platelet deposition during ischaemia [65, 66]. There is currently no direct therapy for this condition, but preclinical evidence indicates a potential for microbubble-enhanced sonothrombolysis to restore microvascular patency [117]. Although microbubble-enhanced sonothrombolysis has been tested clinically for large vessel recanalization, it is yet to be tested with regards to microvascular recanalization and restoration of cerebral perfusion. Additionally, the ideal microbubble type or dosage for enhanced recanalization of large or small vessels has not yet been determined. A recent study performed in rats demonstrated that microbubble-enhanced sonothrombolysis with Sonovue microbubbles could restore microvascular patency after MCA recanalization [117]. This study demonstrated an increase of microvascular patency after Sonovue-enhanced sonothrombolysis compared to sonothrombolysis alone. For this chapter, I hypothesised that a new microbubble formulation, BR38 – designed to circulate longer than Sonovue in the cerebral circulation – could also restore the perfusion deficits that can occur after stroke. This study was designed to determine differences in efficacy of BR38 and Sonovue at equivalent doses to the earlier study [117]. Additionally, lower microbubble doses of both Sonovue and BR38 were tested to determine if a dose response existed. These two microbubble formulations were also compared against tPA-therapy to determine any benefit over current standard treatment. This study was designed as an efficacy study and is one of only two studies investigating reperfusion with microbubble-enhanced sonothrombolysis. As such, a short sacrifice time was required to determine the immediate effect of microbubble-enhanced sonothrombolysis on microvascular perfusion. Longer term safety and functional studies were not included in this study, but would be required if this therapy was to be considered for clinical reperfusion for stroke.
At the time of thesis submission, the work in this chapter was submitted to *Plos One* for peer review and publication.


The following statement has been accepted by all co-authors to this study. Signed statements are presented in Appendix A.

*We, the co-authors, attest that Research Higher Degree candidate, Amelia Tomkins, contributed to the paper/publication entitled *Sonothrombolysis with BR38 microbubbles improves microvascular perfusion in a rat model of stroke* as outlined below:*

- 0% Conception and design of the experiments
- 0% Surgery and experimental work
- 70% Micro-CT analysis and interpretation of findings
- 70% Writing the paper and critical appraisal of the content
SONOTHROMBOLYSIS WITH BR38 MICROBUBBLES IMPROVES MICROVASCULAR PATENCY IN A RAT MODEL OF STROKE

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3.2 ABSTRACT

Background. Early recanalization of large cerebral vessels in ischaemic stroke is associated with improved clinical outcome, however persisting hypoperfusion leads to poor clinical recovery despite large vessel recanalization. Limited experimental sonothrombolysis studies have shown that addition of microbubbles during treatment can improve microvascular patency. We aimed to determine the effect of two different microbubble formulations on microvascular patency in a rat stroke model.

Methods. We tested BR38 and SonoVue® microbubble-enhanced sonothrombolysis in Wistar rats submitted to 90 minute filament occlusion of the middle cerebral artery. Rats were randomised to treatment (n=6/group): control, rt-PA, or rt-PA+3-MHz ultrasound insonation with BR38 or SonoVue® at full or 1/3 dose. Treatment duration was 60 minutes, beginning after withdrawal of the filament, and sacrifice was immediately after treatment. Vascular volumes were evaluated with microcomputed tomography.

Results. Total vascular volume of the ipsilateral hemisphere was reduced in control and rt-PA groups (p<0.05), but was not significantly different from the contralateral hemisphere in all microbubble-treated groups (p>0.1).

Conclusions. Microbubble-enhanced sonothrombolysis improves microvascular patency. This effect is not dose- or microbubble formulation-dependant suggesting a class effect of microbubbles promoting microvascular reopening. This study demonstrates that microbubble-enhanced sonothrombolysis may be a therapeutic strategy for patients with persistent hypoperfusion of the ischaemic territory.
3.3 INTRODUCTION
Transcranial ultrasound combined with microbubbles augments thrombolysis in clinical studies and experimental animal models of acute intracranial arterial occlusion [87-89, 117]. Application of transcranial color-coded duplex sonography (TCCS) in conjunction with IV recombinant tissue-type plasminogen activator (rt-PA) and microbubbles reduces time to recanalization and increases the total numbers of patients with recanalization [88, 89]. In addition to large vessel recanalization, this therapy has the potential to increase perfusion of the cerebral microvasculature in areas of hypoperfusion [117].

Microbubbles are ultrasound contrast agents used diagnostically and, when administered with rt-PA and ultrasound, have the potential to improve thrombus dissolution [96]. Although the mechanism of thrombus dissolution by ultrasound and microbubbles is not fully understood, it has been previously hypothesised that administration of microbubbles dramatically lowers the threshold for ultrasound-induced cavitation that causes mechanical stress on thrombi, leading to destabilisation and subsequent thrombus dissolution [96, 98]. Hence, microbubbles lower the energy requirements for producing sonothrombolysis thereby increasing the lytic activity of ultrasound [98].

Despite successful recanalization of large arteries, the affected ischaemic territory may remain hypoperfused. This is likely due to a “no reflow phenomenon” of the microcirculation whereby fibrin deposits and cell detritus occlude small cerebral vessels during the stagnant flow conditions of ischaemia [65, 66]. While large vessel recanalization is an important therapeutic approach for stroke, growing evidence suggests that reperfusion is a better predictor of good clinical outcome [11, 61, 68]. The diagnostic ultrasound contrast agent, SonoVue®, containing sulfur hexafluoride microbubbles, has been shown to safely re-establish microcirculatory patency completely in an experimental study of stroke when administered in conjunction with TCCS and rt-PA [117]. Treatment with rt-PA alone did not restore microvascular patency after middle cerebral artery (MCA) recanalization [117]. The present study was conducted to evaluate the treatment effects of the diagnostic agent SonoVue® and a new microbubble agent, BR38, with regards to reversal of perfusion deficits after stroke caused by occlusions of the microcirculation. BR38 is especially designed for usage in therapy of acute intracranial artery occlusion. Details of the respective properties and characteristics of SonoVue® and BR38 are outlined in Table 3-1.
Table 3-1. Properties of SonoVue® and BR38 Microbubbles

<table>
<thead>
<tr>
<th></th>
<th>SonoVue®</th>
<th>BR38</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gas</strong></td>
<td>100% sulfur hexafluoride (SF₆)</td>
<td>35% perfluorobutane (C₄F₁₀), 65% nitrogen (N₂)</td>
</tr>
<tr>
<td><strong>Stabilizer</strong></td>
<td>phospholipid</td>
<td>phospholipid</td>
</tr>
<tr>
<td><strong>Charge</strong></td>
<td>negative</td>
<td>neutral</td>
</tr>
<tr>
<td><strong>Microbubble diameter</strong></td>
<td>2.5 µm (0.7 – 10.0 µm)</td>
<td>1.4 µm (&lt;1.0 – 6.0 µm)</td>
</tr>
<tr>
<td><strong>Resuspension with saline</strong></td>
<td>5.0 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td><strong>Number of microbubbles in resuspension</strong></td>
<td>4 x 10^8</td>
<td>4 x 10^8</td>
</tr>
<tr>
<td><strong>Full dose</strong></td>
<td>4 doses i.v., 15 min intervals</td>
<td>4 doses i.v., 15 min intervals</td>
</tr>
<tr>
<td></td>
<td>10 µl of resuspension with 90 µl saline</td>
<td>10 µl of resuspension with 90 µl saline</td>
</tr>
<tr>
<td><strong>1/3 dose</strong></td>
<td>4 doses i.v., 15 minute intervals</td>
<td>4 doses i.v., 15 minute intervals</td>
</tr>
<tr>
<td></td>
<td>33.3 µl of full dose with 66.7 µl saline</td>
<td>33.3 µl of full dose with 66.7 µl saline</td>
</tr>
<tr>
<td><strong>Volume per application</strong></td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td><strong>Total volume administered</strong></td>
<td>0.4 ml</td>
<td>0.4 ml</td>
</tr>
</tbody>
</table>

3.4 METHODS

3.4.1 Animal preparation
All procedures were performed in accordance with institutional guidelines and the German 5 animal protection legislation and were approved by the Regierungspraesidium Darmstadt 6 animal care and use committee (Approval number Az. B2/257). Rats were administered 5 mg/kg Carprofen (Rimadyl™, Pfizer, Germany) subcutaneously 30 minutes prior to surgery, anesthetized with 5% isoflurane and maintained during surgery via a facial mask with 2-3% isoflurane in air (0.5 L/min). Rectal temperature was maintained at 37.0°C (± 0.25°C) with a circulating water heating pad.
A total of 44 male Wistar Unilever rats (Harlan Winkelmann, Germany) weighing 275-350 g underwent 90 minute right hemispheric MCA occlusion as previously described [117]. Briefly, the right common carotid artery was exposed and a silicone-coated nylon suture (4-0) was inserted and advanced proximally until mild resistance was felt. This indicated that the suture tip had reached the anterior cerebral artery, occluding blood flow to the right MCA (18-22 mm beyond the carotid bifurcation). At 90 minutes post-occlusion, recanalization of the MCA was established by withdrawing the suture. Success of occlusion was confirmed by Laser Doppler Flowmetry (LDF) (OxyFlo 2000®; Oxford Optronix, England) of the regional cerebral blood flow (rCBF). For LDF, the skull was exposed after local anaesthesia with 2% Lidocaine (Xylocaine®, AstraZeneca, Germany). rCBF was measured as described by Soehle et al [165] at 15 locations above the MCA territory measured pre-occlusion (baseline) and post-thread insertion to confirm occlusion (Figure 3-1). LDF values were recorded as blood perfusion units (BPU) prior to suture insertion and occlusion was confirmed by a drop in BPU to <1000.

3.4.2 Experimental groups
Immediately following withdrawal of the occluding filament at 90 minutes, animals were randomised to six treatment groups (n=6/group): control, rt-PA, or rt-PA+3-MHz ultrasound insonation with BR38 or SonoVue® at full or 1/3 dose (Table 3-1). An ultrasound or tPA+ultrasound group were not included because these were previously compared to microbubble treatment [117]. Randomisation was done in a blinded manner so the surgeon was unaware of treatment allocation. Treatment duration was 60 minutes.

![Figure 3-1. Experimental timeline.](image)

Microbubble-enhanced sonothrombolysis was tested in a model of 90 minute MCA occlusion. Regional cerebral blood flow (rCBF) was measured by laser doppler flowmetry to confirm successful occlusion (post-occlusion). Randomisation to treatment group occurred post-recanalization and immediately pre-treatment onset. rt-PA was administered every 5 minutes (dashed lines) in all treatment animals. Microbubbles (SonoVue or BR38) were administered at 15 minute intervals (▼). Control and rt-PA treatment groups received equal volumes of saline for rt-PA and microbubbles. Continuous ultrasound was applied for 60 minutes in conjunction with rt-PA and microbubble infusion.
Full dose SonoVue® was calculated by comparing the microbubble concentration in the blood volume of humans with the concentration in the blood volume of rats. The bubbles per ml of full dose BR38 equates to the bubbles per ml of full dose SonoVue® (Table 3-1). Treatment agents were resuspended in saline and administered via the tail vein. To permit dosing of microbubbles through the same catheter, 10 mg/kg rt-PA (Actilyse®; Boehringer Ingelheim, Germany) was delivered by small bolus injection every 5 minutes over 1 hour. The first injection was equivalent to a 10% loading dose. SonoVue® and BR38 (Bracco Suisse SA, Switzerland) were administered at four time points (Figure 3-1). Animals in control and rt-PA groups received equal volumes of saline.

A 3-MHz diagnostic ultrasound probe was placed 40 mm above the skull (B-mode, color-Doppler functions switched on, maximum output (mechanical index of 1.7)) (Sonos 7500; Philips Ultrasound, USA). The distance between the skull and ultrasound probe was bridged with ultrasound gel (Sonosid®; Asid Bonz, Germany). The beam was aligned to expose the entire brain (spectral Doppler sample volume placed in the midbrain (57 mm)). In the control and rt-PA groups, the probe was positioned but not turned on.

### 3.4.3 Post-mortem preparation
Animals were deeply anesthetized post-treatment and transcardially perfused with heparinized saline. Ligations were placed at the origin of the aortic arch, between the left common carotid and subclavian artery, on the innominate artery and the left external carotid artery. A radiopaque agent (Microfil®; Flow Tech, USA) was infused through the aortic arch for the in situ preparation of the cerebral vessels, the brains were harvested and fixed in formalin until analysis.

### 3.4.4 Micro- and nano-computed tomography (CT)
The X-ray system has previously been described in detail [180] (SkyScan® 1072_80kV; Belgium). The method of analysis was as previously described [117] with a few modifications. Briefly, a coronal aligned section of 3 mm thickness was cut out of each brain for detailed scans of the region with an isotropic voxel size (11.6 µm side length), encompassing the MCA territory (Figure 3-2). The total contrast agent volume represents the sum of all pixels marked as contrast agent after thresholding. The total contrast agent volume equals the total vascular volume (mm³). Regions of interest (ROI) were manually determined in the right and left hemispheres to encompass striatum and cortex using Analyze™ 9.0 (Biomedical Imaging Resource, Mayo Clinic, USA). These regions were chosen as they are the most commonly affected regions in this stroke model. ROI templates were applied to each scan that were equally sized for cortex or striatum and positioned equidistant from the midline in respective regions and hemispheres (Figure 3-2). The vascular volume fraction (VVF) of each hemisphere was the sum of the cortical and striatal ROIs for that hemisphere, and represented a fraction of the total vascular volume. Nano-CT scans were performed on 2 samples (control and BR38 full dose) to illustrate changes
in small arteries in the cortical region at high resolution. A cylindrical sample with a diameter of 2 mm was scanned using nano-CT (Skyscan 2011, Bruker Micro-CT, Kontich, Belgium) with a spatial resolution of 2.5 µm isotropic voxel side length. The nano-CT system has previously been described in detail [181].

### 3.4.5 Histology

After micro-CT analysis, brains were trimmed using a matrix, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin. Microscopic examination was performed by a veterinary pathologist experienced in brain tissue histology in a blinded fashion. As typical infarcts are not expected so early in the development of the lesion, the acute ischaemic changes (AIC) were characterized by vacuolation of the nervous tissue and/or degenerating neurons. Areas with AIC were measured using NIS Elements software (version 1.10, Nikon). AIC volumes were calculated from the section encompassing the MCA territory.

---

**Figure 3-2. Micro-CT scan for vascular volume.** Representative scans of the 3 mm slice encompassing the MCA territory (A) from a control and a BR38 full dose treated rat. Cortical (B) and striatal (C) regions of interest (dashed boxes in A) were analysed in each hemisphere to quantify vascular volume. These scans demonstrate reduced vascular volume in both cortical and striatal regions of the ipsilateral hemisphere (right panels) of the control rat, while demonstrating normal microvascular perfusion after BR38 microbubble-enhanced sonothrombolysis.
3.4.6 Inclusion and exclusion criteria
Animals were included if post-occlusion regional cerebral blood flow (rCBF) declined to <1000 BPU. Prespecified exclusion criteria were: unstoppable bleedings during surgery (n=1), perforation of the skull bone during laser Doppler flowmetry (LDF) preparation, inability to advance the occluding filament more than 18 mm beyond the carotid bifurcation, subarachnoid haemorrhage (SAH) caused by the occlusion (n=2), obliteration of the tail vein during treatment delivery (n=2), or insufficient Microfil® perfusion as judged by perfusion of the contralateral hemisphere (n=3). These criteria were not associated with treatment effect and each excluded animal was replaced.

3.4.7 Statistical analysis
Statistical analysis was performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, US) and Graphpad Prism 6 (Graphpad Software, Inc. CA, USA). Micro-CT of VVF in hemispheres were analysed using two-sided, paired t-test. LDF post-occlusion is presented as a percentage of pre-occlusion baseline. Differences between groups were analysed by one-way ANOVA with Bonferroni post-hoc analysis for multiple comparisons. Acute ischaemic changes were analysed using Kruskal-Wallis with Dunn’s multiple comparison test. Data are presented as mean ± s.d. and \( p < 0.05 \) was considered significant.

3.5 RESULTS
Occlusion of the MCA by filament insertion resulted in an rCBF drop to 59.2 ± 13.0% of baseline for all animals (n=36). There were no significant differences between groups (\( p > 0.05 \)): Control 55.0 ± 6.6%, rt-PA 55.1 ± 13.9%, BR38 full dose 71.3 ± 3.3%, BR38 1/3 dose 64.2 ± 10.7%, SonoVue® full dose 51.5 ± 16.1%, and SonoVue® 1/3 dose 58.1 ± 15.7%.

3.5.1 Micro- and nano CT
The VVF of control and rt-PA treated groups was significantly reduced in the infarcted hemisphere compared to the corresponding contralateral hemisphere (58% and 60% respectively, \( p < 0.05 \)). The VVF of the ipsilateral hemisphere of all microbubble-sonothrombolysis groups was not significantly different from the contralateral hemisphere (Figure 3-3). Figure 3-4 is an illustrative nano-CT scan showing higher resolution of the cortical region of a control and BR38 full dose animal to demonstrate greater microvessel patency in the microbubble-sonothrombolysis treated animal.
**Figure 3-3. Vascular volume of the ipsilateral hemisphere.** Total vascular volumes (VV) of the ipsilateral hemisphere quantified from micro-CT scans are presented as a percentage of the vascular volume of the contralateral (left) hemisphere. Control and rt-PA ipsilateral total VV were significantly reduced from contralateral total VV. *p<0.05.

**Figure 3-4. Nano-CT scan of cortex.** Nano-CT images illustrating the “no reflow phenomenon” in an untreated animal after the withdrawal of the MCA filament (A), and reperfusion of the microvasculature after BR38 (full dose) sonothrombolysis.
Histological examination was performed on control, rt-PA and BR38 full dose groups. One animal was excluded from analysis due to too many post-mortem artifacts (rt-PA group). AIC were observed in all animals and were mostly detectable within the 3 mm brain section encompassing the MCA territory (striatum and/or cortex). The AIC volume was 15.1 ± 7.9 mm$^3$, 32.0 ± 19.2 mm$^3$ and 43.3 ± 26.4 mm$^3$ for the BR38 full dose, rt-PA and control group respectively (Figure 3-5) with a significant difference between control and BR38 full dose groups ($p=0.044$). The presence of the radiopaque agent, Microfil, within the blood vessels made examination for haemorrhage difficult, however haemorrhage was observed in 3 animals. Slight focal haemorrhage around a thrombosed vessel was observed in two animals: rt-PA (n=1) and BR38 full dose (n=1) groups. Another animal (rt-PA group) exhibited slight to moderate multifocal SAH as well as minimal to slight multifocal perivascular haemorrhage within the ischaemic tissue. Reanalysis of VVF and AIC excluding the animal with SAH made no difference to significance.

**DISCUSSION**

We have demonstrated improved microvascular patency in a stroke model of cerebral “no reflow” following microbubble-enhanced sonothrombolysis. We found no difference in the effectiveness of two microbubble formulations, BR38 and SonoVue®, irrespective of dosage. Our data demonstrate that deficits of microvascular patency can persist after recanalization of major cerebral arteries and that rt-PA alone is not sufficient to improve this deficit. Currently, there are

![Figure 3-5. Volume of ischaemic lesion.](image-url)
no specific therapies for microvascular occlusion, yet incomplete reperfusion occurs in approximately a quarter of patients with successful recanalization [11, 182]. Clinical reports now indicate that reperfusion, rather than recanalization, is a better predictor of good clinical outcome after acute ischaemic stroke [11, 61, 68]. Hence, development of therapies that target both large- and micro-vessel occlusions may prove beneficial for improving patient outcome. Microbubble-enhanced sonothrombolysis could be a potential candidate.

Sonothrombolysis with BR38 microbubbles has been demonstrated to lyse large thrombi in vitro [159], but its effect on restoring microvasculature patency was unknown. Consistent with our previous micro-CT analysis of SonoVue®-enhanced sonothrombolysis [117], this study indicates that microbubble-enhanced sonothrombolysis may be beneficial beyond targeting the main thrombus, by also improving microvascular patency. This could be of particular benefit to patients whose main artery recanalizes but in whom hypoperfusion of the ischaemic territory persists. However, the onset time of treatment in our study limits translation of these results. This model was chosen because microthrombosis is known to occur after recanalization [117]. Clinically, sonothrombolysis after recanalization, when recanalization is achieved with tPA, is not feasible given the increased haemorrhage risk. But the results of this study provide evidence that microbubble-enhanced sonothrombolysis restores microvascular patency and should prompt further investigation into the potential clinical application of this strategy.

It was expected that BR38 microbubbles would improve microvascular patency to a greater degree because BR38 circulate for longer and are smaller in size than SonoVue®, therefore potentially reaching smaller vessels of the brain. Yet, microvascular patency was improved to the same extent, regardless of microbubble formulation or dosage. Clinical testing of various microbubble formulations (Levovist®, Definity®, SonoVue®) has demonstrated increased large vessel recanalization and improved functional outcome [87-89, 161]. Our findings correlate with two previous clinical studies comparing microbubble formulations [183] and dosages [87] that demonstrated comparable outcomes of recanalization rates and time to recanalization between treatment groups. This, together with our results, suggests a class effect of microbubbles for enhancing sonothrombolysis in both small and large vessels. Specific microbubble design or dosage may be less important. However, we did not see complete return to contralateral values of vascular volume, suggesting that despite microbubble therapy, there is still a small degree of persistent microvascular occlusion. It is unknown whether different ultrasound parameters, or longer duration therapy may return these vascular volumes to contralateral values completely, but the results of this study indicate that further investigation is warranted.

In this study we observed occlusion of the penetrating arterioles (Figures 3-2 and 3-4). Low perfusion pressures within penetrating arterioles due to capillary occlusion could cause
incomplete perfusion of the contrast agent, overestimating total occlusion. Yet, a clear ipsilateral
deficit is observed indicating that occlusions are related to the stroke. Our results suggest
microbubble-enhanced sonothrombolysis can restore vascular filling but the exact location of
thrombosis would require further investigation. Occlusion of arterioles has been implicated as a
possible cause of lacunar infarction and evidence suggests occlusion of single penetrating
arterioles results in significant neuronal death [184]. While it was not our intention to study
lacunar infarction, with more preclinical testing, microbubble-enhanced sonothrombolysis may
be a potential therapeutic for this condition.

Histological assessment of acute ischaemic changes demonstrated reduced lesion size following
microbubble-enhanced sonothrombolysis and restoration of vascular volume. This correlates with
clinical evidence that although large vessel recanalization is associated with good outcome,
reperfusion may be a more accurate predictor of final infarct volume [11, 61, 68]. Although
histological results are in agreement with Micro-CT results, some limits do apply. The presence
of Microfil and post-mortem tissue artifacts made the histological analysis challenging and in the
section encompassing the MCA territory, some areas could not be evaluated for ischaemic lesion
due to the nature of the sectioning process for micro- and nano-CT analysis. Microfil perfusion
was required for analysis of our primary outcome, forsaking vascular histology that might identify
exact sites of thrombosis within arterioles and capillaries. Additionally, nano-CT provides higher
resolution images and better definition of the microvessels, as illustrated in Figure 3-4,
however because of the superiority of micro-CT for imaging the entire MCA territory without
further manual sectioning of the tissue, it was chosen for quantification. Differences between
microbubble formulations or dosages may have been more apparent on nano-CT had it been
suitable for quantifying the whole MCA territory, since larger capacitance vessels may
disproportionately influence total vascular volumes.

We saw no difference in degree of haemorrhage in the BR38 microbubble group compared with
rt-PA only, correlating with clinical stroke studies of high-frequency ultrasound microbubble-
enhanced sonothrombolysis that report no significant increases of sICH rates [83]. It appears that
the concerns regarding excessive rates of bleeding first raised in the TRUMBI clinical trial [110],
largely pertain to the use of low-frequency ultrasound [110, 115] and high-dose microbubbles
[87]. This study is limited for assessing haemorrhage, specifically due to small numbers and the
animal model. Caution should also be observed when comparing rat to human data for
sonothrombolysis safety. The causes of haemorrhage related to ultrasound are likely to differ with
respect to skull size and insonation field size. Because this was a study of reperfusion efficacy,
correlation of these findings with safety outcomes should be performed in larger animals.
Additionally, TCCS and higher dose microbubbles have caused increased haemorrhage rates for
clinical large vessel sonothrombolysis [84, 87]. While the results of this study should prompt
further study of sonothrombolysis for microvascular occlusion, safety outcomes should be assessed before clinical testing.

Our study indicates that microbubble-enhanced sonothrombolysis may restore perfusion deficits that persist even after large vessel recanalization in a model of acute ischaemic stroke by restoring microvessel patency. This was observed in all microbubble-treated animals irrespective of microbubble formulation or dosage. This finding indicates the potential of this treatment strategy for future clinical use in patients with persistent occlusion of the microvasculature, for which there is currently no direct therapy.

3.7 ADDITIONAL INFORMATION

3.7.1 Acknowledgments and funding sources
We would like to acknowledge the technical assistance of Mrs Gunhild Martels in micro-CT scanning and analysis.

Ms Amelia Tomkins was supported by a Heart Foundation Australia post-graduate scholarship cofunded by the National Stroke Foundation Australia, and by a Hunter Medical Research Institute Travel Scholarship funded by Mrs Jennie Thomas. Dr Neil Spratt was supported by an Australian National Health and Medical Research Council Career Development Fellowship (APP1035465). The study was in part supported by Bracco Suisse SA, Geneva, Switzerland and in part from the Faculty of Human Medicine, Justus-Liebig University, Giessen and the German Research Foundation (NST 162/291-1 FUGG).

3.7.2 Author contributions
Study design was by MN, TG, MKaps and GAK. Samples were prepared by NS, AJT, MJ and MY. Micro-CT data collection and analysis was by AT, MKampschulte, and MN. Histology and analysis was by J-MH and CB. Data interpretation was by NS, AT, J-MH, CB, MKaps, NJS, TG and MN. The manuscript was prepared by NS, AT, MKampsctule, GAK, NJS and MN.

3.7.3 Competing interests
JMH and CB are employees of Bracco Suisse SA, part of Bracco Group. All other authors declare no conflicts of interest.
Chapter 4

4 SONOTHROMBOLYSIS OF EXTRACRANIAL CAROTID ARTERY OCCLUSION WITH VARIABLE STENOSIS

4.1. Study aims, rationale and hypotheses ................................................................. 74

Publication: *Thrombolytic recanalization of carotid arteries is highly dependent on degree of stenosis, despite sonothrombolysis* ................................................................. 76

4.2. Abstract ............................................................................................................... 77

4.3. Background ....................................................................................................... 78

4.4. Methods ............................................................................................................ 78

  4.4.1. General animal details ..................................................................................... 78

  4.4.2. Model of carotid artery occlusion with stenosis .............................................. 78

  4.4.3. Statistical analysis ......................................................................................... 79

4.5. Results ............................................................................................................... 82

4.6. Discussion ......................................................................................................... 84

4.7. Additional information .................................................................................... 85

  4.7.1. Funding sources ............................................................................................ 85

  4.7.2. Author contributions .................................................................................... 85

  4.7.3. Competing interests .................................................................................... 85
4.1 Study Aims, Rationale and Hypotheses

In this chapter, I describe a model of carotid artery occlusion with variable stenosis that was used to test the effect of stenosis on thrombolysis and sonothrombolysis. The specific aims of this study were:

**Primary aim:** to determine the effect of the degree of stenosis on thrombolytic recanalization rates.

**Secondary aim:** to determine whether sonothrombolysis enhances tPA recanalization rates in carotid artery occlusion

**Rationale and hypotheses**

Acute ischaemic stroke is not always due to occlusion of cerebral arteries. Acute occlusion of the carotid artery can cause cerebral ischaemia, often occurring in tandem with an intracranial occlusion. Thrombolysis with tPA is only ~10-30% effective in patients with carotid occlusions and is an independent predictor of neurological worsening [9, 21, 69]. Occlusion of the carotid artery is commonly caused by stenosis of the vessel due to atherosclerotic plaque. Plaque rupture can cause thrombosis and vessel occlusion. It is known that a severe carotid stenosis increases a patient’s risk for stroke, but occlusion with a mild underlying stenosis is likely more common. I hypothesised that the degree of underlying stenosis would play a key determinant role in the efficacy of thrombolysis with tPA. To test this, I developed a model of extracranial carotid thrombosis with vascular stenosis, designed for testing recanalization. In this model, the ICA was ligated to prevent cerebral ischaemia (which is highly variable from extracranial occlusions) and to allow accurate monitoring of occlusion and recanalization along a single section of artery. Model design is discussed in more detail within this chapter.

While sonothrombolysis has been shown to be effective at recanalizing intracranial vessels, there are limitations of this therapy related to the skull. For instance, while low frequency ultrasound is effective for clot lysis *in vitro*, transcranial application causes significant increases in the incidence of symptomatic intracerebral haemorrhage [110]. This is most likely due to the propagation of standing waves within the skull [104]. Additionally, power is attenuated through the skull bone by as much as 90% [102, 104, 105]. The idea of using sonothrombolysis for extracranial occlusions, should allow for adjustment of ultrasound parameters to optimise thrombolysis without the negative impacts of the skull. I hypothesised that in a model of carotid artery occlusion, with differing degrees of stenosis, ultrasound could enhance the recanalization effects of tPA thrombolysis.
At the time of thesis submission, the work in this chapter was accepted for publication in the *Journal of the American Heart Association*.


The following statement has been accepted by all co-authors to this study. Signed statements are presented in Appendix A.

*We, the co-authors, attest that Research Higher Degree candidate, Amelia Tomkins, contributed to the paper/publication entitled *Thrombolytic recanalization of carotid arteries is highly dependent on degree of stenosis, despite sonothrombolysis* as outlined below:*

- 60% Conception and design of the experiments
- 95% Surgery and experimental work
- 90% Analysis and interpretation of findings
- 70% Writing the paper and critical appraisal of the content
THROMBOLYTIC RECANALIZATION OF CAROTID ARTERIES IS HIGHLY DEPENDENT ON DEGREE OF STENOSIS, DESPITE SONOTHROMBOLYSIS

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4.2 Abstract

Background: Stroke associated with acute carotid occlusion is associated with poor effectiveness of tissue plasminogen activator (tPA) thrombolysis and poor prognosis. Rupture of atherosclerotic plaques resulting in vascular occlusions may occur on plaques causing variable stenosis. We hypothesised that degree of stenosis may affect recanalization rates with tPA. Ultrasound + tPA (sonothrombolysis) has been shown to improve recanalization for intracranial occlusions but has not been tested for carotid occlusion. Our primary aim was to determine thrombolytic recanalization rates in a model of occlusion with variable stenosis, with a secondary aim to investigate sonothrombolysis in this model.

Methods and Results: Rat carotid arteries were crushed and focal stenosis created (25% baseline Doppler flow) with a silk-suture tie invoking thrombosis and occlusion. To model mild or severe stenosis, the tie was released pre-treatment or left in place. Animals were treated with tPA (10 mg/kg) or tPA + ultrasound (2-MHz) in each stenosis model (n=7/group). Recanalization was assessed by Doppler flow. Thrombolytic recanalization rates were significantly higher in mild stenosis groups (71% vs 0% with severe stenosis; \( p<0.0001 \)). Recanalization rates were not significantly higher with additional ultrasound in either model.

Conclusion: In this model, the degree of carotid stenosis had a large effect on thrombolytic recanalization. Sonothrombolysis using standard parameters for intracranial sonothrombolysis did not increase recanalization. Further testing is warranted. The degree of underlying stenosis may be an important predictor of thrombolytic recanalization and clinical correlation of these findings may provide new approaches to treatment selection for patients with carotid occlusion.
4.3 BACKGROUND

Carotid artery occlusion can lead to devastating consequences such as stroke. Patients with carotid occlusion-associated stroke have high rates of death and disability but treatments are limited. Successful recanalization with intravenous tPA, the current stroke thrombolytic, is only achieved in 10-30% of patients with carotid occlusion[9, 21, 69]. Although a severe carotid stenosis predicts a greater risk of stroke for any individual[185], the population frequency of mild stenosis is greater. Hence, acute occlusion of the carotid artery that leads to stroke may occur with varying degrees of carotid stenosis. We hypothesized that the degree of underlying stenosis of carotid artery occlusion may be an important predictor of successful thrombolytic recanalization. Given the low efficacy of tPA in carotid athero-thrombomatic occlusion, approaches to enhance its effect are required. Ultrasound + tPA (sonothrombolysis) appears promising for improving intracranial recanalization[84], but has not been tested in extracranial carotid artery occlusion. Therefore, our primary aim was to determine whether the degree of carotid stenosis affected rates of sustained recanalization with thrombolysis at 4.5 hours post-occlusion. Our secondary aim was to determine whether ultrasound enhances sustained tPA recanalization rates in carotid occlusion.

4.4 METHODS

4.4.1 General animal details

Experiments were approved by the Animal Care and Ethics Committee of the University of Newcastle, Australia (Approval No. A-2010-128) and performed in compliance with requirements of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes [186]. Male Wistar rats (n=28; 311-417 g; Animal Resources Centre, Perth, Australia) were anesthetised with 5% Isoflurane and maintained with 1.5-2% in 30/70% O₂/N₂. Rectal temperature was maintained at 37°C with a feedback controlled heat mat (Faculty of Health Workshop, University of Newcastle, Australia). Heart rate, respiration and oxygen saturation were monitored throughout surgery.

4.4.2 Model of carotid artery occlusion with stenosis

To create carotid artery occlusion with variable degrees of stenosis, a modification of the Folts model was used [151]. A timeline and surgical schematic are presented in Figure 4-1. The right carotid arteries were exposed and a 20-MHz, 0.8 mm Doppler flow probe (Iowa Doppler Products, USA) was positioned over the external carotid artery, distal to the intended injury/stenosis site. Flow was monitored audibly through the Doppler instrumentation and visually via LabChart software (ADInstruments, Australia). Since the primary outcome was recanalization of the occluded common carotid artery, the internal carotid artery was ligated. This was done to avoid the potential confounding effects of variable strokes that may result from thromboembolism. A 5-0 silk suture with a double throw tie was placed around the common carotid artery at the
intended stenosis site, but not tightened. Using haemostats guarded with tape, the common carotid artery was crushed 3 times (30 second crush, 30 second rest) to disrupt the endothelium, exposing pro-thrombotic factors. Crushes were made over and directly adjacent to the suture. Following the third crush, a stenosis was created by tightening the suture over the site of injury, reducing blood flow by 75% of pre-crush baseline flow. Flow was monitored for cyclic patterns until complete occlusion was achieved. Complete occlusion was confirmed by cessation of audible flow and irregular flow trace (Figure 4-2A,B). Up to 2 additional crushes were made if: 1) continuous flow was maintained for 10 minutes after the crush cycle, or 2) if cyclic flow patterns including occlusion/recanalization events were observed for 30 minutes post-crush with no complete occlusion.

To model mild or severe stenosis, the tie was loosened (mild) or left in place (severe) after 45 minutes of stable occlusion. If flow spontaneously returned pre-treatment (n=1), the vessel was re-crushed and re-observed for 60 minute occlusion. For each stenosis protocol, animals were randomised to tPA, or tPA + ultrasound. tPA (10 mg/kg, Alteplase, Boehringer Ingelheim, Australia) was administered intravenously via the femoral vein at 60 minutes post-occlusion (10% bolus over 1 minute, remainder over 1 hour). For ultrasound groups, the skin was sutured, covered with Tegaderm™ (3M, North Ryde, Australia) and the ultrasound probe positioned over the occlusion using a laboratory-manufactured spacer containing ultrasound gel. Insonation began with the start of tPA delivery, continuing for 2 hours (2-MHz, 720 mW/cm², 25 mm depth, sample volume 10 mm; EZ-Dop®, DWL Compumedics, Germany). Recanalization was monitored at 30 minute intervals after tPA onset until 4.5 hours post-occlusion. Recanalization was confirmed by audible flow return and a regular LabChart flow trace (Figure 4-2A,C).

4.4.3 Statistical analysis
Baseline time for analysis was 60 min post-occlusion, immediately prior to tPA administration. Recanalization was reported as sustained when a return of flow was observed and remained until the final observation time (4.5 hours post-occlusion). Rates of sustained recanalization (number of animals with sustained recanalization from the total cohort) were compared using log-rank test with differences considered significant if $p<0.05$ (Graphpad Prism 6). Investigation of degree of stenosis was performed by pooling tPA and tPA + ultrasound groups per stenosis model (Figure 3B). Effect of treatment on sustained recanalization was analysed for each stenosis model.
Figure 4-1. Experimental timeline [A] and surgery schematic [B] of carotid artery occlusion with stenosis. Branching arteries were cauterised (superior thyroid and occipital arteries), and the internal carotid artery (ICA) was ligated to prevent embolism to the brain. A flow probe was placed over the external carotid artery (ECA) and baseline flow monitored for 5 minutes (depicted in blue in [B]). The stenosis suture was placed around the common carotid artery (CCA) but not tightened. Three 30 second crushes ([A] red) were made over and immediately adjacent to the suture with 30 second rest periods between crushes. The suture was tightened to reduce flow by 75% and flow was monitored for occlusion. Following 45 minutes of stable occlusion, the stenosis suture was either loosened to create mild stenosis or left in place for a severe stenosis. Treatment began 60 minutes post-occlusion. Flow was monitored every 30 minutes post-treatment onset for any signs of recanalization ([A] green arrows).
Figure 4-2. Example flow trace of crush, occlusion and recanalization. [A] Doppler Flow recorded via LabChart (ADInstruments, Australia) demonstrating crush injury (▲), stenosis (at -10 min), flow decrease to occlusion (grey shading B), and recanalization (grey shading C). [B] Flow decrease to occlusion, correlating with grey shading labelled B in [A]. At low flow rates, audible signal was a better indicator of flow due to “noise” of the flow trace, as can be seen around the point of occlusion in [B]. [C] Recanalization correlating with grey shading labelled C in [A].
4.5 RESULTS

No animals were excluded. One animal died prior to final observation (mild stenosis-tPA group). This animal had fluctuating body temperatures throughout surgery and high temperatures (>39°C) leading up to its death. It was included in the primary analysis and re-analysis excluding this animal did not change the reported results.

Exact flow values relative to baseline are not presented because closing the surgical site for ultrasound insonation affected the Doppler flow trace relative to baseline, preventing accurate judgement of the degree of recanalization. However, flow return was clearly discernible irrespective of skin closure (Figure 2C). Therefore, in the interests of accuracy, we chose sustained recanalization as the marker of recanalization success, rather than attempting to quantify percentage recanalization.

The carotid artery of all animals had been occluded for 60 minutes at tPA onset. High rates of sustained recanalization were observed in the mild stenosis model compared to severe stenosis ($p<0.0001$; Figure 3A,B). In the mild stenosis-tPA group, all animals exhibited recanalization. Sustained recanalization was observed in 5/7 rats, and the remaining 2 animals had recanalization/reocclusion. Reocclusion occurred within 30 minutes of recanalization onset in both cases. In all, initial recanalization occurred between 30-90 minutes post-treatment onset. In severe stenosis-tPA, no recanalization was observed.

Sonothrombolysis had limited effects on recanalization in either mild or severe stenosis groups (Figure 3A). In mild stenosis-tPA + ultrasound, sustained recanalization occurred within 30 minutes of treatment onset in 5/7 rats, as was also seen without the addition of ultrasound ($p=0.67$). Recanalization/reocclusion occurred in 2/7 of severe stenosis-tPA + ultrasound rats, but none had sustained recanalization (Figure 3A).
Figure 4-3. Carotid artery recanalization in the setting of mild or severe stenosis. Recanalization rates are expressed as the percentage of animals recanalized per group (n=7/group). Recanalization was measured every 30 minutes post-treatment onset for 240 minutes (4.5 h post-occlusion). [A] Recanalization/reocclusion events for all animals with mild or severe stenosis treated with tPA alone or tPA + ultrasound (n=7/group). [B] Sustained recanalization (recanalization at endpoint) in mild (open triangle) and severe stenosis models, pooled treatment (*p<0.0001, Hazard Ratio=16.7(4.2-67.4, 95% confidence interval); n=14/group). Presence of ultrasound made no significant difference to the rates of sustained recanalization in either mild or severe stenosis models.
4.6 DISCUSSION

This study demonstrates a strong effect of the degree of carotid stenosis on thrombolytic recanalization after thrombotic carotid occlusion. Sustained recanalization rates were >70% with mild stenosis, compared to 0% with severe stenosis. These findings have potentially important clinical implications. If the degree of carotid stenosis has similar effects clinically and new imaging techniques enable degree of stenosis to be assessed, this could guide the choice of thrombolysis versus endovascular treatment for stroke patients with acute carotid occlusion. The conventional “rat dose” of tPA, 10 mg/kg, was chosen based on its common use in the literature. There is evidence that this dose exerts a maximum effect[187] and further dosage increases do not enhance recanalization[188]. This study did not demonstrate a benefit of sonothrombolysis over tPA alone for extracranial carotid occlusion.

The results provide evidence of the influence of stenosis on thrombolysis as well as providing a preclinical model for testing thrombolytics in extracranial carotid occlusions. Correlation with clinical data is, however, needed to confirm this effect in patients. There are differences between our acute stenosis model and patients with carotid stenosis and/or occlusion. In patients, stenosis due to atherosclerosis is generally due to chronic plaque build-up, while our model is an acute stenosis. Our model is not an absolute replicate of the human condition of atherothrombotic carotid stenosis and occlusion, yet it is an important step forward for experimental models of carotid occlusion. It mimics the stenotic narrowing of the artery, reduced blood flow, endothelial damage and exposure of subendothelial matrix proteins that promote thrombosis and cause carotid occlusion. Previous models of carotid occlusion have used ex vivo prepared clots injected into the vessel[189], or other non-physiological forms of thrombosis induction, such as FeCl₃[190]. Additionally, none of these models have investigated stenosis as a factor of thrombolytic efficacy. The results of our study indicate a clear difference in recanalization response to thrombolytic therapy with differing degrees of stenosis. It is this finding that should prompt further investigation of this effect clinically, and whether techniques can be developed to quantify the degree of underlying carotid stenosis. Our experimental data suggest that this may prove useful in the treatment allocation of patients with carotid occlusion-associated stroke – particularly in centres that may need to transfer patients for endovascular therapies.

In this study we used the standard ultrasound parameters used for transcranial sonothrombolysis[80, 84], however even in the absence of skull attenuation, there did not appear to be a large additive benefit over tPA alone. Ultrasound power is attenuated through the skull by as much as 99%[104, 105]. Yet evidence that higher power increases clot lysis[118] indicates that power attenuation likely limits the efficacy of intracranial sonothrombolysis. Additionally, there is evidence that low frequency ultrasound facilitates clot lysis[77, 118]. However, for intracranial occlusion, low frequency ultrasound has the potential to cause
the propagation of standing waves within the skull causing intracerebral haemorrhage\cite{104, 110}. In the setting of carotid occlusion, where there is not the risk of standing waves and brain bleeding, lower frequency and/or higher ultrasound power may be more effective for thrombolysis. This proposition requires further testing.

Our data suggest that severe carotid stenosis is a likely explanation for at least some of the failed tPA recanalization in patients with carotid occlusion. In our model, as in human athero-thrombotic carotid occlusion, the bulk of the thrombus forms distal to the stenosis, thereby limiting drug delivery to the thrombus. This is likely to be different to intracranial occlusions, which in Western populations are predominantly thrombo-embolic in non-stenosed vessels. Advanced computed tomography, magnetic resonance and carotid duplex imaging can evaluate both intraluminal flow and vessel wall characteristics and are also now being used to evaluate patient suitability for reperfusion therapies in acute stroke. The ability to determine the degree of carotid stenosis in patients with carotid occlusion may help guide the choice of therapy for patients with this devastating form of stroke.

4.7 ADDITIONAL INFORMATION

4.7.1 Funding sources
AT was supported by a National Heart Foundation/National Stroke foundation postgraduate scholarship. NS was supported by an NHMRC career development fellowship, #1035465.

4.7.2 Author contributions
AT, CN, CL and NS contributed to study design. Experimental work and data collection was performed by AT, RH and DP. Data analysis and interpretation and manuscript preparation was performed by AT and NS.

4.7.3 Competing interests
None
Chapter 5

5 TISSUE PLASMINOGEN ACTIVATOR FOR PRECLINICAL STROKE RESEARCH

5.1. Study aims, rationale and hypotheses
Publication: Tissue plasminogen activator for preclinical stroke research: Neither “rat” nor “human” dose mimics clinical recanalization in a carotid occlusion model

5.2. Abstract

5.3. Introduction

5.4. Results

5.5. Discussion

5.6. Methods

5.6.1. Animals

5.6.2. Carotid artery occlusion

5.6.3. Treatment groups

5.6.4. Recanalization

5.6.5. Statistics

5.7. Additional information

5.7.1. Acknowledgements and funding sources

5.7.2. Author contributions

5.7.3. Competing interests
5.1 Study Aims, Rationale and Hypotheses

In this chapter, I describe a model of carotid artery occlusion used to test the recanalization efficacies of increasing tPA doses. The specific aim of this study was:

To investigate recanalization rates of tPA doses ranging from the conventional human dose (0.9 mg/kg) to the rat dose (10 mg/kg) in a model of physiologically formed clots.

Rationale and hypotheses

There is a growing field of research into thrombolytic enhancers, yet it is not known what dose of tPA most effectively mimics clinical recanalization rates. Previous preclinical studies report high recanalization rates in different clot models with the conventional rat dose of tPA. However, for the testing of thrombolytic enhancers, high rates of recanalization with tPA alone does not allow room for improvement with alternative therapies. The rat dose of tPA is based on evidence that the rat fibrinolytic system is 10-fold less sensitive than the human system [152]. Studies have been published that demonstrate increasing tPA doses reduce infarct volumes [153, 191]. While possible that smaller infarcts relate to earlier or more complete recanalization, this hypothesis can only be inferred from these studies. Whether the current rat dose of tPA generates recanalization rates in a model of natural thrombosis that mimic clinical rates is yet to be determined. I hypothesised that in a model of naturally forming clot, a more clinically relevant dose of tPA for recanalization would lie between the traditional rat and human doses that are conventionally used. Although this study does not directly investigate the overall aim of this PhD – testing sonothrombolysis – a “human equivalent” tPA dose is important to consider for any future preclinical testing of sonothrombolysis, or any thrombolytic enhancer.
At the time of thesis submission, the work in this chapter had been accepted for publication in *Scientific Reports*.


The following statement has been accepted by all co-authors to this study. Signed statements are presented in Appendix A.

We, the co-authors, attest that Research Higher Degree candidate, Amelia Tomkins, contributed to the paper/publication entitled *Tissue Plasminogen Activator for preclinical stroke research: Neither “rat” nor “human” dose mimics clinical recanalization in a carotid occlusion model* as outlined below:

- 60% Conception and design of the experiments
- 95% Surgery and experimental work
- 90% Analysis and interpretation of findings
- 70% Writing the paper and critical appraisal of the content
TISSUE PLASMINOGEN ACTIVATOR FOR PRECLINICAL STROKE RESEARCH: NEITHER “RAT” NOR “HUMAN” DOSE MIMICS CLINICAL RECANALIZATION IN A CAROTID OCCLUSION MODEL

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5.2 Abstract

Tissue plasminogen activator (tPA) is the only approved thrombolytic therapy for acute ischaemic stroke, yet many patients do not recanalize. Enhancing thrombolytic efficacy of tPA is a major focus of stroke research. Traditionally, a “rat dose” of 10 mg/kg has been used in rodent models. Recent studies suggested that the clinical “human” dose (0.9 mg/kg) may better mimic clinical recanalization. These studies only compared the rat and clinical doses, and so we aimed to test recanalization efficacy of multiple tPA doses ranging from 0.9 to 10 mg/kg in a model of endothelial injury and vessel stenosis. The common carotid artery of rats was crushed and stenosed to allow in-situ occlusive thrombus formation (Folts model of ‘physiological’ thrombus). Intravenous tPA was administered 60 minutes post-occlusion (n=6-7/group). Sustained recanalization rates were 0%, 17%, 67% and 71%, for 0.9, 1.8, 4.5, and 10 mg/kg, respectively. Median time to sustained recanalization onset decreased with increasing dosage. We conclude that 10 mg/kg of tPA is too effective, whereas 0.9 mg/kg is ineffective for lysis of occlusive thrombi formed in situ. Neither dose mimics clinical tPA responses. A dose of 2x the clinical dose is a more appropriate mimic of clinical tPA recanalization in this model.
5.3 INTRODUCTION

Tissue plasminogen activator (tPA) is the only approved thrombolytic therapy for acute ischaemic stroke. Early recanalization of occluded vessels is associated with improved clinical outcome, yet less than 50% of all stroke patients treated intravenously with tPA will successfully recanalize [9]. In the setting of carotid artery occlusion, tPA is even less effective, with recanalization rates of 10-30% [21, 69]. There is a great need for improved therapies for stroke and one approach has been to enhance thrombolysis with adjuvant therapies such as sonothrombolysis [84]. For any new or adjuvant thrombolytic therapy, rigorous preclinical testing should occur and aim to mimic the clinical conditions of ischaemic stroke and tPA efficacy. There has been controversy regarding what a “human equivalent” dose of tPA is for preclinical research – particularly in rodents, in which most such studies are performed.

Traditionally, a dose of 10 mg/kg tPA has been used for rodents. This “rat dose” was based on an in vitro study from the early 1980’s that demonstrated that the fibrinolytic system of rats is 10-fold less sensitive than humans [152]. Two recent comparisons of rat and clinical doses in rats [158] and mice [155] both indicated that the clinical dose was a better mimic of the clinical situation [155, 158]. Additional doses were not compared in these studies. The typical method for determining a “human equivalent” dose of any therapeutic utilizes a conversion based on body surface area of humans to the target species [192]. For rats, this conversion requires multiplication of the human dose (0.9 mg/kg) by 6.2, indicating a “human equivalent” dose of 5.58 mg/kg tPA for rats. However, this conversion does not take in to account additional factors that may affect the fibrinolytic process other than body size, and is not generally used when converting doses of tPA for stroke research.

Multiple methods of forming experimental thrombi exist for preclinical stroke models. Formation of thrombi in situ or ex vivo and the presence or absence of added pro-thrombotic factors, such as thrombin and/or CaCl₂, leads to variability of final thrombus composition. All of these factors play a key role in the overall thrombolytic susceptibility of the thrombus and therefore the variability of tPA efficacy between studies [130, 138]. Ideally, a preclinical model for testing stroke thrombolytics and thrombolytic enhancers should use thrombi that closely mimic human stroke thrombi and have similar recanalization rates.

For this study, we developed a method of physiological thrombus formation by endothelial injury and stenosis of the carotid artery in rats. To determine which tPA dose best reflects clinical recanalization rates in this model, we aimed to investigate sustained recanalization rates of varying doses of tPA ranging from the clinical dose to the traditional rat dose. Time to sustained recanalization was a secondary outcome.
5.4 Results

To test the thrombolytic efficacy of varied doses of tPA on a physiological thrombus we used a rat model of carotid occlusion with a mild underlying stenosis. Recanalization was monitored every 30 minutes post-tPA delivery to 4.5 hours post-occlusion. Sustained recanalization, defined as recanalization without reocclusion, was observed in 0% of 0.9 mg/kg treated rats (0/6), 17% of 1.8 mg/kg treated rats (1/6), 67% of 4.5 mg/kg treated rats (4/6), and 71% of 10 mg/kg treated rats (5/7) (Fisher’s exact test, \( p = 0.015 \), Figure 5-1). Recanalization/reocclusion was observed in 2 animals, both in the 10 mg/kg dosage group. In pilot experiments we found that recanalization was easily confirmed (as in Figure 5-3B). However accurate quantification of the degree of recanalization was not possible because coupling of the flow probe to the vessel with saline caused fluctuations in the baseline of the flow trace (data not shown). We did not see evidence of major changes in flow once vessels did recanalize. Therefore, in the interests of accuracy, we chose sustained recanalization as the marker of recanalization success based on dose, rather than attempting to quantify percentage recanalization.

![Figure 5-1. Recanalization rates at varying doses of tPA.](image)

Data presented as the percentage of animals with sustained recanalization per group. Fisher’s exact test, \( p = 0.015 \)
Median times to recanalization (interquartile range) from start of tPA treatment were 210 (210-210) minutes for 0.9 mg/kg treated rats, 210 (210-210) minutes for 1.8 mg/kg treated rats, 65.5 (26-210) for 4.5 mg/kg treated rats, and 34 (27-210) minutes for 10 mg/kg treated rats (Log Rank Test, \( p = 0.017 \), Figure 5-2). Earliest recanalization onset was 25 minutes (10 mg/kg), with no recanalization occurring beyond 87 minutes (27 minutes after tPA treatment end) in any group.

No animals were excluded. One animal died just prior to the final observation (10 mg/kg group). This animal had fluctuating body temperatures throughout surgery and high temperatures (>39°C) leading up to its death, with no other explanation found. It was included in the primary analysis. Re-analysis excluding this animal gave a recanalization rate of 83% (instead of 71%) for this group, but made no material difference to the primary findings.

5.5 Discussion

In this study, we found that the traditional rat dose of tPA (10 mg/kg) was highly effective for carotid artery recanalization (71% recanalization rate). This is far superior to what is achievable in clinical stroke, where recanalization rates are <50% for middle cerebral artery (MCA) occlusion\(^1\) and only 10-30% for occlusions of the carotid arteries [21, 69]. The clinical dose of
tPA (0.9 mg/kg) caused no recanalization in our model, which is also not reflective of the clinical situation. We found a 2x clinical dose (1.8 mg/kg) to better reflect clinical recanalization rates. We observed a possible ceiling effect at doses at and above 4.5 mg/kg (5x clinical dose). Our findings are in keeping with previous work showing that the rat fibrinolytic system is less sensitive than the human system. However, they suggest that the 10-fold difference in sensitivity found in vitro [152] may overestimate the in vivo situation.

A “human equivalent” dose of tPA for preclinical research that results in recanalization rates reflective of the clinical setting has not previously been determined. We chose a range of doses spanning across those used in previous studies, with multiples of the clinical dose (i.e. 2x and 5x the clinical dose). Although the thrombolytic efficacy of tPA has been well established clinically, its efficacy is suboptimal [9] and the growing field of research studying thrombolytic enhancers requires a preclinical dose of tPA that reflects the clinical response rates. Recent studies comparing clinical to rat dose reported the clinical dose to be a better clinical mimic, but these studies did not investigate additional doses [155, 158]. The high recanalization rates we saw with the 10 mg/kg “rat” dose are similar to rates reported in other studies using this dose (67-100% in various stroke models [154-157]). These rates are not reflective of clinical recanalization and are particularly unsuited to studies of thrombolytic enhancer therapies, since there is little scope for additional benefit. Additionally, we found a possible ceiling effect of tPA dose at 4.5 mg/kg, with no significant increase in recanalization rates at the higher dose. Such an effect has also previously been reported for doses above 10 mg/kg [188]. For a “human equivalent” tPA dose, our study indicates that a 2x clinical dose (1.8 mg/kg) in this model with 17% recanalization rates best reflects clinical recanalization of carotid artery occlusion for which clinical rates are between 10-30% [21, 69]. To achieve the higher end of this clinical range, a dose between 1.8 mg/kg and 4.5 mg/kg may be necessary. However, this highlights that the previously accepted doses are not ideal to mimic clinical rates. In other situations, such as MCA occlusion, clinical recanalization rates are higher than carotid occlusion [9]. Differing clot compositions and co-morbidities also affect recanalization efficacy with tPA [193, 194]. It is likely that tPA doses required to mimic clinical recanalization rates will differ with regards to the model choice, incorporating clot type, co-morbidities, and species. We recommend that researchers aiming to find improved thrombolytic or recanalization therapies over tPA alone, need to determine the “human equivalent” dose for use in their chosen model that generates recanalization rates that correlate with the clinical conditions being tested.

We chose a model of naturally forming thrombi in order to create as ‘physiological’ a model as possible. This clot composition is the likely explanation for the complete lack of recanalization we saw with clinical dose tPA, in contrast to previous studies. Such studies have tended to use spontaneously formed thrombi, or other clot types with high sensitivities to tPA thrombolysis.
Thrombus composition is well known to affect the efficacy of tPA lysis [130, 138]. Our model, based on the Folts method, produces physiological thrombi that are platelet rich [151]. Platelet rich clots are histologically better mimics of clinical thrombi [141] and are more resistant to thrombolysis than many other experimental thrombi [133, 134, 195].

Recanalization was chosen as the primary outcome for this study because it is the major effect by which tPA causes clinical improvement [9]. In rat models of stroke, recanalization is not always reported due to difficulties in directly monitoring recanalization intracranially in the rat. The extracranial nature of our chosen model allowed direct real time monitoring of recanalization. We observed earlier onset time to recanalization with increasing doses of tPA. With early recanalization being predictive of good clinical outcome [9], it is beneficial for enhancer therapies to not only increase the rates of recanalization, but to reduce the time to sustained recanalization onset. At higher doses, recanalization onset is already early (median time 34 minutes for 10 mg/kg), leaving little room for improvement if testing alternate therapies. Functional outcome was intentionally not included in this study because there is already convincing evidence that tPA produces good functional outcome in rodents [9, 153]. We know from clinical studies that functional improvement is highly correlated with recanalization of the occluded artery. The purpose of this study was to determine whether the previously accepted “rat dose” causes recanalization rates too high to provide any scope for improvement when testing thrombolytic enhancers.

In conclusion, we have found that both rat and clinical doses of tPA are not reflective of clinical recanalization rates in a model of naturally forming thrombus. The rat dose was above that producing a “maximal” effect of recanalization. The no response observed with clinical tPA dose confirms that the rat fibrinolytic system is less sensitive to humans, but not to the 10-fold degree previously accepted. Neither dose appeared ideal for testing thrombolytic enhancers. For this model of carotid occlusion in rats, we propose a 2x clinical dose (1.8 mg/kg) to be best reflective of clinical recanalization rates.

5.6 Methods

5.6.1 Animals

This study was approved by the Animal Care and Ethics Committee of the University of Newcastle, Australia (Approval No. A-2010-128) and performed in compliance with requirements of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes [186]. Male outbred Wistar rats (n=25; 338-433 g; Animal Resources Centre, Perth, Australia) were anesthetised with 5% Isoflurane, and maintained with 1.5-2% in 30/70% O2/N2 through a nose cone and rectal temperature was maintained at 37°C with a feedback controlled
heat mat (Faculty of Health Workshop, University of Newcastle, Australia). Heart rate, respiration and oxygen saturation were monitored throughout surgery.

5.6.2 Carotid artery occlusion

To create a physiological thrombus to occlude the carotid artery, a modification of the Folts model [151] was used with a mild underlying stenosis. The right carotid arteries were exposed and the internal carotid artery ligated to avoid risk of thromboembolism to the brain. Resultant strokes from carotid thromboembolism are highly variable, as in stroke patients, and could potentially confound experiments. A 20-MHz, 0.8 mm Doppler flow probe (Iowa Doppler Products, USA) was positioned over the external carotid artery to monitor blood flow. Baseline flow was recorded for 5 minutes prior to injury using LabChart 7 software (ADInstruments, Australia). The common carotid artery was crushed three times (30 seconds with 30 second rest intervals, Figure 5-3A) using haemostats guarded with tape to disrupt the endothelium, exposing pro-thrombotic factors. A double looped 5-0 silk suture was placed over the site of injury, and tightened to create stenosis following final crush. This stenosis reduced blood flow by 75% of pre-crush baseline flow. Flow was monitored for cyclic flow patterns until complete occlusion was achieved (Figure 5-3A). An additional 30 second crush was made over the stenosis if: 1) continuous flow was maintained for 10 minutes after final crush, or 2) if cyclic flow patterns were observed for 30 minutes post-crush with no complete occlusion. No more than two additional crushes were performed in any animal. The tie was loosened 45 minutes after stable occlusion to establish a mild stenosis. Flow spontaneously returned pre-treatment in two animals. In one, the vessel was re-crushed, while the other reoccluded spontaneously within minutes. Sixty minute stable occlusion was again monitored before treatment.

5.6.3 Treatment groups

At 60 minutes post-occlusion, animals were intravenously administered tPA (Alteplase, Boehringer Ingelheim, Australia) at the clinical dose (0.9 mg/kg, n=6), 2x the clinical dose (1.8 mg/kg, n=6), 5x the clinical dose (4.5 mg/kg, n=6), or the rat dose (10 mg/kg, n=7) as a 10% bolus over 1 minute, with the remainder infused over 1 hour.

The 0.9 mg/kg and 10 mg/kg groups were part of a separate study in which they had been randomised to either tPA or tPA+ultrasound treatment (tPA+ultrasound groups not presented here). The additional animals were randomised to either 1.8 or 4.5 mg/kg tPA for this study.
**Figure 5-3. Doppler flow of crush and occlusion with recanalization.** Representative example of Doppler flow indicating crush injury and stenosis followed by occlusion (A). Grey shaded bars indicate 3x 30 second crush cycles, including a recrush after 10 minutes of continuous flow. Triangles (▼) indicate tightening of silk suture to create stenosis and achieve 75% flow reductions. Dashed horizontal lines indicate baseline flow of 57 cm/s (A) and 25% baseline flow of 14.25 cm/s (A and B). Recanalization was observed at 130.6 minutes (A and B). The red box in (A) indicates the area zoomed in for (B) to show clear change from occluded “noise” to flow trace, representing recanalization. Recanalization was also confirmed by the return of audible Doppler flow correlating with flow trace.
5.6.4 Recanalization
The primary outcome for this study was recanalization rates for each group. Recanalization was monitored every 30 minutes after tPA onset until 4.5 hours post-occlusion and presented as the total number of animals with sustained recanalization at experiment end per group. The end point of 4.5 hours was chosen based on the clinical time window for tPA treatment inclusion. Recanalization was reported as sustained when recanalization occurred and flow remained until the final observation time. Recanalization/reocclusion was reported when flow was observed at one time point and no flow observed at the next.

A secondary outcome of time to sustained recanalization was determined retrospectively from LabChart files by a blinded observer and was reported as the time that the flow trace returned to normal waveform post tPA treatment start, with the flow trace continuing as normal to experiment end (Figure 5-3B).

5.6.5 Statistics
Sustained recanalization rates at endpoint were analysed for statistical significance using Fisher’s exact test. Time to sustained recanalization onset was analysed by survival analysis and log-rank test. Statistical significance was considered to be a \( p \)-value <0.05.

5.7 ADDITIONAL INFORMATION

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5.7.2 Author contribution statement
AT, CL and NS were responsible for study design. Experimental procedures were performed by AT and RH. Data analysis and manuscript preparation was by AT and NS. All authors reviewed the final manuscript.

5.7.3 Competing interests
The authors declare no competing financial interests
Chapter 6

6 DISCUSSION

6.1 General discussion ................................................................. 99
6.2 Sonothrombolysis for reperfusion ............................................. 101
6.3 Characteristics of stroke for “personalised medicine” ................. 103
   6.3.1 Identifying clot composition ........................................... 104
   6.3.2 Identifying carotid artery stenosis with associated occlusion ... 105
6.4 Alternatives to sonothrombolysis for enhanced revascularisation .... 106
   6.4.1 Alternate thrombolysis .................................................. 107
   6.4.2 Endovascular revascularisation ...................................... 108
6.5 Conclusions ............................................................................ 109

6.1 GENERAL DISCUSSION
For this thesis, I aimed to test sonothrombolysis (± microbubbles) for recanalization and reperfusion in different models of stroke in rats. Clinical trials of sonothrombolysis have been primarily limited to intracranial large artery occlusions. Yet stroke can be caused by occlusions of a variety of vessels that supply the brain, including the extracranial carotid arteries. Occlusion of the microvasculature may also occur due to “no reflow” after large artery occlusion and recanalization. Hence, I modified and developed different models of stroke to test sonothrombolysis in these conditions, as well as in a traditional intracranial occlusion (MCA occlusion). Additional factors that were hypothesised to affect sonothrombolytic success were incorporated into my studies, including the occluding clot composition and the degree of underlying stenosis in carotid occlusion. In these models, I found limited success of sonothrombolytic recanalization. PRC were completely resistant to lysis in a model of MCA occlusion, even with microbubble-enhanced sonothrombolysis. The degree of carotid stenosis was related to thrombolytic success whereby recanalization was achieved in the presence of a mild stenosis, but not in the presence of a severe stenosis. No increase in recanalization was observed with the addition of ultrasound in these carotid stenosis models. These findings raise doubts regarding sonothrombolysis for recanalization of large vessel occlusions. Microbubble-enhanced sonothrombolysis, however, restored microvascular patency after large vessel recanalization. In this model, tPA alone was insufficient to restore vessel patency. This finding indicates a potential for sonothrombolysis to improve reperfusion. These findings suggest that clot composition, microvascular occlusion and vessel stenosis are all potential contributors to...
sonothrombolytic efficacy for vessel recanalization and reperfusion. Finally, I tested a range of tPA doses to determine a “human equivalent” dose to mimic clinical recanalization rates. I found this dose to be lower than the conventionally used rat dose, indicating that prior studies have likely overestimated thrombolytic efficacy. In my study, the traditional rat dose produced maximal recanalization rates, which has also been observed at this dose in other studies [154-157]. Future research of any thrombolytic enhancer for recanalization should select lower tPA doses that mimic the clinical situation, thereby allowing room for improvement with the target therapy or intervention.

The results of this thesis come at an important time for sonothrombolysis research for stroke, with the recent large scale, multicentre phase III clinical trial – CLOTBUST-ER – being terminated early due to futility [196]. This is the first large-scale clinical sonothrombolysis trial for stroke and with just over half of the intended cohort recruited (467/824), interim analysis deemed it highly improbable that the primary outcome (functional recovery at three months) would be significantly different in the sonothrombolysis group compared to the group receiving tPA treatment alone. The analysis of the CLOSTBUST-ER results will not be published until next year, however when looking at the study design [197], there may be a number of reasons that this trial did not show an improvement in patient outcome with sonothrombolysis. In particular, selection criteria was based primarily on a high NIHSS score (≥10), described as severe strokes, with no imaging criteria in the primary analysis to confirm a salvageable penumbra or the presence of an arterial occlusion. It is therefore highly probable that a significant proportion of included patients had strokes whereby the infarct core was too large to allow any significant level of improvement. Additionally, if an arterial occlusion was not present (if, for example, it had spontaneously lysed), a treatment designed to lyse an occluding clot would not have any beneficial effect. In a recent trial of tenecteplase, a new generation tPA, researchers included patients based on these criteria: a salvageable penumbra defined by CT perfusion, and an associated arterial occlusion as defined by CT angiography. In selecting patients most likely to benefit from treatment, Parsons et al demonstrated improved reperfusion, recanalization and, most importantly, patient outcome in the tPA control group, above the levels generally reported in the literature [35] (This trial will be discussed further in section 6.4.1.). This idea of selecting patients for “personalised medicine” is certainly an important idea for stroke, a condition which is highly variable amongst patients. Treating a patient to salvage a penumbra that does not exist is futile, as is treating with clot busting drugs when no clot is present and may explain the lack of improvement with sonothrombolysis for CLOTBUST-ER. This thesis has also indicated that PRC are resistant to sonothrombolysis and it is highly probable that a proportion of CLOTBUST-ER patients had clots that were not amenable to lysis. The results presented in my thesis suggest that large vessel recanalization with sonothrombolysis is not effective over tPA alone. Adding this to
the recent termination of CLOTBUST-ER, previous ideas of the potential of sonothrombolysis for stroke therapy should be reconsidered. However, my results of sonothrombolysis of microvascular occlusion suggest its potential as a reperfusion therapy. For this discussion, I will first discuss the positive results of sonothrombolysis for reperfusion and then discuss the idea of “personalised medicine” by focusing on the findings of this thesis: identifying clot composition in situ and vascular stenosis. Finally, given the findings of this thesis that arterial recanalization with sonothrombolysis is not effective, and given the termination of the CLOTBUST-ER trial, I will discuss some potential alternatives to sonothrombolysis for revascularisation.

6.2 Sonothrombolysis for Reperfusion

My studies reveal that microbubble-enhanced sonothrombolysis can restore microvascular patency, where tPA alone does not (Chapter 3). This study highlights the potential of this intervention for a condition that leads to poor patient outcome [11, 61, 68]. Occlusion of the cerebral microvasculature is not currently directly treated, but there is growing evidence that reperfusion is a better predictor of good outcome than recanalization alone [11, 61, 68]. This means that reopening the large vessel does not guarantee good patient outcome. If the small vessels remain occluded, the potentially salvageable penumbra will not be saved. Recent studies have reported that as many as 23% of patients with recanalization had no reperfusion [11, 61]. Given that these studies report more than half of all patients do not reperfuse, there is an indication for identifying and targeting reperfusion to improve patient outcome. Although these studies do not confirm the causes of perfusion deficits, microvascular occlusions due to the “no reflow phenomenon” are a likely cause. My results suggest a potential benefit of microbubble-enhanced sonothrombolysis for reperfusion. The potential of microbubble-enhanced sonothrombolysis for microvascular occlusion is yet to be tested clinically. If patients presenting with microvasculature occlusion can be identified early then therapy, such as microbubble-enhanced sonothrombolysis, could be initiated to directly target reopening of the occluded microvasculature.

To translate microbubble-enhanced sonothrombolysis to the clinic, patients with occlusion of the microvasculature need to be identified. There are multiple post-mortem techniques that can identify microvascular occlusion and perfusion deficits in preclinical studies, such as micro-CT as described in Chapter 3. However clinically, imaging for microvascular occlusion requires the infusion of contrast agents and is indirect and often inferred or assumed. This is based on contrast stagnation or “washout” delay with angiographic techniques or perfusion deficits identified by CT-perfusion imaging or MRI-diffusion weighted imaging [61, 68, 198, 199]. Although these techniques do not definitively confirm microvascular occlusion (perfusion deficits may also be caused by vessel constriction, or areas of stagnant flow), they have been successfully utilized to demonstrate increased reperfusion correlating with good patient outcome following thrombolytic
therapy [200]. Although these imaging techniques are likely to be suitable to confirm microvascular occlusion, if the perfusion deficits are not caused by fibrin and platelet deposition, microbubble-enhanced sonothrombolysis may not be of any benefit for reperfusion. To confirm microvascular occlusion, targeted-microbubbles (as discussed in section 6.3.1) could be utilized for imaging to distinguish between clots in the microvasculature versus stagnant flow or vascular constriction [201]. Specifically targeting fibrin, which is generated when clots form, or targeting activated platelets to identify platelet accumulations indicative of thrombosis, may lead to better identification of microvascular occlusions. There is also potential for these targeted microbubbles to further enhance sonothrombolysis for microvascular occlusions at the sites to which they accumulate, although most reports do not show increased efficacy over non-targeted microbubbles [202-204]. This is not surprising given my results in the microvasculature, in which a high dose microbubbles with ultrasound insonation did not enhance microvascular patency over lower doses.

My study (Chapter 3) is one of only two studies investigating microbubble-enhanced sonothrombolysis of the microvasculature [117]. The findings suggest that microbubble-enhanced sonothrombolysis is a promising strategy to enhancing reperfusion, but the limited preclinical data means much is still to be determined before clinical translation. In my study and the study by Nedelmann et al [117], rats were treated after mechanical recanalization of the MCA. Clinically, recanalization is mostly achieved with tPA. Yet, microvascular occlusion identified post-tPA recanalization may not allow for subsequent sonothrombolysis due to the increased bleeding risks associated with additional tPA infusion. Nedelmann et al demonstrated that sonolysis (ultrasound without tPA) was not beneficial in this model, indicating that ultrasound clot lysis for microvascular occlusions requires the infusion of tPA [117]. There is evidence that tPA therapy can induce coagulation activation and fibrin formation out to 3 days after stroke [205]. If there is the potential for a benefit of sonothrombolysis of the microvasculature several hours after initial tPA (e.g. evidence of a salvageable penumbra on CT imaging), then additional tPA might be considered. The reperfusion efficacy of microbubble-enhanced sonothrombolysis prior to large vessel recanalization is not yet known. There could also be a benefit of microbubble-enhanced sonothrombolysis for patients with spontaneous recanalization and no reperfusion. It has been reported that as many as 32% of stroke patients spontaneously recanalize within 24 hours of stroke onset [9, 61]. If no large vessel occlusion is confirmed on pre-thrombolysis CT or MR imaging, but persistent microvascular occlusion and a salvageable penumbra are observed, then microbubble-enhanced sonothrombolysis may be of therapeutic benefit to these patients. Another approach to recanalization of large arteries is endovascular intervention, particularly utilising clot retrieval devices (discussed further in section 6.4.2) [37-41]. Associated with endovascular
recanalization, the MR CLEAN\textsuperscript{7} trial reported reperfusion in 59\% of patients [37]. Although the EXTEND-IA\textsuperscript{8} trial reported a probability of reperfusion at >90\%, differences between trials are likely related to patient selection criteria, whereby EXTEND-IA used imaging to select only patients with salvageable penumbra [38]. The lower rates of reperfusion in MR CLEAN and other trials not selecting based on penumbral imaging, could be related to microvascular occlusions or no salvageable penumbra due to later treatment and infarct progression. Given the data that reperfusion is a better predictor of good outcome than recanalization [11, 61, 68] and that “time is brain”, then it should be considered that both recanalization and reperfusion should be targeted for the best patient outcome. In these endovascular trials, most patients received i.v. tPA prior to endovascular intervention for recanalization. Ultrasound insonation of the occluded microvasculature and microbubble infusion during this early tPA treatment period could potentially enhance the chances of reperfusion. If recanalization of the large artery does not occur during the period of tPA infusion, patients could then be allocated to endovascular intervention. Improving reperfusion early should increase the number of patients with salvageable penumbras and increase the number of patients eligible for endovascular therapy when penumbral imaging is utilized for selection. This idea may also suggest a hypothesis that microbubble-enhanced sonothrombolysis of the microvasculature could be used as standard treatment when the large artery occlusion is identified as receptive to thrombolysis and microvasculature occlusion exists: i.e. tPA may recanalize the large artery, while the microbubble-enhanced sonothrombolysis will increase chances of reperfusion. Yet, with only preclinical data of the efficacy of microbubble-enhanced sonothrombolysis to restore microvascular occlusion (Chapter 3 and [117]), more study is required to determine the effects on infarct sizes and functional outcomes before testing clinically.

6.3 CHARACTERISTICS OF STROKE FOR “PERSONALISED MEDICINE”

Stroke is a variable condition and as such it is unlikely that a single treatment will be the answer for all patients. Identifying characteristics of the disease that could impact on treatment success may allow allocation of patients to the most appropriate therapy or intervention for their individual situation. Additionally, a better understanding of these characteristics that lead to failed thrombolysis could assist in developing alternate revascularisation therapies and strategies. As previously discussed, patient selection for therapy is already being implemented into clinical trials with advanced imaging to confirm salvageable penumbra and associated occlusions [35, 37-39]. My thesis was designed to test the efficacy of sonothrombolysis over tPA alone, but has indicated that clot composition and/or the degree of an underlying stenosis may contribute to tPA efficacy.

\textsuperscript{7} Multicenter Randomized Clinical trial of Endovascular treatment for Acute ischemic stroke in the Netherlands

\textsuperscript{8} Extending the Time for Thrombolysis in Emergency Neurological Deficits - Intra-Arterial
and subsequent patient outcome. Can selection criteria for patient treatment be extended further to incorporate the identification of the occluding clot composition, and/or the degree of any underlying carotid stenosis, that may contribute to the rate of thrombolytic success?

6.3.1 Identifying clot composition and size
In this thesis, I report that PRC are resistant even to microbubble-enhanced sonothrombolysis (Chapter 2) suggesting that clot composition is a likely contributor to thrombolytic resistance. If high platelet content is a contributing factor to failed recanalization, then imaging for the components of occluding clots in situ could identify patients who may or may not respond thrombolytic therapy. Identification of occluding clots with a high platelet content could lead to more rapid allocation of patients to alternative treatment strategies to reopen the occluded vessel, such as endovascular approaches [37-39]. Computationally, occluding stroke clots differ in their proportions of platelets, fibrin and red blood cells [74] ultimately affecting the overall clot structure and therefore, thrombolytic susceptibility. Several imaging approaches have been investigated for their ability to determine clot compositions in situ [16, 129, 175]. While standard imaging is still not accurate enough to clearly distinguish differing clot compositions, advances are being made to determine the compositions of occluding thrombi. With non-contrast CT, decreasing clot attenuation (as measured by Hounsfield units (HU)) is consistent with increasing proportions of platelets, atheroma and cellular debris [175]. It has also been revealed that clots with lower HU are more resistant to tPA lysis [174], consistent with my findings that PRC are resistant to sonothrombolysis. Higher HU of a clot is associated with a higher erythrocyte component due to a linear correlation with the amount of haemoglobin present [16]. Additionally, the hyperdense vessel sign on CT and blooming artifact on MRI, can also indicate clots of higher mean percentage of erythrocytes [129, 206]. Other components of the clot (including platelets and fibrin) and the structural differences (such as fibrin fibre thickness [131]) that affect thrombolytic efficacy are not identified by the hyperdense vessel sign or blooming artifact. While these studies are a step towards identifying clot composition in situ, the signals are absolute and the technology does not yet exist to conclusively distinguish one clot type from another – i.e. platelet-rich from fibrin-rich. Microbubble contrast agents targeted to the platelet and/or fibrin components of clots could also aid in the identification of clot composition. Signal intensities from targeted microbubbles are greater than the signal intensities of non-targeted microbubbles due to more microbubbles binding to clots [204, 207, 208]. Hence, specifically targeting the activated platelets of a clot could give an indication of the platelet content. It has been demonstrated that platelet- and fibrin-targeted microbubbles bind to PRC and platelet-poor clots in differing amounts, and at higher quantities than untargeted microbubbles [209]. However, whether the total number of microbubbles bound is related to the proportions of platelets or fibrin within the clot and whether this is quantifiable in situ, has not yet been explored. Hence, identifying thrombolytic responders
from non-responders based on clot composition, while theoretically beneficial for patient selection for treatment, is not yet achievable. If the components of clots are major players in thrombolytic success, then advancing this technology for diagnostic purposes will benefit stroke therapy.

Additional to clot composition, clot size, known to be a predictor of thrombolytic success [210, 211], should be considered when evaluating thrombolytic susceptibility. My study of PRC in a thromboembolic stroke model used 25 mm clots, a common clot length for rat stroke models [156, 158, 212, 213]. Yet Figure 2-4A shows clots of enormous size relative to the rat brain. Clinically, the use of non-contrast CT and CT angiography can allow evaluation of clot length in most stroke patients and studies have found that clots longer than 8-15 mm are unlikely to thrombolysse [210, 214]. There is certainly a clear ratio difference between an 8-15 mm clot in a human brain and a 25 mm clot in a rat brain. For this thesis, preliminary experiments of PRC <5 mm (data not presented) generated highly variable infarction due to variable clot lodgement within the cerebral vasculature. I determined that longer clots produced less variability and chose a 25 mm clot based on data that erythrocyte-rich [156, 158] and fibrin-rich [212, 213] clot types between 20-50 mm are susceptible to thrombolytic recanalization. A limitation of my study was that a direct comparison of different clot compositions or clot sizes was not made. The purpose of the study was to test microbubble-enhanced sonothrombolysis in a clinically relevant model of stroke with the intention of testing this potential recanalization therapy for patients that will not respond to tPA alone. Direct comparisons of clot types have been made in other studies where PRC are reported as more stable and more resistant to thrombolysis than other clot types [133, 134, 140]. Hence, PRC are better mimics of clots that do not respond to current therapy. Additionally, with increasing concentrations of platelets, PRC are compositionally more similar to human stroke thrombi [141]. Indeed, my preliminary study (reported as Study 1 in Chapter 2) appeared to present a clot that was stable, although produced some spontaneous recanalization that mimicked clinical rates [9]. The complete lack of recanalization in any group in Study 2 suggests a potential role of both composition and size to sonothrombolytic failure. Combining the information of clot size and clot composition in a patient presenting with stroke may also indicate their susceptibility to thrombolytic therapy.

6.3.2 Identifying carotid artery stenosis associated with occlusion
My studies demonstrate that the degree of stenosis associated with a carotid artery occlusion corresponds with thrombolytic success (Chapter 4). Thrombolysis with tPA has been shown to be less effective for extracranial carotid occlusions than for cerebral artery occlusions [9, 21, 69]. Identifying the degree of stenosis may allow the allocation of patients to appropriate therapies, such as thrombolysis for occlusion with mild stenosis, or surgical intervention for severe stenosis. However, current imaging techniques can identify stenosis or occlusion but not both together.
Additionally, distinguishing between a sub-occlusive severe stenosis and a complete occlusion cannot be achieved with all imaging modalities. Imaging of vessel wall characteristics and the identification of carotid plaques may provide indication of the presence of a stenosis in occluded vessels. Antonopoulos et al identified plaque characteristics associated with carotid occlusion by duplex ultrasonography. While the authors did not determine a quantifiable degree of stenosis in the occluded ICAs, plaque characterisation as echolucent or echogenic may be indicative of stenosis grade underlying an occlusion. Echolucent plaques, composed of large lipid cores and thin fibrous caps, were reported as more often associated with carotid artery occlusion [215]. In non-occluded ICAs, echolucent plaques were also more associated with severe stenosis (>70%). Echogenic plaques, which were more commonly associated with milder stenosis (<70%) of non-occluded vessels, were identified in 28% of occluded ICAs. This study demonstrates the possibility of utilising current diagnostic imaging for identifying underlying stenosis but would require further definition to quantify the degree of stenosis in occluded vessels. Advanced MR imaging is also now being utilised to classify vessel wall pathologies, identifying the degree of stenosis and the characteristics of the carotid plaques in non-occluded vessels [216-218]. MRI can identify complex plaques at high risk of rupture in non-occluded vessels by identifying lipid-rich necrotic cores, thinned or ruptured fibrous caps, and intraplaque haemorrhages. In doing so, this imaging modality provides more information regarding the plaque than just degree of stenosis. However, information regarding the underlying plaque characteristics of occluded arteries were not presented in these studies. Interestingly, MRI has revealed complex plaques that are “non-stenotic” but are at risk of rupture [216, 218]. My studies demonstrate that acute, locally forming thrombi in response to endothelial disruption can be lysed by tPA when a mild stenosis is present and that a severe stenosis underlying an occlusion will not thrombolise. The ability to identify the degree of an underlying stenosis of carotid occlusions could lead to better triage of patients to therapy. Currently, patients with carotid artery occlusion are treated with tPA with no knowledge of an underlying stenosis. In endovascular centres, these patients would be allocated directly to surgery to reopen the vessel. If patients outside of these centres with occlusion and severe stenosis could be quickly identified then, where possible, they could be transported to an endovascular centre. However, most of the world population does not have access to such facilities. Additionally, if a carotid occlusion with mild or no stenosis is identified as a likely tPA-responder, it might be more cost-effective to keep the patient in the smaller centre, as well as removing the surgical risks to the patient. This is also in light of new generation thrombolytics that appear more effective at recanalization than the current tPA, alteplase (discussed further in section 6.4.1.).

My studies did not find a benefit of sonothrombolysis over tPA alone, likely due to the high rates of recanalization with tPA alone leaving no room for improvement with ultrasound (Chapter 4).
The results of Chapter 5 indicate that the conventional rat dose of tPA is too high to mimic clinical recanalization rates in the carotid occlusion model. Hence, there may be justification for further testing of sonothrombolysis for extracranial carotid artery occlusions. The hypothesis that limitations associated with transcranial sonothrombolysis (power attenuation, standing waves causing haemorrhage) are negated for extracranial occlusions should allow testing of alternate ultrasound parameters that may result in benefit of this therapy. Ultrasound is cheaper and more widely available for use diagnostically than other imaging modalities, and Antonopoulos et al demonstrate the potential of ultrasound to distinguish between plaque types [215]. To be able to utilize ultrasound both diagnostically and therapeutically for extracranial occlusions could improve patient outcomes in smaller centres where endovascular surgery is not an option.

6.4 ALTERNATIVES TO SONOTHROMBOLYSIS FOR ENHANCED REVASCULARISATION

Given that only a third of all patients will fully recover or recover with minimal disability after a stroke event, improving current stroke therapy is a key focus of stroke research [2, 70]. The aim for this thesis was to investigate sonothrombolysis as a potential enhancer of the thrombolytic effect of tPA. Yet, with the limited success of sonothrombolysis observed in my studies, and the recent termination of the large-scale CLOTBUST-ER trial, the benefit of sonothrombolysis for recanalization over tPA alone should be questioned. Recanalization of the occluded arteries is the best known method of improving patient outcome after stroke, yet tPA itself is suboptimal at restoring perfusion in all patients [9]. Hence, alternate approaches to reopening the vessels may be required. Additionally, the evidence that PRC do not lyse by conventional or enhanced thrombolysis, suggests that more aggressive methods of revascularisation are needed. Recent endovascular trials provide evidence that such approaches may be beneficial for patients with “unlysable” clots.

6.4.1 Alternate thrombolytics

A potential limiting factor of sonothrombolysis may in fact be due to limitations of alteplase (tPA). Sonothrombolysis is ultimately reliant on the presence of tPA as it does not directly cause clot lysis, instead facilitating the process by improving tPA access to the clot and more tPA binding sites (outlined in section 1.2.1). If alteplase limits sonothrombolysis, what alternatives are available? Numerous other thrombolytics have been trialled for stroke thrombolysis (urokinase, streptokinase etc.), with varying successes and often limited by increased bleeding. However, recent studies have supported the potential benefits of a new generation tPA, tenecteplase, over alteplase [35, 200, 219]. A recent trial reported that tenecteplase treatment increased reperfusion, recanalization and good clinical outcome at 24 hours far beyond the capabilities of alteplase (reperfusion: 79.3 ± 28.8 versus 55.4 ± 38.7%, p=0.004; recanalization: 88% versus 68%, p=0.05; improvement in NIHSS score: 8.0 ± 5.5 versus 3.0 ± 6.3, p<0.001;
tenecteplase versus alteplase, respectively) [35]. Recanalization rates with tenecteplase were also higher than reported recanalization rates with sonothrombolysis (with alteplase) [80, 82, 87, 220], although no direct comparisons of tenecteplase and sonothrombolysis have been made. Tenecteplase is 14-fold more fibrin specific than alteplase, inducing faster and more complete clot lysis and less bleeding complications [221]. Importantly, in light of my PRC findings, tenecteplase has an 80-fold greater resistance to inhibition by PAI-1 [222] – a significant factor in the lytic resistance of PRC. While the benefits of tenecteplase for anterior cerebral occlusions have been investigated [35, 200, 219], its potential for extracranial carotid occlusion is unknown. Indeed, patients with carotid artery occlusions were specifically excluded from the trial by Parsons et al [35]. Clinically, carotid occlusions are less responsive to tPA and these patients likely benefit more from endovascular intervention. However, the vast majority of stroke patients worldwide do not currently have access to endovascular centres. With the evidence that tenecteplase is more effective than alteplase, and with the potential to modify ultrasound parameters for extracranial carotid occlusions (due to the removal of the risks associated with the skull), combining these two interventions could lead to a more effective therapy for carotid occlusion that is more readily available to patients. The results of Chapter 4 of this thesis show thrombolytic success of carotid occlusions with mild stenosis. Although there was no added benefit observed with sonothrombolysis over tPA alone, recanalization rates were high and the results of Chapter 5 indicate that a lower dose of tPA better mimics clinical recanalization rates for this condition. These combined findings indicate that this model would be suitable for future preclinical testing of tenecteplase for extracranial carotid occlusions, as well as the testing of alternate ultrasound parameters for sonothrombolysis. Additionally, tenecteplase’s effect on microvascular occlusions has not been directly investigated. However, the CT perfusion data of the Parsons et al trial [35] demonstrating significant reperfusion over alteplase could indicate microvascular recanalization. Whether the reperfusion effect I observed preclinically with microbubble-enhanced sonothrombolysis could clinically match, or exceed the reperfusion effect generated by tenecteplase, is yet to be determined. Yet, given the high reperfusion rates of tenecteplase, and the dramatic clinical recovery observed, it is likely that sonothrombolysis of the microvasculature will not have any greater effect. The clinical trials of tenecteplase demonstrate that alternate thrombolytics may achieve better outcomes over the conventional tPA, alteplase. It remains to be determined whether tenecteplase efficacy is also limited by the characteristics of stroke that limit alteplase’s thrombolytic success, such as clot composition or vessel stenosis as highlighted in this thesis.

6.4.2 Endovascular approaches to revascularisation

An alternative to tPA for revascularisation is an endovascular approach, primarily by surgical clot retrieval (mechanical embolectomy). Given the findings of my studies, patients with PRC or
severe stenosis underlying a carotid occlusion could likely benefit more from endovascular intervention than from thrombolysis. Until very recently, the benefits of endovascular intervention remained controversial with regards to benefit over risk [220]. Yet, five studies published in 2015 have become “game-changers” in stroke therapy [37-41], with four of these studies being stopped early due to superior efficacy of intervention [38-41]. In this thesis, I describe that PRC did not lyse with enhanced-sonothrombolysis and, as previously discussed, clot composition likely plays an important role in thrombolytic success. If patients are identified early with clot compositions that cannot be lysed, endovascular clot retrieval may be the best option for these patients. Additionally, I have described that a severe stenosis underlying a carotid occlusion will not allow thrombolysis and recanalization. Although extracranial carotid occlusions were excluded from the EXTEND-IA and the SWIFT-PRIME trials [38, 41], such occlusions were included in the others [37, 39, 40]. Despite higher baseline numbers of patients with extracranial ICA occlusions in the intervention groups in MR CLEAN and REVASCAT [37, 40] (equal numbers in the ESCAPE control and intervention groups [41]), all outcomes still favoured the intervention. Hence, patients identified with occlusions that may not respond to thrombolysis could benefit from endovascular intervention. However, despite the huge success of these trials, there are limitations. In particular, there are currently far too few centres worldwide that can routinely carry out endovascular intervention and the majority of patients worldwide will not have access to them. There are also limited trained interventionalists for such a technique. If patients need to be moved to an alternate facility for endovascular intervention, identification needs to be rapid to allow minimal delay in recanalization and reperfusion onset. Indeed, the MR CLEAN study reported that no patient was outside 30 min from an interventional centre. But how relevant is this for all stroke patients in countries, such as Australia, in which distances between centres can be vast? Additionally, based on the patient selection criteria reported in the trials, a large proportion of patients will be excluded from treatment. In the EXTEND-IA trial, for example, <1% of all ischaemic stroke patients screened were included in the study. However, this highlights the importance of selection criteria. For the EXTEND-IA trial, reperfusion was achieved in 100% of the ischaemic territory (median value, versus 37% with tPA only) and recanalization at 24 hours was observed in 94% of patients (compared with 43% of controls) [38]. Selection criteria of the characteristics of stroke that will allow recanalization and reperfusion is

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9 Solitaire With the Intention For Thrombectomy as PRIMary Endovascular Treatment
10 RandomizEd Trial of reVascularizAton With Solitaire FR® Device Versus Best mediCal Therapy in the Treatment of Acute Stroke Due to anTerior Circulation Large Vessel Occlusion Presenting Within 8 Hours of Symptom Onset
11 Endovascular Treatment for Small Core and Anterior Circulation Proximal Occlusion with Emphasis on Minimizing CT to Recanalization Times
clearly of high importance to future stroke research and needs to be further defined to maximise patient outcome in all situations of stroke.

6.5 CONCLUSIONS
In conclusion, this thesis raises doubts regarding the overall efficacy of sonothrombolysis ± microbubbles as a recanalization therapy for stroke. I observed no improvement of recanalization over tPA alone in studies of large vessel occlusion. However, there may be potential for its use as a reperfusion therapy to restore microvascular perfusion after large vessel recanalization.

The overall findings of my studies were:

- That PRC are resistant to microbubble-enhanced sonothrombolysis [Chapter 2],
- That microvascular patency can be restored with microbubble-enhanced sonothrombolysis after large vessel recanalization, irrespective of bubble type or dose [Chapter 3],
- That the degree of underlying stenosis underlying acute carotid artery occlusion determines thrombolytic success for recanalization [Chapter 4], and
- That the “human equivalent” dose of tPA, that mimics clinical recanalization rates, is lower than the conventionally used “rodent dose”, but higher than the clinical dose [Chapter 5].

Since the commencement of this thesis, the landscape for stroke therapy has dramatically changed. In particular, recent endovascular trials have revealed significant benefit to patient outcome over tPA alone [37-41], as has the more fibrin-specific thrombolytic, tenecteplase [35]. Additionally, reperfusion has now been shown to be a better predictor of good patient outcome than recanalization [11, 61, 68], highlighting the need to consider both outcomes when designing and testing stroke therapies. Given my findings and the recent termination of the CLOTBUST-ER trial [196], sonothrombolysis is unlikely to provide a greater benefit to patients for large vessel recanalization over tPA alone or these alternative interventions (endovascular intervention or tenecteplase). However, there may be a potential benefit of sonothrombolysis for reperfusion therapy.

Despite limited sonothrombolysis success, the findings of this thesis provide important information regarding potential contributors to thrombolytic success that could lead to identifying patients and personalising treatment, such as platelet composition of clots, and the degree of stenosis underlying an occlusion. This thesis also highlights the variable nature of stroke, and I have utilized models designed and developed to mimic clinically relevant clot compositions, occlusive locations (intracranial/extracranial, large vessel/microvasculature), and vessel stenosis. Successful thrombolysis appears to be influenced by these stroke characteristics. Hence, it is
likely that multiple therapeutic and interventional strategies are required to successfully treat all stroke patients. Identifying characteristics of stroke in individual patients could lead to better and more rapid treatment allocation, and thus, will ultimately lead to better patient recovery. Developing and utilizing clinical diagnostic imaging techniques to identify clot composition, microvascular occlusions, and the degree of stenosis *in situ* could identify tPA responders and non-responders, leading to improved patient triage to more effective treatment.
Chapter 7

1 REFERENCES


117. Nedelmann, M., et al., *Combined contrast-enhanced ultrasound and rt-PA treatment is safe and improves impaired microcirculation after reperfusion of middle cerebral artery*


Appendix A

CO-AUTHOR DECLARATIONS
We, the co-authors, attest that Research Higher Degree candidate, Amelia Tomkins, contributed to the paper/publication entitled **Platelet rich clots are resistant to lysis by thrombolytic therapy in a rat model of embolic stroke**, as outline below:

- 60% Conception and design of the experiments
- 90% Surgery and experimental work
- 80% Analysis and interpretation of findings
- 80% Writing the paper and critical appraisal of the content

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- 60% Conception and design of the experiments
- 95% Surgery and experimental work
- 90% Analysis and interpretation of findings
- 70% Writing the paper and critical appraisal of the content

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Chapter 2

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Chapter 5

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Appendix C

SYSTEMATIC REVIEW OF EMBOLIC STROKE MODELS

This systematic review of the embolic stroke literature is ongoing. Data included in the introduction of clot types (Figure 1-10) is generated from the data collected to date. While representative of the most common clot types for rats, the decision tree is not yet inclusive of the whole sample.

The aims of this review are:

- To determine the different clot types used in thromboembolic stroke models
- To determine, based on the information retrieved, a standardised naming system for clot type
- To determine if clot type affects the major stroke outcomes of recanalisation and infarct volume.

The inclusion and exclusion criteria for this review are as follows:

Inclusion criteria

- Original articles
- Models of focal embolic stroke that use a blood clot for embolisation
- Contains a detailed description of clot preparation or clear reference to the original method
- Reports one or more of the following outcomes:
  - Clot composition (i.e. imaging/histological information of exact composition)
  - Recanalisation
  - Infarct size
  - Mortality
  - Haemorrhage
  - Behavioural scores
Exclusion criteria

- Other stroke models
  - Other embolic (not blood clot): macrospheres of silicone, microfil, gold, other; Chemically or photo induced (Photothrombotic, FeCl$_3$, endothelin-1, etc)
  - Non-embolic models: thread occlusion, electrocoagulation, clip occlusion, etc; in vitro, computer simulations, flow models
- Global ischaemia models, lacunar stroke, transient ischaemic attack
- Subarachnoid haemorrhage, traumatic brain injury, spinal cord models
- Human/clinical studies, Fetal, neonatal
- Not cerebral ischaemia
  - pulmonary, cardio, renal, bowel, muscular, retinal, tumor, hind limb
- Review articles, editorials, letters to the editor
- Conference abstracts (these don’t provide enough information regarding clot preparation method)

A search of the databases Pubmed, EMBASE, OVID Medline, and ISI Web of science, was performed on 31 October 2013 using the following search strategy:

((stroke OR “brain infarction” OR “cerebral infarction” OR (ischemia OR ischaemia) OR (“cerebral ischemia” OR “cerebral ischaemia”) OR (“brain ischemia” OR “brain ischaemia”) OR (“focal ischemia” OR “focal ischaemia”) OR (“acute ischemic stroke” OR “acute ischaemic stroke”) “stroke model*” OR “experimental stroke”)

AND (embolic OR “embolic stroke” OR (embolization OR embolisation) OR embolism OR thromboembolic OR “thromboembolic model” OR “thromboembolic stroke” OR thromboembolism OR thrombotic OR “brain embolism”)

AND (animal* OR “animal model*” OR “experimental animal*” OR (rat OR rats) OR wistar OR “sprague dawley” OR “spontaneously hypertensive rat*”))

The number of retrieved references with exclusions and allocations to animal model to date, are presented in Figure A-1.
Figure A-1. Systematic review of preclinical embolic stroke models: studies retrieved to date. Analysis is ongoing. Latest update, 27th January 2015. *Exclusions are based on the criteria as outlined in the text.
Appendix D

ADDITIONAL PAPERS PUBLISHED DURING THE COURSE OF THIS THESIS

Below are the abstracts of additional publications that were co-authored and published during the course of this PhD, but do not form a part of this thesis. Full text articles can be obtained at the relevant journal websites.

Allopregnanolone and Its Precursor Progesterone Do Not Reduce Injury after Experimental Stroke in Hypertensive Rats – Role of Postoperative Temperature Regulation?

Neil J. Spratt1,2, Amelia J. Tomkins1, Debbie Pepperall1, Damian D. McLeod1, Mike B. Calford1,3
1 School of Biomedical Sciences, University of Newcastle, and Hunter Medical Research Institute, Newcastle, Australia, 2 Hunter New England Local Health District, Newcastle, Australia, 3 School of Medicine, The University of Tasmania, Hobart, Australia

Abstract
Allopregnanolone is a neurosteroid synthesized from progesterone in brain. It increases inhibition through modulation of the gamma-aminobutyric acid type A (GABA-A) receptor. Both agents are putative neuroprotectants after ischemic stroke. We sought to confirm their effectiveness in a hypertensive rat stroke model, with intra- and post-operative temperature regulation. The primary study compared allopregnanolone, progesterone or vehicle control treatments, administered 105 minutes after induction of temporary middle cerebral artery occlusion in spontaneously hypertensive rats. Temperature was controlled intraoperatively and a heat mat used in the 6 hours postoperatively to permit animal temperature self-regulation. The primary outcome was infarct volume and secondary outcomes were tests of sensory and motor function. There was no significant effect of treatment on any outcome measure. Given prior reports of GABA-A receptor agonists causing hypothermia, follow-up experiments were conducted to examine postoperative temperature regulation. These did not reveal a difference in postoperative temperature in neurosteroid-treated animals compared to control. However, in all rats maintained postoperatively in ambient temperature, moderate hypothermia was observed. This was in contrast to rats maintained over a heat mat. The lowest mean postoperative temperature was between 34.4–34.9°C in all 3 groups. These data do not support a neuroprotective effect of allopregnanolone or progesterone in ischemic stroke in hypertensives in the setting of normothermia. Given previous evidence of synergy between neuroprotective agents and hypothermia, demonstration of neuroprotective effect of these agents in the absence of postoperative hypothermia would be prudent before consideration of these agents for further clinical investigation.

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Intracranial pressure elevation after ischemic stroke in rats: cerebral edema is not the only cause, and short-duration mild hypothermia is a highly effective preventive therapy

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In both the human and animal literature, it has largely been assumed that edema is the primary cause of intracranial pressure (ICP) elevation after stroke and that more edema equates to higher ICP. We recently demonstrated a dramatic ICP elevation 24 hours after small ischemic strokes in rats, with minimal edema. This ICP elevation was completely prevented by short-duration moderate hypothermia soon after stroke. Here, our aims were to determine the importance of edema in ICP elevation after stroke and whether mild hypothermia could prevent the ICP rise. Experimental stroke was performed in rats. ICP was monitored and short-duration mild (35°C) or moderate (32.5°C) hypothermia, or normothermia (37°C) was induced after stroke onset. Edema was measured in three studies, using wet–dry weight calculations, T₂-weighted magnetic resonance imaging, or histology. ICP increased 24 hours after stroke onset in all normothermic animals. Short-duration mild or moderate hypothermia prevented this rise. No correlation was seen between ΔICP and edema or infarct volumes. Calculated rates of edema growth were orders of magnitude less than normal cerebrospinal fluid production rates. These data challenge current concepts and suggest that factors other than cerebral edema are the primary cause of the ICP elevation 24 hours after stroke onset.

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